Investigation of CYP activity in liver organoids by establishing a liquid chromatography-mass spectrometry method for measuring drug metabolism

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Abstract

Liver organoids arise as tools in drug discovery and toxicity testing as they have the potential to mimic human physiology to a greater extent than the traditional models. However, the organoids are still in their infancy, and there is a need for better characterization through the development of new protocols to determine metabolizing properties. Most drugs are metabolized by cytochrome P450 (CYP) enzymes, and the CYP activity can be evaluated by measuring concentrations of metabolites after drug incubation with organoids. Thus, a liquid chromatography-mass spectrometry (LC-MS) method was developed and validated for the determination of CYP activity in primary hepatocyte spheroids (PHS) and induced pluripotent stem cell (iPSC) derived organoids. Phenacetin, tolbutamide, fluoxetine, and their metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine were used as telltale drugs. The validation criteria were met for both phenacetin and tolbutamide with their metabolites, with a need for a minor adjustment to the limit of quantitation (LOQ) of tolbutamide, but the method for fluoxetine and norfluoxetine could not be validated within the acceptance criteria for validation of a bioanalytical method. Nevertheless, all three metabolites were detected after 24 hours incubation with PHS, which confirmed CYP activity in the organoids, but not in a quantifiable concentration for norfluoxetine. Acetaminophen, and 4-hydroxytolbutamide were also detectable after 6 hours, but only acetaminophen was detected in a quantifiable concentration. None of the three metabolites could be detected after incubation for 24 hours with the iPSC derived organoids. However, the iPSC derived organoids can still have CYP activity, just not enough to provide a metabolite concentration above the detection limit for this method. To sum up, the detection of drug metabolites for all three drugs showed that the PHS had metabolizing properties, and although there is a need for further development, the use of LC-MS to study drug metabolism in organ representations is a viable

approach.

Preface

The work presented in this thesis was performed at the research section for Bioanalytical Chemistry at the Department of Chemistry, University of Oslo. A huge thank you to my supervisors Ph.D. student Frøydis Sved Skottvoll, Professor Steven R.H. Wilson, and Professor Elsa Lundanes, for their continued guidance and support. It has been a priviledge.

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Contents

Abbreviations

ACN	Acetonitrile
ASC	Adult stem cell
CYP	Cytochrome P450
DC	Direct current
DFS	Department of forensic sciences
EMA	European medicines agency
ER	Endoplasmatic reticulum
ESI	Electrospray ionization
FA	Formic acid
FDA	Food and drug administration
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HLM	Human liver microsomes
ICH	International council for harmonication
ID	Inner diameter
ISTD	Internal standard
LC	Liquid chromatography
ME	Matrix effects
MeOH	Methanol

mLOD	Mass limit of detection
MRM	Multiple reaction monitoring
MP	Mobile phase
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NSAID	Non steroid anti inflammatory drug
PHS	Primary hepatocyte spheroid
PSC	Pluripotent stem cell
RP	Reversed phase
RF	Radio frequency
SP	Stationary phase
SRM	Selected reaction monitoring
TQ	Triple quadrupole
UGT	Uridine diphosphateglucuronyltransferase
UV	Ultra violet
W	Working solution
WF1	Working solution fluoxetine 1
WF2	Working solution fluoxetine 2
WISTD1	Working solution internal standard 1
WISTD2	Working solution internal standard 2
WN1	Working solution norfluoxetine 1

WN2	Working solution norfluoxetine 2
WP+A1	Working solution phenacetin and acetaminophen 1
WP+A2	Working solution phenacetin and acetaminophen 2
WT+4HT1	Working solution tolbutamide and 4-hydroxytolbutamide 1
WT+4HT2	Working solution tolbutamide and 4-hydroxytolbutamide 2

1. Introduction

In drug discovery and development, animal testing has been, and still is, a much used method. The use of animal models raises both ethical and biological issues, and hence, the development of new and improved alternative methods is of the essence. One method that is under development is the use of organoids. Organoids are three-dimensional (3D) tissue models typically derived from adult and pluripotent self-organizing stem cells (**Section 1.4**). In 2017, organoids were the "Method of the Year" in Nature Methods [1], and they are expected to become a key tool in biology, and drug discovery, as they may mimic human physiology to a greater extent than traditional cell cultures and even animal models. Nevertheless, organoid development is still in its infancy, thus there is a need for characterization, and further drug metabolism studies to establish and improve the organoids metabolizing properties. Drug metabolism is the chemical alteration of a drug that is absorbed in an organism and usually involves enzymatic activity.

1.1. Drug metabolism and the most common enzymes involved

Most foreign and potentially toxic compounds (xenobiotics) introduced and absorbed into the body are lipophilic substances, and hence not ideal for excretion due to re-absorption in the kidneys or gastrointestinal tract after biliary excretion. When these xenobiotics enters the body, intentional or unintentional, the body tries to convert them into more polar, readily excreted metabolites to avoid accumulation and toxic effect. The conversion can render some xenobiotics more toxic, but also provide a pharmacological effect if the xenobiotic is converted into a pharmacologically active compound, e.g. from a pharmacologically inactive drug (prodrug) into a pharmacologically active metabolite. The conversion is also re-

ferred to as biotransformation or metabolism and consists of several enzymatic pathways. Most xenobiotics are subjected to pathways that constitute phase 1 oxidation and phase 2 conjugation with a water soluble molecule. Metabolism with the phase 1 oxidation is catalyzed by CYP monooxygenase system in the presence of O_2 and H^+ from the co-factor reduced nicotinamide adenine dinucleotide phosphate (NADPH) [2]. Phase 2 conjugation is mainly catalyzed by transferases, where the major enzymes are the transferases uridine diphosphate-glucuronyltransferase (UGT), sulfotransferases, N-acetyltransferases, glutathione S-transferases, and methyltransferases [3]. A monooxygenase system is when a reaction incorporates only one of the oxygens from molecular oxygen, **(Reaction 1)** [4], resulting in an oxidized substrate and a water molecule as a byproduct. Thus the corresponding enzymes are categorized as monooxygenases [5, 6], and a hydroxy intermediate is commonly occurring in the primary oxidation reaction.

$$\mathrm{NADPH} + \mathrm{H^+} + \mathrm{O_2} + \mathrm{RH} \xrightarrow{\mathrm{CYP_{450}}} \mathrm{NADP^+} + \mathrm{H_2O} + \mathrm{ROH} \qquad \{1\}$$

Approximately 75% of all clinically used drugs are metabolized by the CYPs [7, 8], with a wide range of reactions that include oxidation. Examples of some common phase 1 reactions that have oxidation as the primary reaction are sulphoxidation, aromatic- and aliphatic hydroxylation, N-dealkylation, O-dealkylation, and deamination **(Figure 1)** [9].

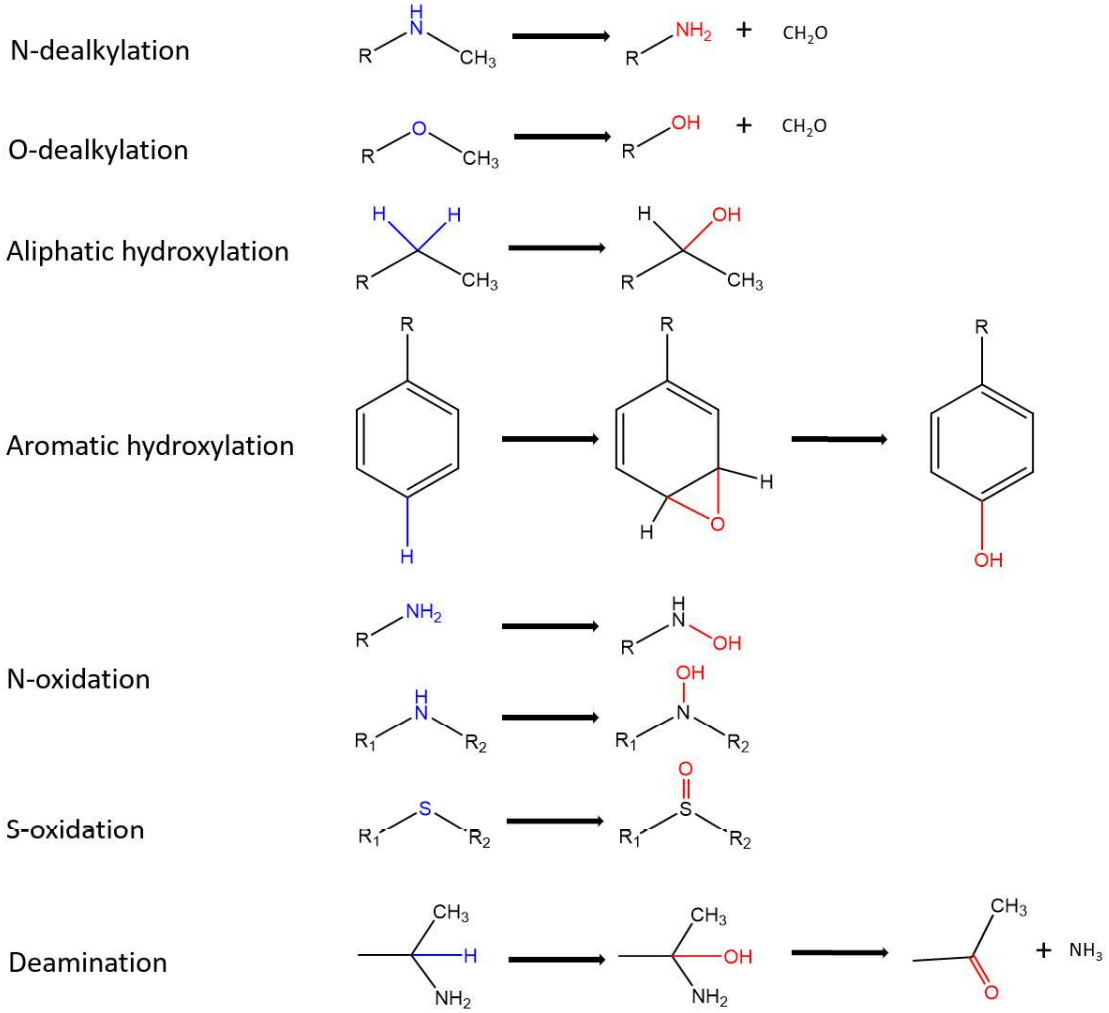

Figure 1: Some of the most common phase 1 oxidative reactions with CYPs. The hydroxy intermediate is commonly occurring in the primary oxidation reaction with the enzymes that are categorized as monooxygenase enzymes. The blue and red coloring indicates where the oxidation reaction occurs. Adapted from [9].

The CYPs are heme containing enzymes, and the name P450 is due to light absorption at 450 nm wavelength when they bond to the ligand carbon monoxide [10, 11]. Three primary CYPs metabolize xenobiotics, CYP1, CYP2, and CYP3 [12], and the major isoforms involved are CYP3A, CYP2C, CYP2D6, CYP1A2, and CYP2E1 [13]. The CYP enzymes are mainly found within the membrane of the smooth endoplasmatic reticulum (ER) of the liver cells called hepatocytes [2,

14], while phase 2 enzymes are predominantly found in the cytoplasm [9]. Phase 2 enzymes are out of the scope of this thesis and will therefore not be further described, but the interested reader is referred to [3] or the chapter on phase 2 enzymes in [9]. Because the majority of CYP enzymes are located in hepatocytes, the liver plays a major role in drug metabolism.

1.2. The liver and its role in drug metabolism

The liver is the largest organ after the skin and takes up most of the upper right quadrant of the abdomen. The liver is anatomically built up of 4 lobules that are collections of hepatocytes in a hexagonal shape. The liver receives blood from two major blood vessels, the hepatic artery that brings oxygenated blood from the heart, and the portal vein that brings nutritious blood from the intestine **(Figure 2A)**. The blood is then mixed in the liver sinusoid, which is a type of vessel surrounded by hepatocytes before it exits through one major hepatic vein back to the heart. The hepatocytes secrete bile into the canalicular, which is the dilated intercellular space between adjacent hepatocytes, and the first channel in the biliary system **(Figure 2B)**. The bile duct together with the portal vein and the hepatic artery, make up a branch that is referred to as the portal triad located at the vertices of the hexagon [15] **(Figure 2 A)**. When the lipophilic drugs are absorbed in the body, they are either largely bound to plasma proteins in the blood or sequestered into fat [16]. Thus, the previously explained biotransformation relies on the capability of the body to convert the lipophilic drugs into more water soluble metabolites more readily excreted by the kidney into the urine. Several tissue and organs are capable of generating water soluble metabolites from some drugs, but the liver is the main site as it is uniquely suited to metabolize lipophilic drugs [16]. The reason is that the pores or holes in the endothelium lining of the sinusoidal blood space are large enough to allow the passage of most plasma proteins. The

space between the sinusoidal vessels and the hepatocytes is called the space of Disse. The drug bonded plasma proteins can passively diffuse from the sinusoid into the space of Disse and consequently come in contact with the hepatocytes plasma membrane. From there, the drugs are transported into the hepatocyte and converted into metabolites before they most often are excreted back into the space of Disse. The alternative is that the generated metabolites are sorted to the canalicular membrane and from there excreted to the bile [16].

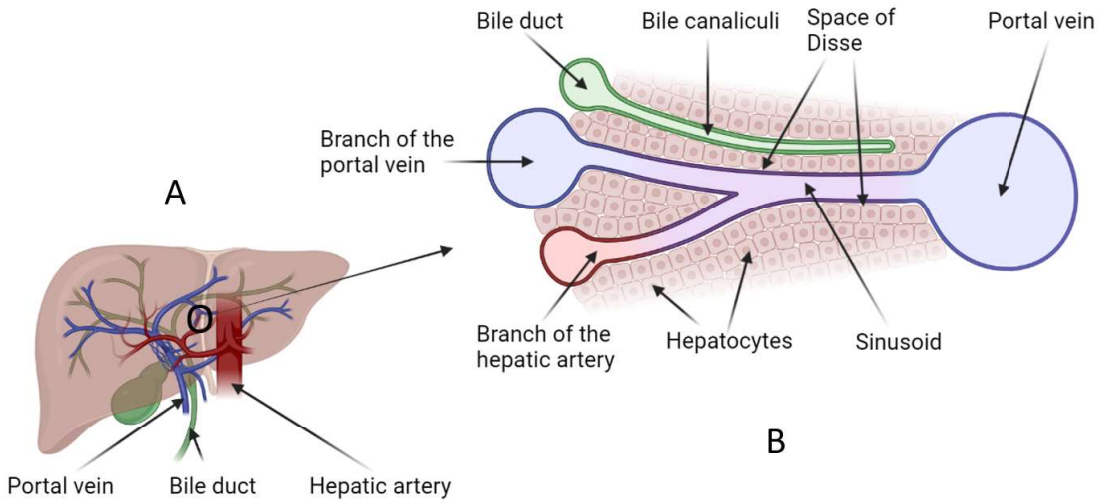

Figure 2: Schematic overview of the drug metabolism in the liver. Bonded to plasma proteins, the acquired drugs are transported to the liver through the portal vein or the hepatic artery(A). Here the drugs comes in contact with the hepatocytic membrane by passive diffusion into the space of Disse. The drugs are then converted into metabolites before being excreted back into the space of Disse or to the bile via the canalicular membrane (B) and exit the liver through the bile duct. Made with BioRender (BioRender.com).

A common approach to estimate in vivo human drug metabolism is the use of human liver-derived models for in-vitro screening assays.

1.3. Golden standards in drug metabolism studies

Several human liver-derived models for in vitro screening assays have been developed during the last few decades, and include perfused liver, liver slices, primary

hepatocytes, cytosol, S9 fractions, supersomes, cell lines, transgenic cell lines, and microsomes [17]. The most accepted standard of the models is the microsomes, especially the human liver microsomes (HLM). HLMs were shown to be fragments or pieces (vesicles) of the ER by Keith R. Porter [18] after Albert Claude [19] discovered how to separate submicroscopic particles of the cell by centrifugal fractionation. The HLMs are extracted by centrifugal fractionation of donated homogenized liver tissue (**Figure 3**). The liver tissue is first homogenized and centrifuged at low g (10,000 x g) for 20 min to separate cell debris (pellet) from the S9 fraction (supernatant) [20]. The HMLs can then be extracted by high speed centrifugation (105,000 x g) for 120 min of the S9 fraction, which separates the S9 fraction into HMLs (pellet) and a cytosolic fraction (supernatant) [20].

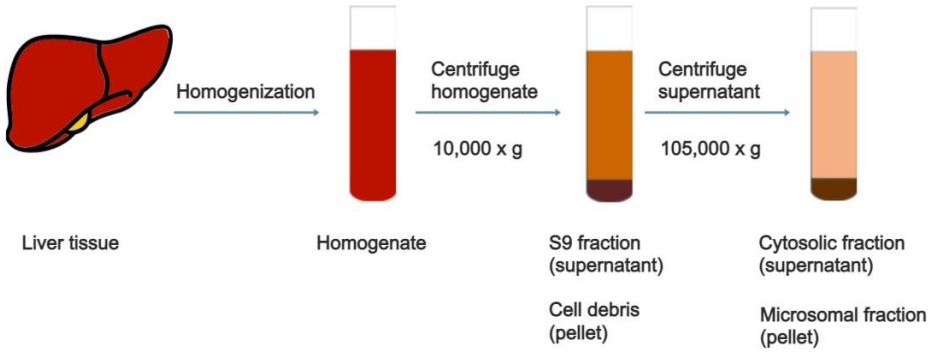

Figure 3: Centrifugal fractionation of homogenous liver tissue is used for extracting the microsomal fraction (i.e. the microsomes). Homogenized liver tissue is centrifuged at low g (10,000 x g) for 20 min to separate the cell debris from the S9 fraction. HLMs can then be extracted by centrifuging the S9 fraction at high g (105,000 x g) for 120 min. Adapted from [20]

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All CYP enzymes can be found in the microsomal fraction [21] with the enzymes active site exposed to the outside (cytosolic side) of the HLM vesicle membrane [21, 22]. The cytosolic pentose pathway primarily supplies the NADPH necessary for oxidative transformation, but due to loss of cytosol during the isolation process, it is necessary to add NADPH or an NADPH regenerating system (usually contain-

ing β-NADP+, glucose-6-phosphate (G6P), and G6P dehydrogenase (G6PDH)) to supply the energy demand of the CYPs needed for enzymatic activity in drug metabolism [17, 23]. The general experimental workflow for determining drug metabolism with HLMs is as follows: The microsomes are incubated together with the selected drug, an NADPH regenerating system, and a phosphate or sodium phosphate buffer with a pH of 7.4. The reaction is initiated when both NADPH and the drug are added to the microsomes. The incubation is performed at 37 °C in a thermal shaker or a shaking water bath for a chosen set of time intervals. The reaction is then terminated by adding a stop reagent such as cold acetonitrile or a small molecule acid or base [24].

In drug metabolism studies, microsomes are the primary choice as a screening model for high throughput assays, but the hepatocytes are increasingly replacing or complementing the microsomes [25]. Hepatocytes directly isolated from liver tissue are called primary hepatocytes and contains a broad complement of metabolizing enzymes and transport proteins that are regulated by the same cellular processes that occur within the liver in vivo [25]. The primary hepatocytes are isolated from donor livers, and are generally seeded in a medium containing fetal bovine serum (FBS), which is a universal growth supplement and enhances the surface attachment ability of the hepatocytes [25–27]. However, protocols for chemically-defined, serum-free conditions have been developed, which is a step towards reducing the use of animals in drug metabolism studies [28] Even though microsomes and primary hepatocytes are the most popular and considered the golden standards when it comes to studying drug metabolism, they lack the full complexity that an organ exhibits. The animal model is considered the golden standard in pre-clinical trials of drug development, however, the unreliability and limitations of animal experimentation have increasingly been acknowledged [29]. The use of the animal model is based on the possibility to predict human re-

sponse to a drug based on the animal response. On average, the studies using animals often fail to accurately predict human responses [30]. Disparities between the response in the animal model and the human model derived from the same disease, and species differences in physiology and genetics, are conditions that explains why the animal model fails to give reliable predictions for human response [29]. In addition, neither, HLMs, primary hepatocytes, nor the animal model are representative of the disease response of a single individual or a specific group of individuals, hence there is a need for new models that can mimic human physiology to a greater extent than the traditional models.

1.4. Three dimensional tissue models can become a key tool in biology, drug discovery, and metabolism studies

An organoid is, as mentioned in **Section 1**, a more complex 3D self organizing tissue model that may mimic human physiology to a greater extent than traditional models, e.g. microsomes. The term organoid means "resembling an organ", and was used as early as 1946 by Smith and Cochrane to describe a case of cystic teratoma [31, 32]. Resembling an organ implies that the organoid must contain more than one cell type of the organ it models, that the organoid should exhibit some function specific to that organ, and the cells should be organized similarly to the organ itself [32]. The organoids can be derived from two main types of stem cells, pluripotent stem cells (PSC), or adult stem cells (ASC) [33, 34]. PSCs are cells that have the ability to develop into a broad set of cell types in the body, while ASCs are cells that are restricted to the organ of origin [35]. Although not referred to as organoid, the first reconstitution of cultured human stem cells to a 3D tissue structure was achieved and described by James Rheinwald and Howard Green as early as 1975 [36]. In more recent advancements in the 3D tissue technology, stem cells exhibit remarkable self organizing properties with resulting organoids

that reflect key structural and functional properties of the organ it resembles [33, 37]. The self organizing pattern is initiated with a differentiation of the stem cells followed by a sorting out of the cells based on adhesive properties **(Figure 4)**. The sorted cells then organize in a spatially restricted lineage commitment that makes up the structure of the organoid [32].

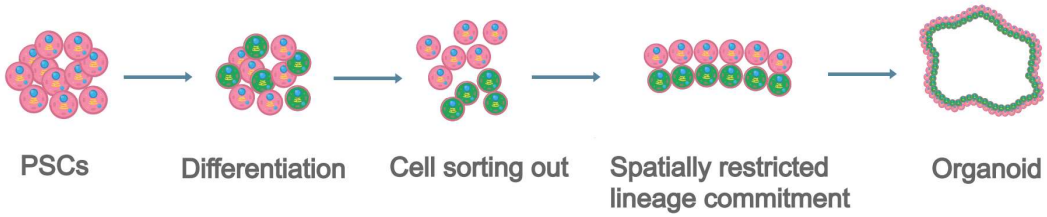

Figure 4: Organoids derived from PSCs. PSC self organizing pattern into 3D tissue models, begins by initial differentiation of the cells, before sorting out the cells based on adhesive properties. The sorted cells then organize in spatially restricted lineage commitments that make up the structure of the organoid. Adapted from [32]. Made with Visme.

The organoids hold tremendous potential for biomedical applications and can be used in several clinical applications like disease modeling, drug screening, host microbe interactions, and regenerative therapy with precision and the possibility for personalized treatment [38]. They also have the potential to replace the animal model in many areas of pre-clinical drug development. Organoid protocols for growing simple intestine, kidney, brain, liver, and lungs amongst others are established [39–43], and a goal in regenerative medicine is to produce organs that can be transplanted into patients [35]. Nevertheless, there are key challenges that need to be addressed, such as better characterization and validation of the organoids as models of human biology through the development of new organoid protocols, and by applying organoids in basic biology and biomedical research [35, 38]. The primary hepatocytes can also be cultured in monolayers or as 3D microtissues and are then termed spheroids [28].

Although the terms spheroid and organoid are often used interchangeably, spheroids are a less complex 3D model than organoids. The main differences between organoids and spheroids are the lack of self organizing and regenerating properties in the cells forming a spheroid [32]. The spheroids are clusters of cells (e.g. primary hepatocytes in liver spheroids) that form a 3D structure by spontaneous aggregation of cells followed by binding of the cell surface to form a compact structure through strong intercellular interactions [44]. Due to the role of the liver in drug metabolism, liver organoids and spheroids are of utmost interest in drug development and metabolism studies. The general experimental workflow of drug metabolism studies with organoids and spheroids has similarities to that of the HLMs. Organoids/spheroids are incubated at 37 °C together with the drug, and the reaction is terminated in the same way as for the HLMs after a selected time point. The main difference is the matrix, which for the organoids and spheroids consists of a cell medium that contains nutrient and antibiotics, and both with FBS, and without FBS [28, 45]. One of the most used antibiotics in the matrix is rifampicin, which induces several drug metabolizing enzymes, and some drug transporter proteins [46]. The cytosolic pentose pathways are intact in the organoids, hence, there are no need for additional NADPH as it is with the use of HLMs.

A fairly recent review article sums up the advancements in 3D in vitro models used for drug validation and toxicity assessment in the past decade [47]. Although 3D tissue models have been studied for several decades, and LC-MS is a frequently used method in metabolism studies with HLMs, none of the referred validation methods used LC-MS to validate drug metabolism with the use of liver organoids as an in vitro model [47, 48]. A separate search within Google Scholar and SciFinder confirm there is a shortage of documentation of the use of LC-MS for validation of drug metabolism with organoids as the in vitro model.

Drugs that are well studied and have known metabolic pathways are key in metabolism studies for new promising models. Although most drugs are mainly metabolized by a specific CYP enzyme, some drugs can be metabolized through more than one CYP metabolic pathway [49]. Drugs that are mainly metabolized by one specific enzyme are often denoted telltale or prototypic drugs, meaning that if the drug is metabolized, it confirms that the specific enzyme is active.

1.5. Telltale drugs for cytochrome P450 enzymes activity

Several drugs have been used in metabolism studies to determine CYP activity, by metabolism studies or drug inhibition studies [50]. Three examples of drugs that are considered telltale drugs because they are preferably metabolized by one specific enzyme are fluoxetine, tolbutamide, and phenacetin. They are from three different groups of drugs, antidepressant, blood-sugar regulator, and nonsteroid anti inflammatory drug (NSAID), respectively.

1.5.1. The antidepressant drug fluoxetine

Fluoxetine is a selective serotonin reuptake inhibitor that has been used as an antidepressant since the late 1980s [51]. Fluoxetine is mainly metabolized to the active metabolite norfluoxetine after a phase 1 N-dealkylation (N-demethylation) (**Figure 5**), a reaction shown to be strongly related to CYP2D6, both in vitro and among healthy volunteers [52, 53]. Fluoxetine has also been reported to be a potent inhibitor of the CYP2D6 enzyme [54, 55]. Thus, based on these previous reports, fluoxetine has the potential to be a good candidate for the determination of CYP2D6 activity in metabolism studies with organoids.

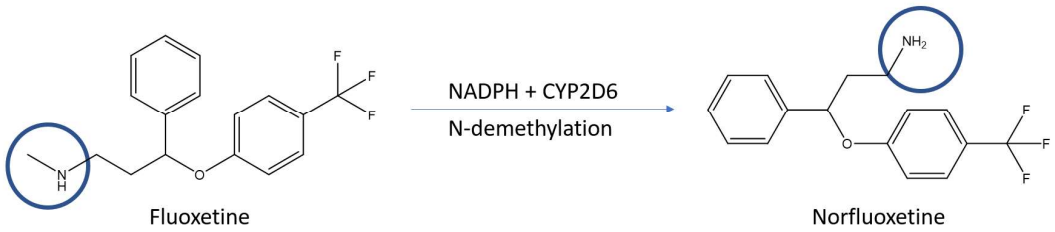

Figure 5: Phase 1 N-demethylation of fluoxetine to its main metabolite norfluoxetine. The main CYP enzyme involved in the metabolism of fluoxetine to norfluoxetine is the CYP2D6 enzyme. In the presence of NADPH, the methyl group attached to the amino group are detached, by a phase 1 oxidative N-demethylation reaction.

1.5.2. The blood sugar regulatory drug tolbutamide

Tolbutamide was the first drug in the group of sulfonylurea (a class of agents that lower blood sugar as a result of increased release of insulin from the pancreas), making tolbutamide a first generation treatment of type 2 diabetes [56]. All the sulfonylurea drugs are derivates of urea [57]. Tolbutamide were commercially introduced to the market in 1956 in Germany, and several of the modern sulfonylureas (generation two and three) are further developments of tolbutamide [56]. Tolbutamide is metabolized by hydroxylation to 4-hydroxytolbutamide by CYP2C9, and is widely accepted as a telltale drug to determine CYP2C9 activity, both in vivo and in vitro **(Figure 6)** [58, 59].

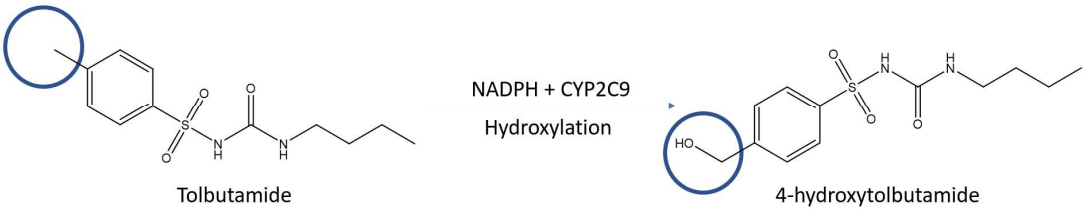

Figure 6: Hydroxylation of tolbutamide to its main metabolite 4-hydroxytolbutamide. The main CYP enzyme involved in the metabolism of tolbutamide to 4-hydroxytolbutamide is the CYP2C9 enzyme. The methyl group on tolbutamide is hydroxylated in the precence of NADPH and CYP2C9 by a phase 1 hydroxylation reaction.

1.5.3. The non steroid anti inflammatory drug phenacetin

Phenacetin was discovered as a byproduct in the production of aniline dye, and was introduced into the medicine as an antipyretic in 1887 [60]. Although the antipyretic effect was soon overshadowed by the discovery of its analgesic effect against several kinds of pain, the original introduction was a part of the discoveries that ushered Germanys early dominance in the synthetic drug and chemical field [60, 61]. Phenacetin was withdrawn from the market in most countries by 1983 due to reports of renal disease and cancer, but it stands as the worlds first synthetic pharmaceutical drug [62]. The main metabolite acetaminophen is commonly known as paracetamol, the most commonly used analgesic and antipyretic worldwide [63], and a dealkylation (O-deethylation) of phenacetin into the active metabolite acetaminophen is widely used as an index reaction for CYP1A2 (**Figure 7**) [64].

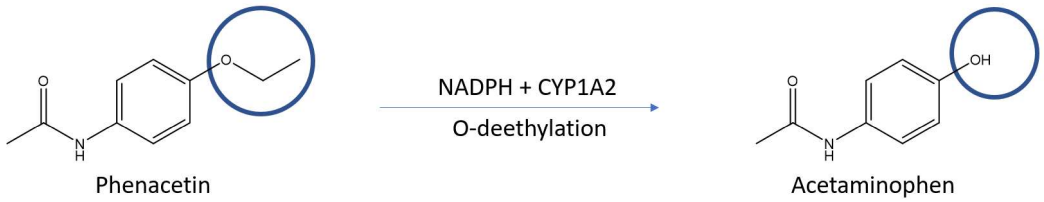

Figure 7: Phase 1 O-deethylation of phenacetin to its main metabolite acetaminophen. The main CYP enzyme involved in the metabolism of phenacetin to acetaminophen is the CYP1A2. With NADPH as a co-factor, the ethyl group are detached and replaced by a hydrogen forming a hydroxygroup.

Two widely used detection methods in metabolism studies with HLMs, are ultraviolet (UV) detection that detects UV light absorbed by the analytes, and MS which detects ions in the gas phase. All three drugs and their respective metabolites have conjugated pi systems that absorb light, and functional groups that are ionizable under the right conditions, thus, making them candidates for detection with both UV and MS.

1.6. Detection techniques often used in metabolism studies

1.6.1. Detection of analytes in liquid phase with ultraviolet detection

The UV detection system typically consists of a light source, a monochromator, and a detector **(Figure 8)**, and operates with a mass limit of detection (mLOD) of 0.1-1 ng [65]. The limit of detection (LOD) is described as the smallest detectable quantity of an analyte which differs significantly from a blank. A signal to noise ratio (S/N) of 3, where the signal is detectable but still too small for accurate measurements are defined as the LOD, while an S/N of 10 refers to the LOQ, which are the smallest amount of analyte that can be measured with reasonable accuracy [66] (p. 102-105).

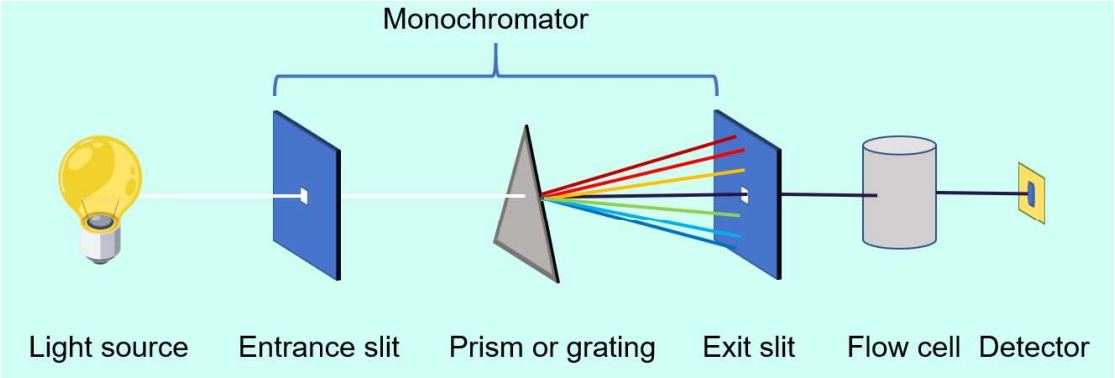

Figure 8: Basic components in a UV detection system. When a light source with a broad spectrum enters the monochromator, a prism or a grating splits the light into single wavelengths. The angle of the prism or grating can be controlled, and making it possible to only let the chosen wavelength pass the exit slit. The light of the single wavelength can then pass through the flow cell and further on to the detector.

The light source is sent through the monochromator where it is split into different wavelengths, and with a filter or grating, the selected wavelengths can be directed through the sample cell. The intensity of the light that goes through the sample cell is recorded in the detector, and a signal is obtained for all compounds that absorb light of the appropriate wavelength. The absorbance (A) can be obtained

according to Beer's law (**Equation 1**),

$$A = \varepsilon bc \tag{1}$$

where ε is the molar absorptivity, b is the path length, and c is the concentration. Thus, the number of molecules, and how effective a molecule absorbs light, determine the extent of light absorption. The maximum absorbance is referred to as the lambda (λ) max, and are normally chosen for spectrophotometric analysis, as that provide the greatest sensitivity for the analysis [66] (p.439). However, there should be kept in mind that UV absorption can be influenced by variations in pH, solvent, temperature, and analyte concentration [67–70].

Although UV detection traditionally used to be the most used LC detector, a more sensitive and selective detector like the mass spectrometer (MS) (mLOD = fg-pg) have improved in regards to selectivity, and resolution, and are more widely used for biological samples, particularly for metabolism studies where more selective or sensitive detection is needed [65, 71].

1.6.2. Detection of analytes in gas phase with mass spectrometry

The basic principle of MS is to separate ions in the gas phase according to their mass to charge ratio m/z, and then perform qualitative and/or quantitative detection. The MS instrumentation has a general layout that consists of an ion source, a mass analyzer, and an ion detector, and can detect most compounds as long as they are ionizable and transferable to the gas phase **(Figure 9)** [65, 72]. Although, in order for the ions to reach the detector, they have to travel through the system without colliding with other neutral gas molecules, hence, another important part of the instrumentation is a pumping system, which provides a high vacuum in the mass analyzer, detector, and sometimes in the ion source. A computer system is

used for data acquisition and to control the MS instrumentation.

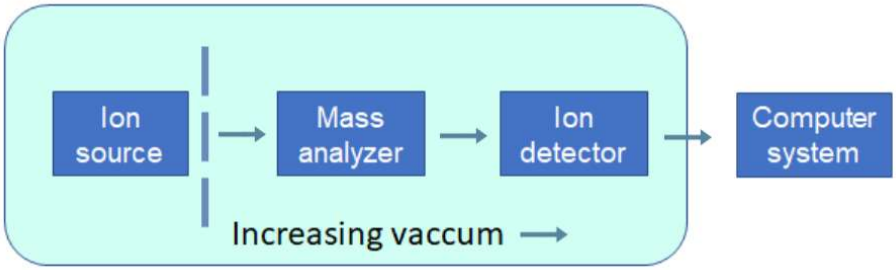

Figure 9: The general layout of a mass spectrometer. The mass analyzer, ion detector and sometimes the ion source operates under high vacuum provided by a pumping system, and are controlled by a computer system that also is used for data acquisition.

There are several mass analyzers [73] that operate with different techniques and principles, but they all separate ions produced in the gas phase according to their m/z [72]. A much used mass analyzer in metabolism studies is the quadrupole.

Quadrupole

Quadrupole mass analyzers are very robust and easy to use [74]. The system consists of four cylindrical or hyperbolic metallic rods assembled in a parallel construction **(Figure 10)**. Both opposite pair of rods is connencted electrically, with radio frequency (RF) and direct current (DC) voltage. This creates an oscillating electric field in the x-y plane with a potential given by **Equation 2 and 3**,

$$+ = \mathrm{U} - \mathrm{V}\cos\varpi\mathrm{t} \tag{2}$$

$$- = -\mathrm{U} - \mathrm{V}\cos\varpi\mathrm{t} \tag{3}$$

where U is the DC voltage and $\mathrm{V}\cos\varpi$ is the amplitude of the RF voltage. The ions enter the electrical field in the z direction, and start oscillating in the y and x direction due to the potential applied to the rods. By applying only RF, a wide

range of m/z ions can traverse through the quadrupole with a stable oscillating trajectory. A quadrupole with only RF applied is often used as an ion focusing component or ion guide. If both RF and DC voltage is applied, only ions of a specific m/z value can traverse through the quadrupole, while ions with smaller or larger m/z have unstable trajectories and will be lost due to collisions with the rods [65, 75]. The mass range can be adjusted by increasing the RF and DC while maintaining their ratio constant. The increase of RF and DC with a constant ratio will result in ions of increasing m/z reaching the detector. When the ions make contact with the detector, a signal is generated and recorded by the computer system. The signals are then displayed graphically as a mass spectrum that shows the relative abundance of the signal according to their m/z ratio [76].

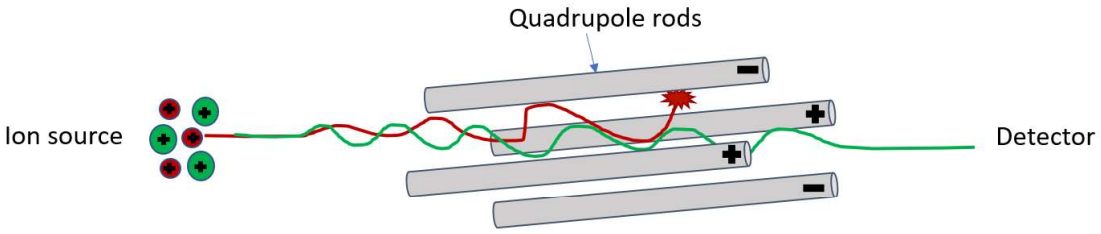

Figure 10: Quadrupole mass analyzer. The quadrupole consists of 4 metallic rods where each opposite pair share the same RF and DC voltage. The rods act as a filter that allows ions with a specific m/z to traverse through, while the rest have unstable trajectories and collides with the rods.

The quadrupole mass analyzers are described as filter or scanning instruments, and are often used as an ion focusing component in many instruments. A single mass analyzer has limited possibilities for specificity, quantification, and separation of ions with similar m/z in a complex sample like the biological samples in metabolism studies, but sensitivity and selectivity in quantification studies can be improved by the use of a triple quadrupole (TQ) (tandem MS) [74].

Triple quadrupole

Tandem MS (MS/MS) is a technique where two or more mass analyzers are coupled together and selected ions (precursor ions) are fragmented into product ions from a collision with an inert gas, e.g. argon. In regards to the quadrupole, MS/MS can be achieved by coupling three quadrupoles together (Q1-Q3) into a TQ, where Q1 and Q3 acts as mass filters (both RF and DC), and the middle one functions as an ion guide and collision cell (only RF) [77]. All three quadrupoles can be operated individually, which gives the MS the ability to be operated in different modes. The main scan modes are the precursor ion scan, product ion scan, neutral loss scan, and the selected reaction monitoring [77]. In selected reaction monitoring (SRM), Q1 is used to select a precursor ion with a given m/z, which undergoes fragmentation in Q2 before Q3 is used to select specific fragments and guide them through Q3 to the detector [78, 79]. If more than one precursor ion is selected, it is referred to as multiple reaction monitoring (MRM) [79]. MRM is often the method of choice when working with drug molecules and their metabolites because MRM is a highly selective and sensitive mass spectrometry technique that can selectively quantify multiple compounds within complex matrices. The SRM scan mode process is shown in **Figure 11**.

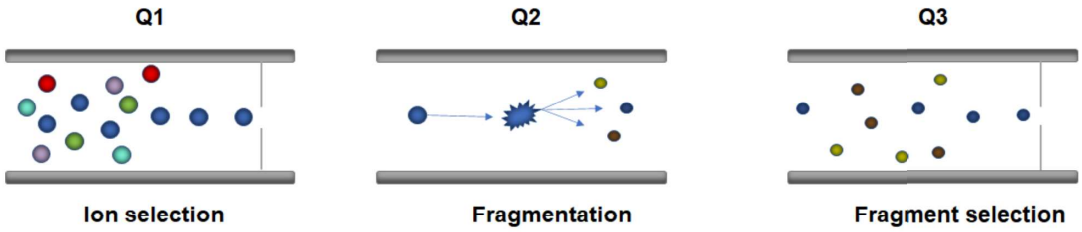

Figure 11: The SRM mode in MS/MS. One or more precursor ions are selected to pass through Q1 and into Q2 that acts as a collision cell. Here, the ions are fragmented in collision with an inert gas. A selection of fragments is then allowed to pass through Q3, and reach the detector.

The MS detects ions in the gas phase, thus ions in the liquid phase from biological

samples need to be transferred to the gas phase. An ion source placed prior to the MS can be used to introduce the ions to the MS, as well as turn ions in the liquid phase to ions in the gas phase. However, not all ion sources are capable of generating gas phase ions from liquid phase ions, but electrospray ionization (ESI) is an ion source capable of turning liquid phase ions into gas phase ions.

Electrospray ionization of analytes prior to mass spectrometer detection

ESI is a frequently used ionization method that was developed in the 1980s [80]. ESI ionizes liquid phase analytes and transfers them into the gas phase. The ESI can be operated in positive or negative mode (alternation between positive and negative mode is also possible for some ESI sources), meaning it generates cations in positive mode and anions in negative mode. The ESI is a soft ionization method, where the term soft means that there is a limited amount of analyte molecule fragmentation during the ionization, and hence, information about the unfragmented molecule can be achieved [81, 82]. The ESI can also be used to generate gas phase ions from moderately polar to polar molecules [81]. The ESI is a three step process that involves the dispersal of a fine spray of charged droplets, evaporation of the solvent, and then release of ions from the highly charged droplets. The ionization process begins when a voltage is applied between the solution in the capillary and a counter electrode, which can be the MS inlet. The applied voltage together with a nebulizing gas (e.g. N_2) at the capillary outlet (emitter), forms a taylor cone that disperses the sample liquid into charged droplets. A drying gas (often the same as the nebulizing gas) is then added to evaporate the solvent, resulting in highly charged droplets, followed by decomposition of the droplets due to surface charge density into much smaller charged droplets. This decomposition continues until higher generation droplets lead to observable gas phase ions [76, 83, 84]. **Figure 12** depicts the transformation of liquid into droplets until the ionization

is complete as the ions become observable.

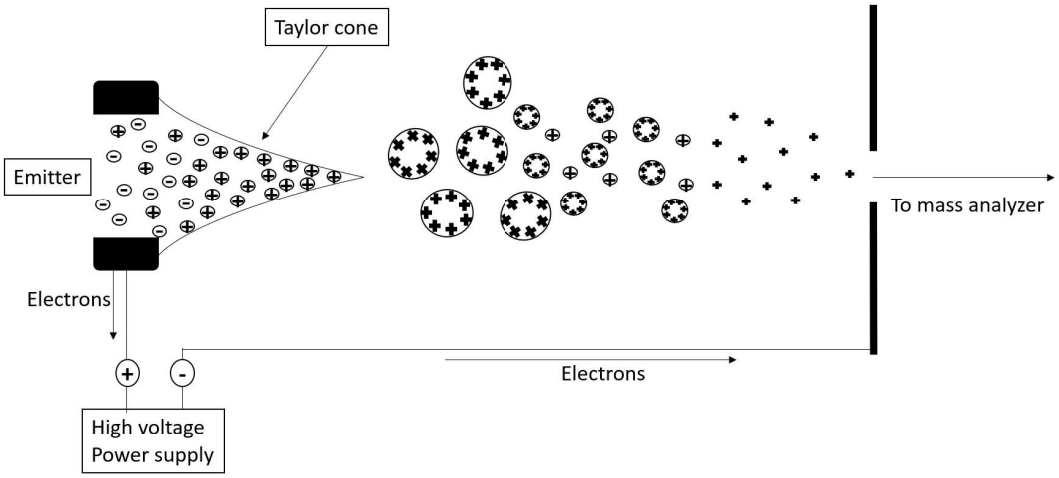

Figure 12: The formation of highly charged gas ions in ESI operated in positive mode. When applying a voltage between the solution and a counter electrode (MS) a taylor cone is formed at the tip of the emitter, which disperse the sample liquid into fine droplets. The solvent in the droplets evaporate and creates higher and higher charged droplets that eventually lead to observable gas ions. Adapted from [85]

.

Although the MS separates molecules based on their m/z, an increase in detection sensitivity and selectivity can be achieved by performing a separation step prior to the MS. A pre separation with LC also provides a retention time as a secondary identifier to the m/z, and could also reduce the chance of interfering compounds and matrix effects such as ion suppression or ion enhancement in complex matrices [86].

1.7. Liquid chromatography as an additional separation prior to detection

LC is a separation method that separates compounds transported through a column by a mobile phase (MP) based on the interaction between the molecules and a

stationary phase (SP) in the column. The interaction could be based on characteristics like compound hydrophobicity, polarity, or size, and determine the migration through the column and thus the elution and retention time (t_R) for each compound. Typical instrumentation setup consists of pump(s), injector (manual or automatic), column(s), a detector, and a data handling device [65] **(Figure 13)**.

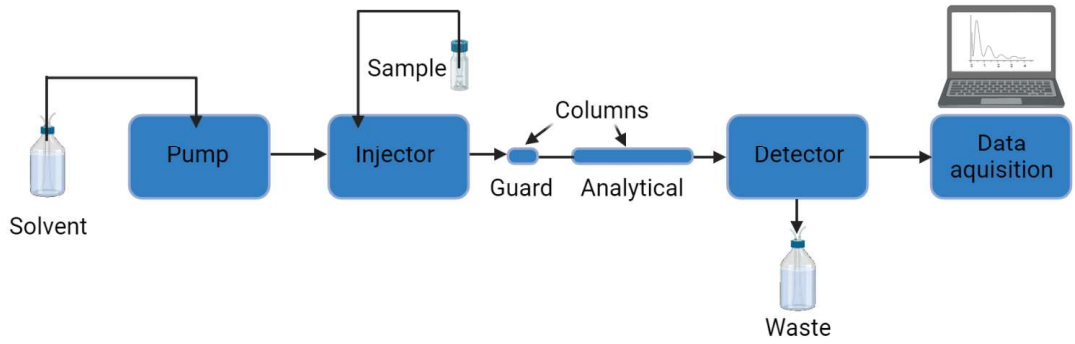

Figure 13: Schematic overview of a typical instrumentation setup for an LC system. The typical LC system consists of pump(s), an injector, column(s), a detector, and a data handling device.

The retention order of multiple compounds in a complex sample is determined by the separation principle applied. The separation principle is also chosen based on the characteristics of a compound. Reversed phase (RP) chromatography is a separation principle that has proved to be a most useful analytical tool, particularly for the analysis of biological samples [87, 88]

1.7.1. Reversed phase as a chromatographic separation principle

RP is a chromatographic principle that separates the analytes according to their increasing hydrophobicity [65] and is often the preferred choice when dealing with biological samples. In RP, the SP is a hydrophobic chain (often C18 or C8) chemically bonded to totally porous silica particles **(Figure 14)**, as apposed to normal phase where the SP is a hydrophilic phase [65]. This means that hydrophobic

compounds have stronger interaction with the SP, and hence longer retention time than more hydrophilic compounds on an RP column.

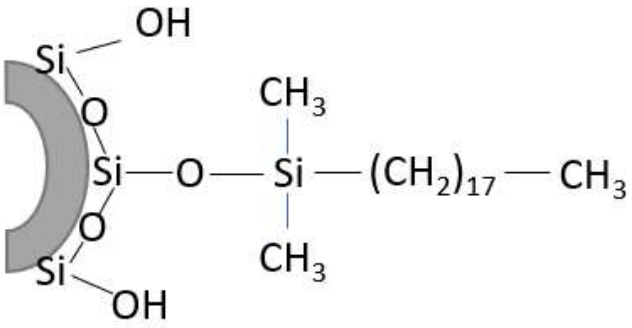

Figure 14: C_{18} RP SP. The SP in RP chromatography is a hydrophobic chain, here illustrated with C_{18}, chemically bonded to silica particles. Adapted from [65]

Throughout the years, new and modified RP chromatography SP have been introduced to provide more separation power, resulting in a market of over 600 brands of RP columns with modifications to end-capping, base deactivation, and polar embedding as some of the possible modifications [89, 90]. End-capping of an RP column by trimethylsilylation of the silica reduces silanol interaction, and protects the silica support from dissolution [91]. Base deactivation means that the column is specially prepared for analysis of basic drugs [92]. Polar embedding means that there is a polar group near the beginning of the carbon chain which can interact with the silanol group, and are typically used for highly aqueous MP [93]. The MP in RP is typically a mixture of an aqueous and organic solvent, with a buffer or acid for pH control. The amount of organic solvent is based on the hydrophobicity of the compounds, hence, gradient elution is often used when dealing with several compounds. To prevent the risk of the hydrophobic chains getting dewetted, resulting in lower loading capacity and reduced retention, the solvent gradient should have an organic compound mixed in from the start, 5 % acetonitrile or methanol are typically used [65]. To clean and prepare the column after each solvent gradi-

ent elution, the recommendations are that at least a 10 % increase in an organic solvent for 2-3 column volume should be performed before returning to the starting mixture. The column should then be allowed to re-equilibrate for a minimum of 10 column volumes before the next injection, although recent studies have shown that fewer column volumes can be sufficient [94]. When using a solvent gradient, the system has a mixer that can be placed before or after the pumps to mix the MPs. Each LC system has an individual dwell volume that is known as the volume between a mixing chamber and the column inlet [66] (p. 701). The dwell volume often leads to a time delay in the solvent gradient. An unretained analyte, or solvent molecule travels through the column in the shortest time possible (t_M), and can be used to adjust the retention time (t'_R) for a retained analyte [66] (p.612). In conventional LC, the inner diameter (ID) of the column is often between 3-5 mm, and the narrow bore version have IDs of approximately 2 mm [95]. Reduction of column ID to improve signal intensity, and reduced particle size for improved efficiency, can be beneficiary when coupled to a concentration sensitive detector (e.g. ESI-MS). [96, 97]

1.7.2. Chromatographic benefits from reducing the inner diameter of the column, and the size of the particles compared to the conventional standards

The use of a column with a smaller ID results in less radial dilution (**Figure 15**) which is a benefit when dealing with trace analyses and small sample volumes, which are often the case with biological samples. By using a column with a smaller ID, and keeping the same injection volume and analyte concentration, a stronger signal can be detected. The maximum volume that can be injected onto the column without extra band broadening depends on the elution strength of the sample solvent compared to that of the MP [65].

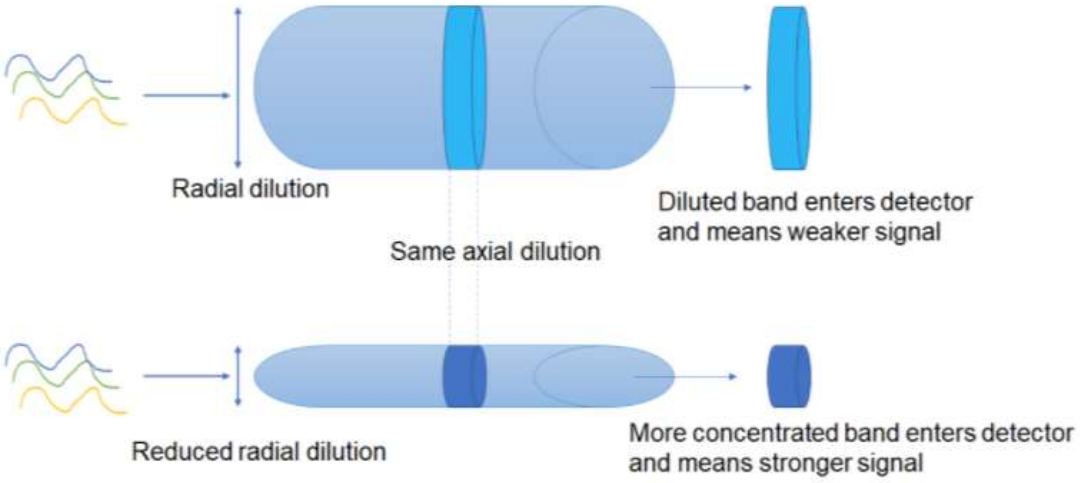

Figure 15: A column with a more narrow ID will give less radial dilution, resulting in a stronger signal. In a column with a larger ID, the radial dilution will give rise to a weaker signal than with the use of a more narrow ID. They will have the same axial dilution but a more concentrated band with less radial dilution giving rise to a stronger signal in the detector with the use of a more narrow ID.

The van Deemter equation **(Equation 4)** is widely used in the evaluation of column efficiency, and describes the plate height (H) as a function of linear velocity (μ) [65, 97],

$$H = A + \frac{B}{\mu} + C\mu \tag{4}$$

where A, B, and C represents eddy dispersion, longitudinal diffusion, and axial dispersion respectively [97]. The conventional columns are typically packed with 3 or 5 µm totally porous particles, but the use of sub 2 µm particles enables faster separations without loss of chromatographic efficiency **(Figure 16)** [65]. Reduced particle size will though, increase the back pressure in the system, and therefore requires pumps that can handle a higher pressure.

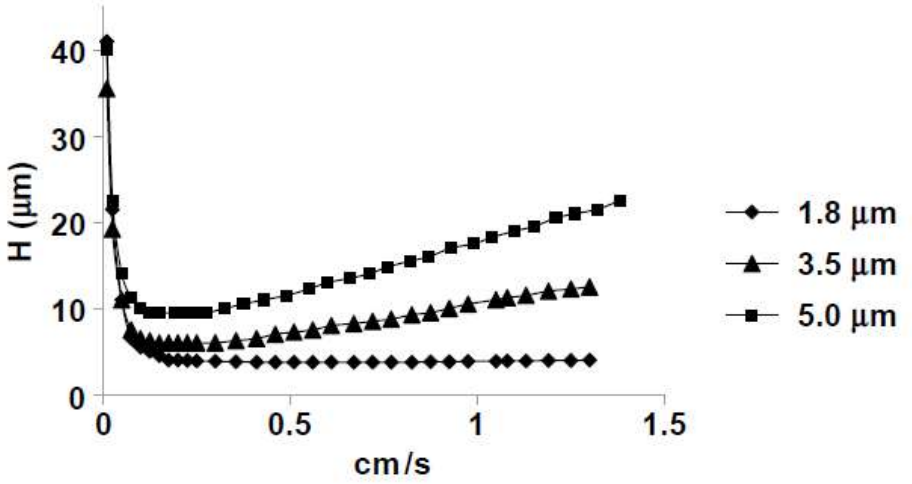

Figure 16: Van Deemter curve with plate height as a function of linear flow for particles of different sizes. A higher flow rate can be used with the same efficiency when using smaller sized particles. Copied from [65]

1.7.3. Special considerations regarding solvent when using ultraviolet and mass spectrometry detection

Special considerations regarding solvent when using both UV and MS detection, are the purity of the solvent. The purity of the solvent is of importance to minimize interferring compounds. With UV detection, the impurities compete with the analyte for light absorption and must be minimalized. The purification is often referred to as percent transmittance of light. A difference in absorbance between two MPs (e.g. water and organic) in UV detection with the use of a solvent gradient program, can cause some drift in the baseline [98]. With the use of LC-MS, the volatility is important because the ions go from liquid phase under high pressure to gas phase under high vacuum in an interface between the LC and the MS. The interface often contains the ionization source or the ionization source is the interface (like the ESI). The absence of impurities are also important for MS in addition to volatility, as they can cause matrix effects like ion suppression which

are rarely repeatable.

1.8. A look into previous reports for the three telltale drugs in metabolism studies

The three drugs phenacetin, tolbutamide, and fluoxetine, together with the metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine, have all previously been used in drug metabolism studies involving HLM and/or primary hepatocytes with both LC-UV [99–101] and LC-MS instruments [102–104]. The most commonly used organic MP was acetonitrile (ACN), and the chromatographic principle was RP with a C_8 or C_{18} RP column. The use of an internal standard (ISTD) to correct for imprecision due to the sample preparation and/or method performance [105] was also commonly occurring. The ion source used with LC-MS was most commonly ESI set to positive ionization, but tolbutamide and 4-hydroxytolbutamide were also detected from negative ionization [106].

1.9. Bioanalytical methods validation guidelines from The Food and Drug Administration

The united states Food and Drug Administration (FDA) is a government agency that regulates, inspects, and reviews production facilitys that makes products which are regulated by the agency. This includes drug manufacturers, and FDA approval can be crucial to companies that are involved in developing new drugs. An important aspect in the process of developing new drugs, are the measurement of drug concentrations in biological matrices, such as serum, plasma, blood, urine, and saliva. The European Medicines Agency (EMA) are the european counterpart to FDA in many ways. Both FDA and EMA have previously established guidelines and recommendations for validating bioanalytical methods to achieve reliable

results, although with some differences [107], but in 2019 they both published the M10 guidelines provided by the International Council for Harmonication (ICH) as the standard guideline. The guidelines describe how to perform a validation, and include selectivity, specificity, matrix effects, calibration curve range (LOQ, responce function), accuracy, precision, and analyte stability in matrix and stock solutions as elements that should be included in a full validation of a bioanalytical chromatographic method, [108, 109]. The acceptance criteria for each element are shown in **Table 1**.

Table 1: Overview of acceptance criteria for bioanalytical method validation. The acceptance criteria for validation of a bioanalytical method for selectivity, specificity, matrix effects, calibration curve and range, accuracy and precision, carry over, dilution integrity, and stability, as described by FDA and EMA. Adapted from [108, 109]

Validation element	Acceptance criteria
Selectivity	Responses detected and attributable to interfering components should not be more than 20 % of the analyte response at the LOQ and not more than 5 % of the ISTD response in the LOQ sample for each matrix.
Specificity	Responses detected and attributable to interfering components should not be more than 20 % of the analyte response at the LOQ and not more than 5 % of the ISTD response in the LOQ sample.
Matrix effects	The accuracy should be within ±15 % of the nominal concentration and the precision (relative standard deviation (% RSD)) should not be greater than 15 % in all individual matrix sources
Calibration curve and range	The accuracy of the back-calculated concentrations of each calibration standard should be within ±20 % of the nominal concentration at the LOQ and within ±15 % at all the other levels.
Accuracy and precision	The overall accuracy at each concentration level should be within ±15 % of the nominal concentration, except at the LOQ, where it should be within ±20 %. The precision (% RSD) of the concentrations determined at each level should not exceed 15 %, except at the LOQ, where it should not exceed 20 %.
Carry-over	Carry-over in the blank samples following the highest calibration standard should not be greater than 20 % of the analyte response at the LOQ and 5 % of the response for the ISTD.
Dilution integrity	The mean accuracy of the dilution should be within ±15 % of the nominal concentration and the precision (% RSD) should not exceed 15 %.
Stability	The mean concentration at each quality control level should be within ±15 % of the nominal concentration.

2. Aim of study

Organoids can potentially mimic human physiology to a greater extent than the traditional models (e.g. animals, HLMs, primary hepatocytes), but there is still a need for better characterization, and validated methods for determining their metabolizing properties in the work towards establishing organoids as models of human biology.

Thus, the aim of this study was to develop and validate an LC-MS method to measure if three telltale drugs for CYP activity, phenacetin, tolbutamide, and fluoxetine, would be metabolized into their conventional metabolites, acetaminophen, 4-hydroxytolbutamide, and norfluoxetine in PHS, and iPSC derived organoids. The more traditional model, HMLs, was to be used during development to establish the metabolism of the three drugs.

Initial testing, to achieve chromatographic knowledge about the three drugs and their metabolites, were to be done with the use of LC-UV, as it is considered to be a robust method. **Figure 17** is a graphical overview of the aim and workflow of this study.

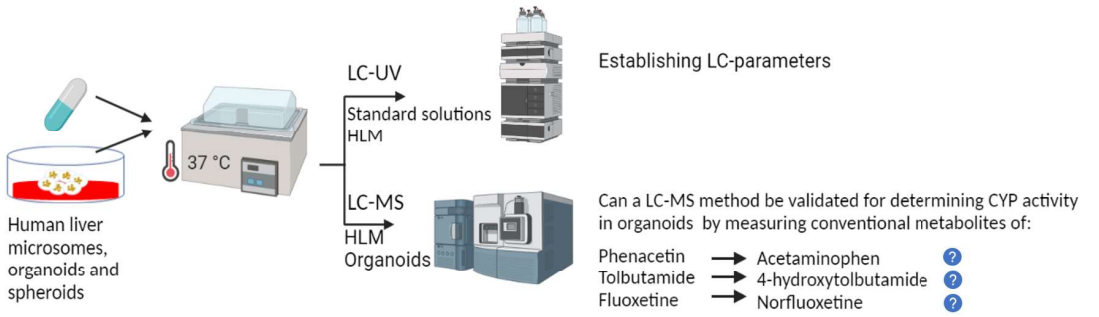

Figure 17: A graphical overview of the aim of study and workflow for this study. A bioanalytical LC-MS method was to be developed and validated for use in determining CYP activity in organoids and spheroids, with the help of HLM and LC-UV during developement.

3. Experimental

3.1. Chemicals

Two types of water were used, type 1 water that was obtained from a Milli-Q® Integral water purification system from Merck Millipore (Billerica, MA, USA), hereafter referred to as water, and HiPerSolv Chromanorm Water HPLC LC-MS grade (VWR chemicals, Radnor, PA, USA) hereafter referred to as MS graded water.

3.1.1. Chemicals used for liquid chromatography-ultra violet detection

HiPerSolv Chromanorm ACN for HPLC was from VWR chemicals, and LiChropur® formic acid (FA) (98-100%, HPLC) came from Merck (Darmstadt, Germany). Thiourea ($\geq$ 99.0%) came from Sigma-Aldrich (St. Louis, MO, USA).

3.1.2. Chemicals used for liquid chromatography- mass spectrometry

HiPersolv Chromanorm methanol (MeOH) for HPLC, LC-MS grade and HiPerSolv Chromanorm formic acid ($\geq$ 99%) were both purchased from VWR. Argon and nitrogen gas with a purity of 5.0 (99.999%) both came from Nippon Gases Norge AS (Oslo, Norge).

3.1.3. In vitro biotransformation models and reagents

Xtreme 200 pool human liver microsomes 0.5 mL 20 mg/mL and RapidStart™ NADPH Regenerating System were purchased from SEKISUI XenoTech (Kansas City, KS, USA), and Corning® Gentest™ NADPH regenerating System came from Corning (Glendale, AZ, USA). Sodium phosphate monobasic (NaH_2O_4P) ($\geq$ 99.0%) was purchased from Merck and sodium phosphate dibasic (Na_2HO_4P) ($\geq$ 98.0%) from Sigma-Aldrich. Cell medium with and without 2% FBS were provided

by Dr. Aleksandra Aizenshtadt at the Centre of Excellence-Hybrid Technology Hub (HUB centre).

3.1.4. Drugs, metabolites, and internal standards

Phenacetin ($\geq$ 98.0%, HPLC), acetaminophen (analytical standard), tolbutamide (analytical standard), 4-hydroxytolbutamide ($\geq$ 98.0%, HPLC), fluoxetine hydrochloride ($\geq$ 98.0%, HPLC) and norfluoxetine hydrochloride ($>$ 97.0%), were all purchased from Sigma-Aldrich. A second norfluoxetine hydrochloride standard ($\geq$ 98%), fluoxetine d5 hydrochloride ($\geq$ 99%), and tolbutamide d9 ($\geq$ 99%) were purchased from Cayman chemicals (Ann Arbor, MI, USA). Phenacetin d5 was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

3.1.5. Cell material

PHS, and iPSC derived organoids were prepared and incubated with drugs by Dr. Aleksandra Aizenshtadt at the HUB centre. The primary heaptic spheroids were prepared according to the protocol from Bell et al. [28], and the iPSC were differentiated toward hepatic spheroids using a modification of a published protocol by Si-Tayeb et al. [110]. The following brief descriptions of the preparations were provided by Dr. Aleksandra Aizenshtadt.

"Cryopreserved primary human hepatocytes (PHH) (Lonza, Lot HUM180201A) were thawed according to vendor protocol. PHH were plated into Elplasia ultra-low attachment plates with microwells (Corning) at the concentration 500 viable PHH/microwell. PHH aggregation was facilitated by short centrifugation (100 x g, 2 min). Spheroids were cultured in Williams E medium supplemented with 2 mM L-glutamine, 1% insulin-transferrin-selenium mix, 10% FBS, 0.1 µM dexamethasone for the first 3 days in culture. From day 4 half of the medium was daily exchanged for the serum-free media with the same formulation."

"Briefly, iPSC were differentiated toward definitive endoderm in IMDM/F12 media containing 1% lipids concentrate, 100 μg/ml transferrin, 3 μM CHIR99021, 50 nM PI-103 and 100 ng/ml activin A for 24h and 100 ng/ml activin A for subsequent 48h. The definitive endoderm cells were treated with 10ng/mL FGF2 and 20ng/mL BMP4 in IMDM/F12 medium supplemented with 1% N2 and 1% B27, then with 5 μM A8301, 20ng/mL HGF, 20ng/mL BMP4 for 3 more days and with 25ng/mL HGF, 1% DMSO for another day. At day 12 cells were detached and aggregated in the agarose U bottom microwells in the presence of 25ng/mL HGF, 0.1 μM dexamethasone, 1 μM forskolin, 0.5% ITS, 100 uM ascorbic acid-2 phosphate (AAP), 1% DMSO, 1% B27 and 1% N2. After formation of spheroids at day 13 media was replaced for William's E media, supplemented with 5% FBS, 20 ng/ml HGF and 10 ng/ml oncostatin M, 1% ITS, 100 μM AAP, 0.1 μM Dexamethasone and 0.5% DMSO. Spheroids were cultured in microwells in William's E media, supplemented with 1% ITS, 0.1 μM dexamethasone, 20 ng/ml oncostatin M, for another 10 days.

3.2. Small instruments and consumables

Weighing was done with an AT200 analytical balance from Mettler-Toledo (Greifensee, Switzerland). Centrifuge tubes (15 and 50 mL) with printed graduations and flat caps were from VWR, and Eppendorf safe lock and protein LoBind tubes (1.5 mL) were from Eppendorf AG (Hamburg, Germany). The two freezers that held -20 °C and -80 °C were from Elektrolux and Arctiko, respectively. For vortexing and pH adjustments, an IKA minishaker and an Accumet AE150 benchtop pH meter with an IKA topolino magnet stirrer, all from Fischer Scientific (Leicestershire, England) was used. Solvent evaporations were done with a Speed-vac^{TM} (Thermo Fisher Scientific, Waltham, MA, USA). Centrifugation was performed using a 5424R centrifuge from Eppendorf. A NanoDrop 2000 spectrophotometer

with the NanoDrop 2000/2000c software (version 1.4.2), both from Thermo Scientific, was used to determine λ max for the analytes. Incubations with microsomes were done in a PHMT Grant-bio Thermo-Shaker, or in a GLS 400 shaking water bath, or a SUB Aqua 5 plus water bath, all from Grant (Cambridge, UK). The incubations done by Dr. Aleksandra Aizenshtadt with the spheroids and organoids were done in a heating cabinet purchased from VWR. For direct injections on the MS to determine MRM transitions, a pump 11 Elite injection pump from Harward apparatus (Holliston, MA, USA) was used with a 250 µL Hamilton® (GR, Switzerland) gastight syringe. The autosampler vials were 0.3 mL PP transparent vials with blue cap from VWR. Degassing of MPs before use were done with a BRANSON (Brookfield, CT, USA) 5510 ultrasonic bath.

3.3. Instrumentation for liquid chromatography with ultraviolet detection

The LC-UV setup was from Agilent technologies (Santa Clara, CA, USA) and was equipped with Agilent ChemStation software (version B.04.03). The LC-UV setup consisted of an 1100 series G1379A micro vacuum degasser, an 1100 series G1376 capillary pump, an 1100 series Agilent/HP G1313A ALS autosampler, and an Agilent 1200 series G1365D MDW multi-wavelength detector. All analyses where performed with a solvent gradient and UV detection. See **Section 3.13.1** for details of the instrument settings.

3.4. Instrumentation for liquid chromatography with mass spectrometry detection

The LC-MS setup was purchased from Thermo Fischer and consisted of a Dionex ultimate 3000 pump, a Dionex 3000 column oven, and a Dionex 3000 autoinjector

equipped with an injector with a maximum injector volume of 25 µL, coupled to a Thermo TSQ Vantage mass spectrometer equipped with a heated electrospray ionization source. The pump, column oven and autoinjector were controlled by Chromeleon Xpresss software (version 6.80SR13), while the MS was controlled by Thermo TSQ Tune Master (version 2.3.0.1214SP3). Thermo Xcalibur Roadmap (version 2.2SP1.48) assumed control of both during runs with the established methods.

All analyses where performed with solvent gradient and ESI-MS (MRM-mode) detection. See **Section 3.13.2** for details of the instrument settings.

3.5. Reversed phase C_{18} columns

The column used for both LC-UV and LC-MS, was a 50 x 2.1 mm Polar Luna® C18 1.6 µm fully porous 100Å LC column from Phenomenex (CA, USA), with a 2.1 mm ID SecurityGuard Ultra UHPL C_{18} polar 1.6 µm fully porous guard column, also from Phenomenex. A 1SR1 Screen 1/16" OD, 1 micron filter from Vici (Houston, TX, USA) was placed prior to the guard column by using plastic tweezers to place the filter, before coupling the columns to the instruments.

3.6. Solutions

Unless otherwise stated throughout the rest of this thesis, stock solutions were the solutions made from dissolving purchased powder, and which were then stored for future use. Validation solutions were the solutions used for validating elements of the method, and calibration solutions were used for calibrating the method for in vitro metabolism studies. Working solutions were the solutions made fresh from stock solutions before each analysis, and were used for initial analyses before validation, or to prepare validation solutions, calibration solutions, or in vitro

samples. Incubation solutions are the inccubated in vitro samle solutions. Solution replicates were denoted n_s, and injection replicates n_i. A solution prepared with two or more analytes in the same solution will be referred to as an in-cocktail solution. All solutions were vortexed for 1 minute before storage or use.

3.6.1. Mobile phases for liquid chromatography analyses

The MPs were prepared in 1 L graduated laboratory bottles. For the LC-UV instrumentation, MP reservoir A (MP A) contained 3% ACN/0.1% FA (v/v) (in water), and MP reservoir B (MP B) contained 90% ACN/0.1% FA ($\geq$ 98-100%) (v/v) (in water). For the LC-MS instrumentation, MP A contained 0.1% FA in water, and MP B contained 0.1% FA ($\geq$ 99%) in MeOH. All mobile phases were degassed by ultrasonic bath for 10 min prior to use.

3.6.2. Analytes and internal standards stock solutions

The stock solutions were prepared in centrifuge tubes or volumetric flasks, before vortexing for 1 minute, and then divided into aliquots in Safe Lock tubes. Stock solutions of the analytes phenacetin, acetaminophen, tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine were prepared by diluting purchased powder in 100% ACN. The purchased powder of the ISTDs phenacetin d5, tolbutamide d9 was prepared the same way. Concentrations and storage conditions for the stock solutions are shown in **Table 2**. A more detailed description of the preparation of stock solutions can be found in **Section A.2**.

Table 2: Stock solutions for analytes and ISTDs The concentrations, and storage conditions for the analytes phenacetin, acetaminophen, tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine, and the ISTDs phenacetin d5, tolbutamide d9, and fluoxetine d5.

Analyte	Concentration (mg/mL)	Storage condition
Phenacetin	1	-20 °C
	5	-20 °C
Acetaminophen	1	-20 °C
Tolbutamide	1	-20 °C
	5	-20 °C
4-Hydroxytolbutamide	1	-20 °C
Fluoxetine	0.5	-20 °C
	1	-20 °C
	5	-20 °C
Norfluoxetine (first/old)	1	-20 °C
Norfluoxetine (second/new)	1	-80 °C
Phenacetin d5	1	-20 °C
Tolbutamide d9	1	-80 °C
Fluoxetine d5	1	-80 °C

3.6.3. Solutions used for matrices and in vitro metabolism studies

A 100 mM phosphate buffer was prepared by dissolving 1.4187 g Na_2HPO_4 in 100.0 mL water (100 mM final concentration) and 0.6002 g NaH_2HPO_4 in 50.0 mL water (100 mM final concentration) in volumetric flasks. The NaH_2PO_4 solution was then added to the Na_2HPO_4 solution dropwise until a pH 7.4 was reached, and then stored in a graduated laboratory bottle with cap in 2-8 °C.

A 1.1 M FA solution was prepared by transferring 212 µL 98-100 % concentrated FA to water in a 5 mL volumetric flask and diluting to the mark with water.

RapidStart NADPH regenerating system solution was prepared by dissolving the

purchased K5000 concentrate in 3.5 mL MS graded water and dividing it into aliquots of 350 µL in LoBind tubes which were stored at -20 °C.

A volume of 1.5 mL HLM matrix were prepared fresh before use in a Lobind tube by mixing 1125 µL 100 mM phosphate buffer, 150 µL NADPH regenerating system, 75 µL HLM 20 mg/mL, and 150 µL 1.1 M FA, and then centrifugation for 10 minutes on 14500 rpm at 4 °C before pipetting out the supernatant which were used as microsome matrix. For solutions without microsomes, 1200 µL 100 mM phosphate buffer was added instead of 1125 µL.

3.7. Solutions used for liquid chromatography-ultraviolet detection

3.7.1. Solutions for limit of quantitation

An in-cocktail working solution of 50 µg/mL with all the analytes present were prepared from diluting thawed stock solutions with phosphate buffer in Safe Lock tubes, and then further diluted to validation solutions of 25, 10, 1, 0.75, 0.5, 0.25, and 0.1 µg/mL with 0.1% FA in phosphate buffer.

3.7.2. Solutions for validation of linearity curve

Stock solutions of all the analytes were thawed and diluted with 0.1% FA in phosphate buffer to prepare six validation solutions (1-6). The consentrations of the validation solutions had a range of LOQ to 50 µg/mL for all the analytes (**Table 3**). A blank was prepared by adding 0.1 % FA to phosphate buffer.

Table 3: Validation solutions for LC-UV. Concentrations for phenacetin (P), acetaminophen (A), tolbutamide (T), 4-hydroytolbutamide (4HT), fluoxetine (F), and norfluoxetine (N) in the validation solutions 1-6.

Solution	1	2	3	4	5	6
P (µg/mL)	0.5	10	20	30	40	50
A (µg/mL)	0.75	10	20	30	40	50
T (µg/mL)	0.75	10	20	30	40	50
4HT (µg/mL)	0.5	10	20	30	40	50
F (µg/mL)	0.75	10	20	30	40	50
N (µg/mL)	0.5	10	20	30	40	50

A thorough description of how to prepare the validation solutions can be found in **Section A.2.1**.

3.8. Solutions used for liquid chromatography-mass spectrometry

3.8.1. Solutions for adapting and optimizing the solvent gradient and detection signal

Working solutions of 0.25 µg/mL fluoxetine, tolbutamide, 4-hydroxytolbutamide, phenacetin, and acetaminophen, and 0.5 µg/mL norfluoxetine were prepared separately and in-cocktail with 0.1% FA in phosphate buffer.

3.8.2. Solutions for determination of limit of quantitation

An in-cocktail working solution containing 250 µg/mL of each analyte was prepared in 100 mM phosphate buffer and 0.1 % FA. Validation solutions of 75, 50, 25, 10, 2.5, 1, and 0.75 ng/mL were then prepared by further diluting the working solution in 0.1% FA in phosphate buffer.

3.8.3. Solutions for initial investigation of matrix effects

Stock solutions of all the analytes were thawed and diluted in solvent, HLM matrix, cellmedium without FBS, and cell medium with FBS according to **Table 4** for low, middle, and high concentrations.

Table 4: The low, middle, and high concentrations used for initial investigation of matrix effects. The low, middle, and high concentrations of the drugs phenacetin (P), tolbutamide (T), and fluoxetine (F), and the metabolites acetaminophen (A), 4-hydroxytolbutamide (4HT), and norfluoxetine (N).

Analyte	P	T	F	A	4HT	N
Low (ng/mL)	8.3	20.8	41.7	8.3	20.8	41.7
Middle (ng/mL)	41.7	104.2	208.3	41.7	104.2	208.3
High (ng/mL)	83.3	208.3	416.7	83.3	208.3	416.7

3.8.4. Solutions used for validation of the linearity curve

The validation solutions were prepared in the same way for all four different matrices used in this thesis, solvent (0.1% FA in 50/50 water/MeOH (v/v)), cell medium with and without 2% FBS, and HLM matrix by thawing stock solutions and diluting with the matrix of choice. Six non-zero validation solutions (1-6) with increasing analyte concentration level were prepared in addition to a blank (matrix), and a zero (blank plus ISTD). The non-zero validation solutions had a range of LOQ to 500 ng/mL for fluoxetine and norfluoxetine, LOQ to 300 ng/mL for tolbutamide and 4-hydroxytolbutamide, and LOQ to 200 ng/ml for phenacetin and acetaminophen **(Table 5)**. In addition, 100 ng/mL of the ISTD phenacetin d5, tolbutamide d9, and fluoxetine d5 were present in the zero, and validation solution 1-6. The set with the solvent matrix was prepared in a mix of equal amount of MP A and MP B, and both of the sets consisting of cell medium, were prepared as described. For the HLM matrix, all dilutions to make the working

solutions prior to the validation solutions were done in phosphatebuffer, and the HLM matrix was only added as the matrix in the validation solutions.

Table 5: Validation solutions for LC-MS. Concentrations for phenacetin (P), acetaminophen (A), tolbutamide (T), 4-hydroytolbutamide (4HT), fluoxetine (F), and norfluoxetine (N) in the validation solutions 1-6.

Solution	1	2	3	4	5	6
P (ng/mL)	2.5	20	50	100	150	200
A (ng/mL)	2.5	20	50	100	150	200
T (ng/mL)	1	50	100	150	200	300
4HT (ng/mL)	1	50	100	150	200	300
F (ng/mL)	10	100	200	300	400	500
N (ng/mL)	50	100	200	300	400	500

The validation solutions were used as calibration solutions with HLM matrix for HLM metabolism studies, and in cell medium without FBS for PHS, and organoid studies. A thorough description of how to prepare the validation solutions, and thus the calibration solutions can be found in **Section A.2.2**.

3.9. Calculations

Calculation of matrix effects were done with slope numbers or the signal area as shown in **Equation 5**

$$\frac{\text{Matrix other than standard}}{\text{Standard matrix}} * 100\% \tag{5}$$

Back calculation of concentrations in solutions or samples were done with the Y equation of the linearity, validation, or calibration curve.

3.10. Determination of limit of quantitation

For both LC-MS and LC-UV, the LOQ was determined by analyzing decreasing analyte concentrations with the established gradient program. The LOQs were determined by visual evaluation of the chromatograms, in addition to calculating RSD to below ±20%. Replicates for the LC-UV were analysed with the solvent gradient program in **Table 7** for n_s=1, and n_i=6, and for the LC-MS they were analysed with the solvent gradient in **Table 9** for n_s=1, and n_i=3.

3.11. Establishing liquid chromatography parameters with ultraviolet detection

3.11.1. Determining lambda max for the drugs and their metabolites

Working solutions of 50 µg/mL were prepared in Safe Lock tubes separately for each drug and their metabolite in water from thawed stock solutions of 1 mg/mL (n_s=1). A volume of 2 µL of the working solution was pipetted directly onto the pedestal of the NanoDrop instrument and measured for each analyte with n_i=3. Water and ACN in the same volumetric ratio as the analytical solutions were used as a blank.

3.11.2. Establishing solvent gradient program and baseline separation of the drugs and their metabolites

A working solution with 50 µg/mL of all the analytes in-cocktail was prepared in a Safe Lock tube by diluting thawed stock solutions with water and 0.1% FA. In addition, one working solution for each analyte separately were prepared in the same way. A 20 min gradient with an increase in MP B from 0-90% was used as a starting point. Adjustments were done to the parameters flow, time, and % MP B (see **Section 3.13.1 Table 7** for the final solvent gradient program). Analyses

were performed for (n_s=1) and (n_i=5) for the analytes in-cocktail, and for each drug and metabolite separately.

3.11.3. Validation of the linearity curve

A validation of the linearity curve was performed by preparing a new set of validation solutions as described in **Section 3.7.2** for 5 concecutive days. Analyses were performed for n_s=1, and n_i=3 analyses with the solvent gradient program in **Table 7**.

3.12. Establishing a method with liquid chromatography-mass spectrometry

3.12.1. Direct injection to determine fragment ions

A working solution with 10 µg/mL of each analyte, and ISTD from thawed stock solutions was prepared in 50/50 ACN or MeOH/water or MS graded water(v/v) with 0.1% FA in Safe Lock tubes, and used for direct injection. The injection pump was set to 10 µL/min during the direct injection, and the MS tune settings were as shown in **Table 6**.

Table 6: MS tune setting for direct injection. Tune settings for temperatures, voltages, gas pressures, and gas consumption for direct injection.

Parameter	Setting
Ion transfer tube temp (°C)	280
Vaporizer temperature (°C)	50
Sheath gas pressure (psi)	10
Auxiliary gas flow (arb.)	5
Spray voltage (V)	+3000/-2500
Typical N_2 gas consumption (L/min)	1

3.12.2. Adapting and optimizing the solvent gradient and detection signal for liquid chromatography-mass spectrometry

Retention order and a solvent gradient program were determined by setting the gradient from LC-UV (**Table 7**) as a starting point, and adjustments were done to the parameters flow, time, and % MP B (see **Section 3.13.2 Table 9** for the solvent gradient program). Analyses were performed for n_s=1, and (n_i=3 for the analytes in-cocktail, and for each drug and metabolite separately with the working soutions described in **Section 3.8.1**.

3.12.3. Evaluation of matrix effects in solvent, cell medium with and without fetal bovine serum, and human liver microsome matrix

One set of validation solutions described in **Section 3.8.4**, were prepared for each of the four matrices used in this study, solvent, cell medium with and without serum, and the one for HLMs. The set with the solvent matrix was prepared in a mix of equal amount of MP A and MP B, and both of the sets consisting of cell medium, were prepared as described. For the HLM matrix, all dilutions to make the working solutions were done in phosphatebuffer, and the HLM matrix was only added as the matrix in the calibration solutions. Analyses were performed with n_s=1, and n_i=3 with the solvent gradient program in **Table 10**.

3.12.4. Validation with cell medium without serum as matrix

A validation of the methods linearity curve were done by preparing a set of the validation solutions described in **Section 3.8.4** in cell medium without FBS for three concecutive days. All validation solutions were repeated for n_s=1, and n_i=3. The run was repeated after 24 hours for each of the calibration solutions sets after being stored at 5 °C. The validation solutions for day 1 were made without FA, and a new set of validation solutions with FA were made from the same working

solutions after storing them at -20 °C overnight. The highest non-zero calibrator were followed by a blank, in addition to an isocratic wash with 80 % MeOH in 0.1 % FA, and a second blank.

3.13. Instrument settings

3.13.1. Instrument settings for liquid chromatography-Ultra violet detection

All the in vitro drug metabolism analyses on the LC-UV system were run at 225 nm, and the solvent gradient program, flow rate, MP composition, and pressure restriction on the system are depicted in **Table 7**. The injection volume was 1 µL.

Table 7: Solvent gradient program for the LC-UV system. The solvent gradient program used for the in vitro drug metabolism studies on the LC-UV system lasted for 11.01 minutes, and ranged from 10-47 %B, with a pressure restriction of 400 bar.

Time (min)	MP B (%)	Flow rate (µL/min)	Max pressure (bar)
0.00	10	300	400
0.50	20	300	400
4.50	23	300	400
9.50	37	300	400
10.00	47	300	400
11.00	47	300	400
11.01	10	300	400

3.13.2. Instrument settings for liquid chromatography-mass spectrometry

MS settings for solvent flow rate and column temperature, in addition to the tune settings vaporization temperature, cycle time, and spray voltage were varied according to **Table 8** during optimization of the signal area.

Table 8: Overview of the parameters and the variation to optimize the detected signal area intensity on the MS. Optimization of the detected signal on the LC-MS were performed by varying the parameters vaporization temperature, spray voltage, cycle time and MP flow.

Parameter	Variation	Intervals
Vaporization temperature (°C)	300-400	50
Spray voltage (+/- V)	2000-3500	500
Cycle time (s)	0.5-0.3	0.05 and 0.025
Column oven temperature (°C)	25 and 40	-
Flow rate (µL/min)	300-400	50

All the in vitro analyses on the LC-MS system were run with an injection volume of 1 µL, column oven temperature of 40 °C, and a solvent gradient program (**Table 9 or 10**), which included a cleaning, and re-equilibration step with 80 % MP B towards the end. The MS tune settings for temperatures, voltages, gas pressures, and cycle time are shown in **Table 11**, with MRM transitions for each drug, metabolite and internal standard shown in **Table 12**.

Table 9: Initial solvent gradient program for the LC-MS system. The initial solvent gradient program used for the in vitro metabolism studies on the LC-MS system lasted for 9 minutes, and ranged from 15-48% MP B in 6 min, with an additional cleaning step at 80% MP B for 1.01 min, followed by 1.39 min to re-equilibrate the column.

Time (min)	MF B (%)	Flow (µL/min)
0.00	15	300
0.50	25	300
2.50	30	300
3.000	45	300
6.00	48	300
6.50	80	300
7.60	80	300
7.61	15	300
9.00	15	300

Table 10: Final solvent gradient program for the LC-MS system. The solvent gradient program used for the in vitro metabolism studies on the LC-MS system lasted for 12.5 minutes, and ranged from 10-48% MP B, with an additional cleaning step at 80% MP B for 2.5 min, followed by 1.5 min to re-equilibrate the column.

Time (min)	MF B (%)	Flow (µL/min)
0.00	10	300
0.50	10	300
1.00	25	300
2.50	30	300
3.00	45	300
8.50	47	300
8.51	80	300
11.00	80	300
11.01	10	300
12.50	10	300

Table 11: Tune settings for the mass spectrometer. The optimized MS tune values for gas pressure, temperatures, voltages, and cycle time for the method on the LC-MS.

Parameter	Value	Parameter	Value
Capillary temperature (°C)	380	Auxillary gas pressure (arb.)	15
Vaporizer temperature (°C)	350	Sheat gas pressure (arb.)	45
Column oven temperature (°C)	40	Ion sweep gas pressure (arb.)	1
Spray voltage positive mode (V)	3500	Collision gas pressure (mTorr)	1
Spray voltage negative mode (V)	3500	Cycle time (s)	0.400

Table 12: Overview of the MRM transition settings. The MRM transitions were obtained by direct injection, and the optimal settings and transitions based on the + 1 precursor ions are listed.

Analyte	Monoisotopic mass	Precursor ion	Fragment ion	MRM collision energy	S-lens value	Polarity
Fluoxetine	309.1340 g/mol	310.135	148.048	6	64	+
			43.827	13	64	
Norfluoxetine	295.1184 g/mol	296.126	133.990	5	56	+
Tolbutamide	270.1038 g/mol	269.107	169.966	19	88	-
			106.002	33	88	
4-Hydroxytolbutamide	286.0987 g/mol	285.063	185.958	21	83	-
			103.962	33	83	
Phenacetin	179.0946 g/mol	180.107	138.073	16	66	+
			110.029	21	66	
Acetaminophen	151.0633 g/mol	152.092	110.031	15	63	+
			92.992	22	63	
Fluoxetine-d5	314.1654 g/mol	315.136	153.911	6	83	+
			43.911	14	83	
Tolbutamide-d9	279.1603 g/mol	278.116	169.978	22	68	-
			105.936	34	68	
Phenacetin-d5	184.1260 g/mol	185.124	143.023	16	57	+
			110.963	25	57	

3.14. In vitro drug metabolism studies with liver models

3.14.1. In vitro metabolism studies with human liver microsomes

The metabolism of the three drugs was studied both individually and in-cocktail, but the samples were prepared with the same following procedure in Protein LoBind or Safe Lock tubes. The three drugs were thawed and diluted in 100 mM phosphate buffer to their respective concentrations of 150 µg/mL phenacetin, 250 µg/mL tolbutamide and 350 µg/mL fluoxetine for LC-UV analysis, and 100 µM for the LC-MS. The three ISTDs were also thawed and diluted to a working solution of 1 µg/mL in phosphate buffer. The HMLs were thawed and diluted to 10 mg/mL in phosphate buffer right before sample preparation each time. Preincubation solutions were made by adding 10 µL 10 mg/mL HMLs (1 mg/mL final concentration) and 10 µL NADPH regenerating system to 70 µL of phosphate buffer, before they were incubated in a water bath or in a thermoshaker at 37 °C for 10 min. The metabolism was initiated by the addition of 10 µL of the working solution of the chosen drug (final concentration of 35 µg/mL fluoxetine, 25 µg/mL tolbutamide, and 15µg/mL phenacetin for LC-UV, and 10 µM of each for LC-MS) and resulted in a final sample volume of 100 µL. After the metabolism was initiated, the sample tubes were vortexed for 5 seconds and incubated in the water bath or thermo shaker for selected time periods (**Table 13**), with n_S=3 for all time periods. The shaking of the thermoshaker was set to 500 rpm, but for the water bath, a manual shaking of the incubated solutions was performed for 30 seconds every 10 min. The metabolism was terminated by the addition of 10 µL 1.1 M FA, and for the LC-MS, 10 µL 1 µg/mL of the three ISTD was also added. The sample tubes were then vortexed for 30 seconds and put on ice. Finally, the sample tubes were centrifuged at 4 °C at 14500 rpm for 10 min. The supernatant (analytical solution) was pipetted out into autosample vials, and kept on ice until analyzis,

or stored at -80 °C if not analyzed the same day. Control samples without HLMs (n_S=3), were prepared simultaneously and by the same procedure for each time period with 80 µL phosphate buffer instead of 70 µL to make up for the absence of HMLs. Validation solutions in HLM matrix were used as calibration solutions. **Figure 18** summarizes the workflow of the drug metabolism study in HLMs.

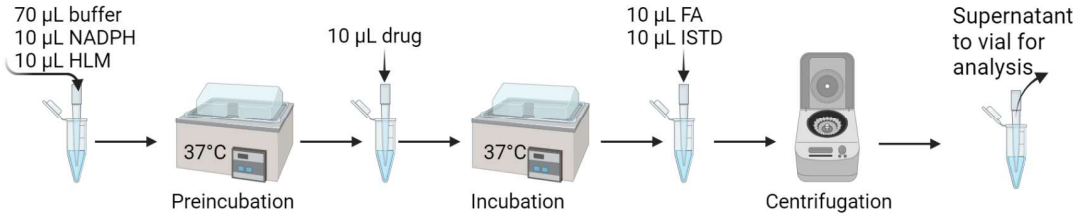

Figure 18: Illustration of the workflow of drug metabolism studies in HLMs. HLMs were incubated with NADP regenerating system, buffer and drugs at 37 °C for different time periods, before adding FA and ISTD, before centrifuging. The supernatant were then used for analysis. Made with BioRender (BioRender.com).

Table 13: Overview of incubation time periods. The three drugs had different selected time periods for period 1-3, but mutual time for time period 4 and 5.

Analyte	Time period 1	Time period 2	Time period 3	Time period 4	Time period 5
Phenacetin	0 min	20 min	40 min	150 min	240 min
Tolbutamide	0 min	60 min	90 min	150 min	240 min
Fluoxetine	0 min	40 min	75 min	150 min	240 min

The incubation with HLMs was done for each drug separately for the LC-UV system, but for the LC-MS system it was done both separately for each time point, and with an in-cocktail mix where solutions for all the time points (0, 20, 40, 60, 90, 120, 150, 240 min) were prepared.

3.14.2. In vitro metabolism studies with induced poluripotent stem cell derived organoids and primary hepatocyte spheroids

Solutions of phenacetin, tolbutamide, and fluoxetine at a concentration of 20 µM each, were prepared separately in cell medium without FBS (serum-free media), and was used for incubation with iPSC derived organoids (approximately 20 organoids) and PHSs. A volume of 50 µL 20 µM phenacetin, tolbutamide, or fluoxetine was added to 3 50 µL organoid culture samples in a 96 well plate, giving a total volume of 100 µL and a drug concentration of 10 µg/mL (n_S=3). Two 96 well plates were prepared in the same way, and then incubated at 37 °C for 6 and 24 hours. A volume of 10 µL 1.1 M FA was added to terminate the reaction and the 96-well was plate stored at -80 °C. On the day of analysis, the 96-well plate was thawed on ice and 10 µL of 10 µg/mL ISTD was added to the incubated solution. The incubated solution with ISTD were pipetted into LoBind or Safe Lock tubes and centrifuged in 4 °C for 10 minutes at 14500 rpm, before the supernatant was transferred to autosample vials. The samples were then diluted 10 times by pipetting 10 µL of the sample into 90 µL of the cell medium matrix with 0.1 % FA in new autosampler vials and then analyzed. Three negative controls were prepared alongside each drug and incubation time. Validation solutions in cell medium without FBS were used as calibration solutions. **Figure 19** summarizes the workflow of the drug metabolism study in PHS and iPSC derived organoids.

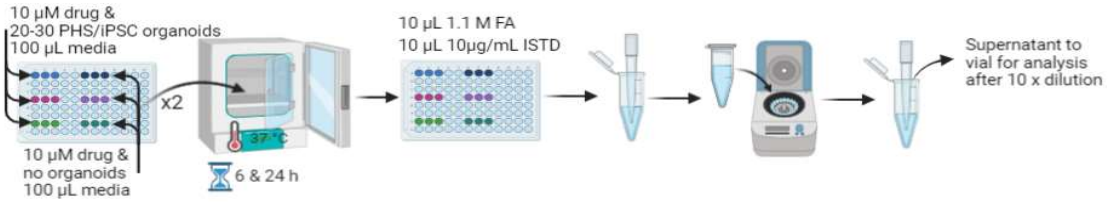

Figure 19: Illustration of the workflow of drug metabolism studies with PHS and iPSC derived organoids. The organoids were incubated for 6 and 24 hours with, and without drugs, before adding FA and ISTD, before centrifuging. The supernatant was used for analysis after 10 x dilution. Made with BioRender (BioRender.com).

4. Results and discussion

The motivation for this study was to determine if drug metabolism by CYP enzymes in well established models like the HLMs, also could be achieved by using a new and more complex model, the liver organoids. Due to their larger complexity, they may be able to mimic human drug response to a greater extent than the current in vitro models, but there is still a shortage of robust protocols and validation of the organoids as an in vitro model. For determining CYP activity in liver organoids, a bioanalytical LC-ESI-TQ-MS (hereafter referred to as LC-MS) method was developed and validated by measuring the metabolites of the drugs phenacetin, tolbutamide, and fluoxetine, i.e. acetaminophen, 4-hydroxytolbutamide, and acetaminophen, respectively. Initial analyses were based on metabolism in human liver microsomes by the use of LC-UV. When the LC-MS method was established and validated, the HLM samples were replaced with samples containing drugs incubated with organoids. The results and discussion section is for that reason divided into two subsections, where the first subsection **(4.1)** covers the work with LC-UV and the second subsection **(4.2)** covers the work with LC-MS. Analyte(s) in this section include the three drugs fluoxetine, tolbutamide, and the three metabolites norfluoxetine, 4-hydroxytolbutamide, and acetaminophen. Sections, figures, and tables denoted **A** followed by a number can be found in the appendix, together with all additional raw data used for calculations and graphical figures.

4.1. Initial chromatography of the analytes with liquid chromatography-ultraviolet detection

To establish a method for the analytes by the RP-LC-UV system, the λ max and a solvent gradient had to be determined and optimized, respectively.

4.1.1. Determining lambda max for ultraviolet detection

To find the most suitable wavelengths for detection of the analytes with the LC-UV system, the λ max in the UV range was determined with NanoDrop 2000. The average λ max values are shown in **Table 14**, together with literature values from Cayman Chemicals (CC).

Table 14: The λ max for the analytes. Table 14 shows the λ max values for phenacetin (P), acetaminophen (A), tolbutamide (T), 4hydroxytolbutamide (4HT), fluoxetine (F), and norfluoxetine (N) that were measured with the NanoDrop 2000, in addition to literature values retrieved from Cayman Chemicals (CC). Fluoxetine and norfluoxetine had several literature values for λ max.

Analyte	P	A	T	4HT	F	N
Nanodrop	245 nm	242 nm	227 nm	227 nm	225 nm	226 nm
CC	250 nm	249 nm	229 nm	229 nm	225 nm	225 nm
					226 nm	227 nm
					268 nm	264 nm
					276 nm	276 nm

Although **Table 14** shows a deviation between the measured values and the literature, the measured λ max values for the analytes are mostly in the same area as the literature value. It is commonly known that deviation in λ max can be caused by variation of pH [67], solvent [68], temperature [69], and concentration [70], and these parameters for the literature value were not known. Therefore, the wavelengths measured with the NanoDrop were used for further analyses.

During the work with the optimal solvent gradient and repeatability of the retention time (Section 4.1.3), there were noticed that the highest signal area intensity seemed to not correspond with the measured lambda max. Hence the area for all the used wavelengths was compared **(Figure 20)**.

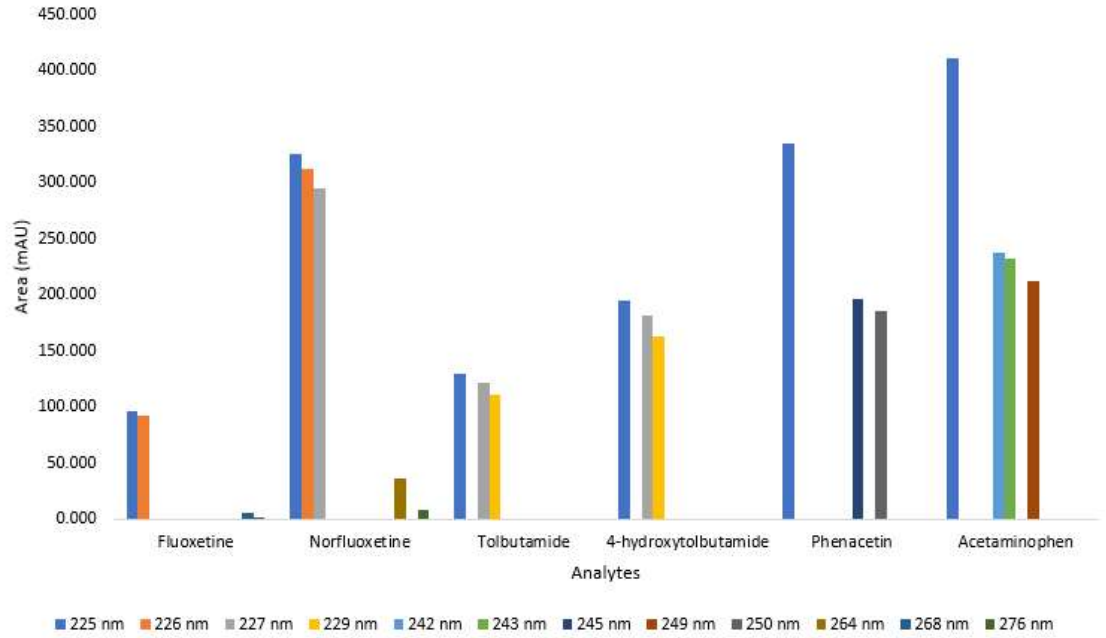

Figure 20: Overview over the signal area at different wavelenghts for all the analytes. A comparison of the signal area for all analytes at all the used wavelenghts shows that 225 nm gives the highest signal for both drugs and metabolites.

Figure 20 shows that 225 nm gives the largest signal area for every analyte on this system. A detection criteria of 225 nm should still give minimal absorbance of UV light in type 1 water, and HPLC graded ACN (transmittance $>$ 97 % according to VWR). The FA has a lower transmittance (transmittance $>$20% according to VWR) though, but since FA was present in the same 0.1% volume regardless of MP composition, FA would impact the baseline consistently at the same wavelength throughout the gradient. The use of only one wavelength would simplify the method, and because thiourea only was used as a t_M indicator and also had absorbance at 225, it was determined to use 225 nm for all analytes.

In summary

The measured λ max with NanoDrop 2000/2000c were determined to be 245, 242, 227, 227,225, 226 nm for phenacetin, acetaminophen, tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine, respectively. However, a second look at the λ max revealed that a wavelength of 225 nm gave the most intense signal area for all the analytes for this specific LC-UV system.

4.1.2. Chosing a column and mobile phases

To determine the optimal MP, and SP for the column, the hydrophobicity, and pH characteristics of the analytes were taken into consideration. All the analytes used in this study are organic, thus the primary choice was an RP SP column. RP is well known to suit separation of organic compounds, and to have high effectivity and repeatability. An RP analytical column successfully used in-house for previous analyses was a 50 x 2.1 mm Polar Luna® C_{18} 1.6 µm fully porous 100Å LC column from Phenomenex, thus it was decided to try that column. Considering the matrices that were used for this study, a guard column and a filter would protect the analytical column from organic matter (e.g. HLM, proteins). Hence, both a C_{18} guard column and a 1 micron filter were placed prior to the analytical column. As for MP, water with an organic modifier are the typical MP for RP. The first two go to organic phases were ACN, and MeOH. Both are compatible with both UV, and MS detection, but ACN are less viscous, thus generates a lower backpressure than MeOH. In addition, the ACN has a higher light transmittance (90% according to VWR) for lower wavelengths (220 nm) than MeOH (65% according to VWR), making ACN more suitable for UV detection. As a pH control, FA was chosen because 0.1% FA is a commonly used compound for pH control, and FA was already present in the incubation solutions as it was used to terminate the metabolic

reaction. The literature also confirmed that the most common MP, and SP used for metabolism studies with LC-UV for the three drugs and metabolites, were C_{18} as an SP, and water with ACN as the organic modifier for the MP [99–101].

In summary

RP was chosen as the chromatographic principle, with a C18 SP in the column, and 0.1% FA in water with ACN as an organic modifier were chosen as the MP.

4.1.3. Establishing baseline separation of the three drugs and metabolites

After the MP and column with SP were chosen, a baseline separation of all the analytes needed to be established. To have the possibility to perform in-cocktail analyses, it was beneficial to establish a baseline separation of all the analytes that could be used. The initial testing was done with LC-UV because it is a robust method to determine retention time and establish baseline separation for multiple analytes. The baseline separation of 50 µg/mL phenacetin, tolbutamide, fluoxetine, and their metabolites acetaminophen, 4-hydroxytolbutamide on the LC-UV system are shown in **Figure 21**. A concentration of 25 µg/mL thiourea was used as t_M indicator here, and for the rest of the work with LC-UV. A gradient dwell time of approximately 4 min was observed, thus, the total run time for each analysis including re-equilibration of the column was set to be 17 minutes.

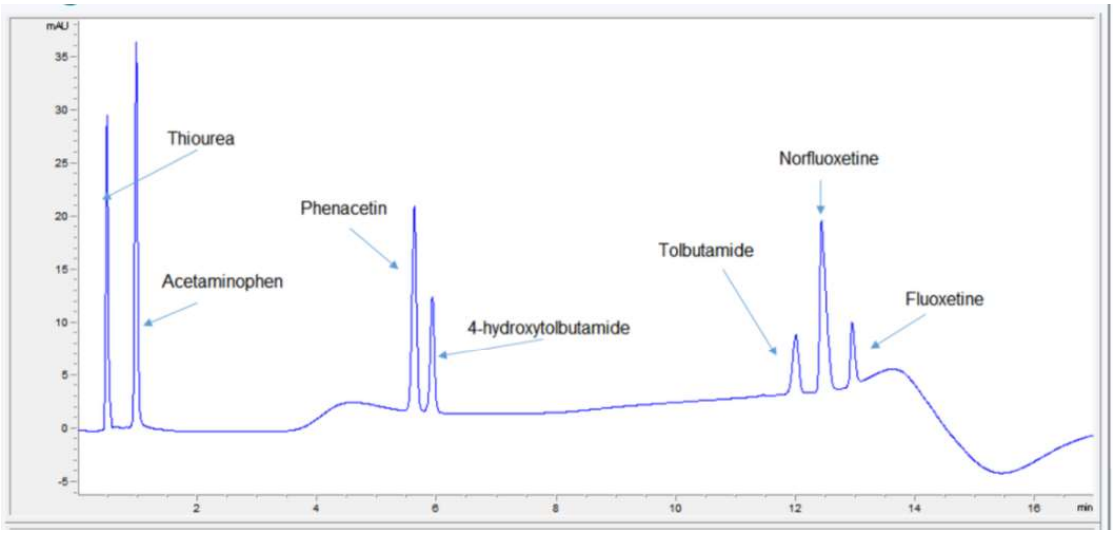

Figure 21: LC-UV chromatogram for 50 µg/mL of all six analytes and 25 µg/mL thioruea. The eluting order from the last to first where, fluoxetine, norfluoxetine, tolbutamide, 4-hydroxytolbutamide, phenacetin, and acetaminophen, with thiourea as the t_M indicator was aquired with a gradient ranging from 10-47% MP B in 11 min. A 50 x 2.1 mm Polar Luna® C18 1.6 µm fully porous 100Å column was used, and the flow rate was 0.3 mL/min. The wavelength were set to 225 nm.

Figure 21 shows that the elution order was as expected with the more hydrophobic drugs eluting after their respective metabolite. The slight increase or drift in the baseline is caused by ACN having a slightly stronger UV absorbance at 225 nm than water [98]. Due to the possibility of interactions between the analytes in-cocktail that could affect the retention time, the individual and in-cocktail retention time were compared. The adjusted average retention times (t'_R) with the standard deviation are shown in **Table 15**.

Table 15: Adjusted retention times (t'_R) for each of the six analytes individually and in-cocktail. The adjusted retention time for each analyte measured both individually and in-cocktail to determine possible effects of interactions. The standard deviations are shown in the parentheses.

Analyte	Separately t'_R	In coctail t'_R	Difference
Fluoxetine	12.281 (± 0.034) min	12.253 (± 0.009) min	0.028 min
Norfluoxetine	11.840 (± 0.009) min	11.713 (± 0.009) min	0.127 min
Tolbutamide	11.485 (± 0.004) min	11.445 (± 0.009) min	0.040 min
4-hydroxytolbutamide	5.395 (± 0.003) min	5.381 (± 0.014) min	0.014 min
Phenacetin	5.099 (± 0.010) min	5.075 (± 0.016) min	0.024 min
Acetaminophen	0.455 (± 0.002) min	0.457 (± 0.014) min	0.002 min

Table 15 shows a difference in retention time of ≤0.1 minutes for all the analytes, which gives a relative standard deviation (RSD %) of ≤ 0.8%, which was determined to be acceptable, thus, in-cocktail solutions could be used.

In summary

Baseline separation of all 6 analytes in-cocktail was established with LC-UV.

4.1.4. Determination of limit of quantitation and initial validation of the linearity curve

The LOQs for the LC-UV system were determined by analyzing in-cocktail validation solutions with concentrations for all the analytes of 25, 10, 1, 0.75, 0.5, 0.25, and 0.1 µg/mL (n_s=1, n_i=6). The LOQs were determined to be 0.75 µg/mL for acetaminophen, tolbutamide, and fluoxetine, and 0.5 µg/mL for phenacetin, 4-hydroxytolbutamide, and norfluoxetine based on visual evaluation of the LC-UV chromatograms (S/N ≥10) (**Figure 22**), and an RSD below 20% **(Section B.1.3, Table B.3)**.

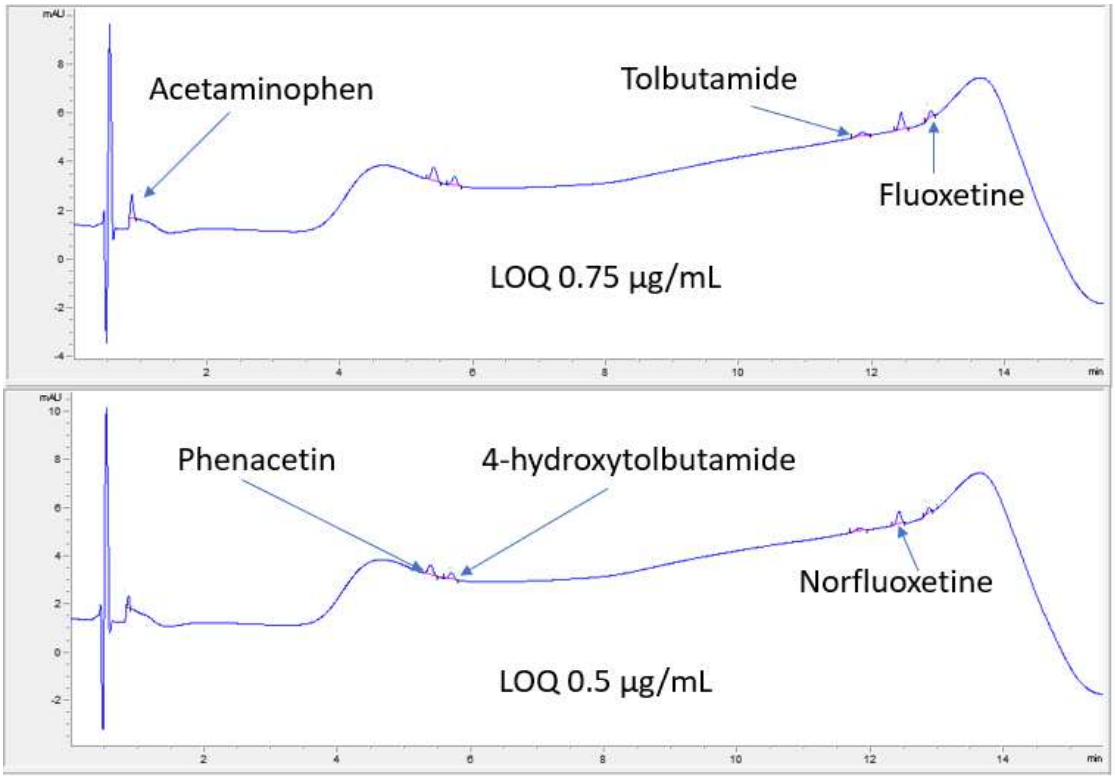

Figure 22: LC-UV chromatogram of the analytes at LOQ concentration for all the analytes at the LC-UV system. The chromatogram was achieved with the same conditions as in **Figure 21**

The LOQ and an additional five solutions with analyte concentration above LOQ (10, 20, 30, 40, and 50 µg/mL) constituted the validation solutions for each analyte. The validation solution with the highest concentration gave at least 100 times the signal area of the LOQ.

An evaluation of repeatability was performed for 5 consecutive days, by evaluating linearity (R^2), retention time, and back calculation. The combined linearity curves are shown in **Figure 23**.

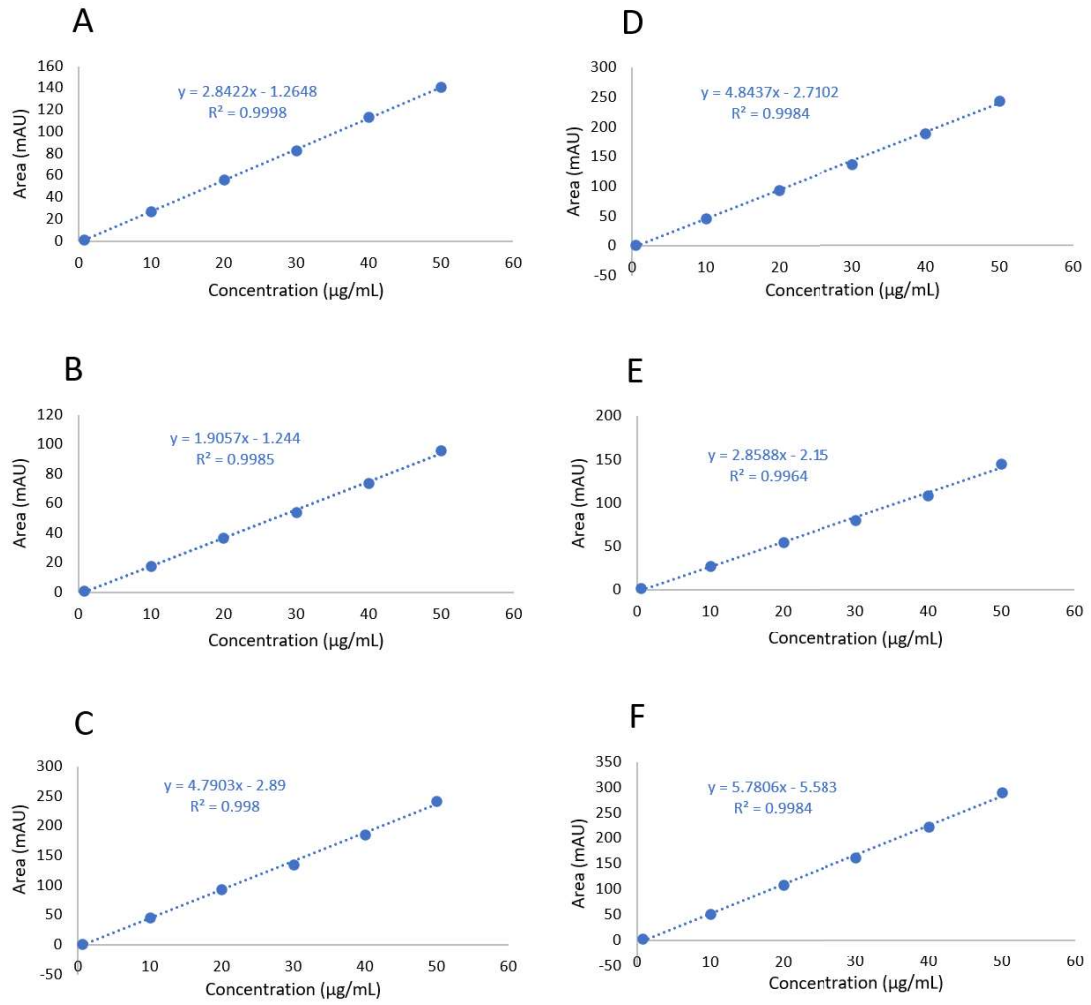

Figure 23: The combined linearity curve for each analyte over 5 consecutive days. Average area over 5 concecutive days as a function of concentration of fluoxetine (A), tolbutamide (B), phenacetin (C), norfluoxetine (D), 4-hydroxytolbutamide (E), and acetaminophen (F).

Figure 23 shows an R^2 above 0.997 for the linearity curve for all the analytes, and they had an RSD below the acceptance criteria of ±15% for each validation solution above LOQ. The acceptance criteria for LOQ is below 20%, and for each separate day, the LOQ RSD was below 20% for all three drugs and metabolites, but the LOQ turned out to not be repeatable inter day for all the analytes. Fluoxetine, norfluoxetine, tolbutamide, and acetaminophen all had an RSD above

20% for the LOQ. The retention time had an RSD below 1% for all analytes except acetaminophen, which had an RSD of 2.3%. Acetaminophen eluted close to thiourea and together with a solvent peak that varied, which made it difficult to accurately integrate the signal area. Consequently, the retention time could be affected, which could be the reason the retention time had greater variation than the 5 other analytes, although still within an acceptable RSD. A back calculation of the established linearity curves was within the acceptance criteria of ± 15% for all the validation solutions above LOQ, except for norfluoxetine that had an RSD of 33-41%. However, when back calculating each of the calibration curve for each separate day for norfluoxetine, the RSD was within the acceptance criteria for all the validation solutions above LOQ for norfluoxetine as well, which could indicate trouble with the stability of the stock solution. None of the back calculations of LOQ were within the acceptance criteria of ± 20% (ranged between 40-57% RSD), except for fluoxetine who had an RSD for LOQ of 9.5%. To make the LOQ solution, the pipetted volume was as low as 1.25 µL, which can give rise to variations. To deal with this issue, a larger volume (>10 µL) could be used to make the LOQ validation solutions, or the concentration of LOQ could be increased.

Previous reports found for LC-UV methods for all three drugs and metabolites [100, 111–114], revealed LOQs down to 2-5 ng/mL, which is better than for the in-house LC-UV system used in this thesis. However, both the injection volumes, columns, and instrumentation varied from that of the in-house system. The linearity was within $R^2 > 0.98$, indicating the possibility of a large range for the linearity within acceptable R^2.

In summary

The LOQ was determined to be 0.75 µg/mL for fluoxetine, tolbutamide, and acetaminophen, and 0.5 µg/mL for norfluoxetine, 4-hydroxytolbutamide, and phenacetin, and a linearity curve with a range from LOQ to 50 µg/mL was validated in regards to repeatability of retention time and signal area within the acceptance criteria, except for repeatability of the signal area inter day of the LOQ.

4.1.5. Metabolism studies with human liver microsomes for ultra violet detection

The developed LC-UV method was used for measuring the signal area of the metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine from the metabolism of 15 µg/mL phenacetin, 25 µg/mL tolbutamide, and 35 µg/mL fluoxetine after incubation with 0.5 mg/mL HLMs. Calibration curves were not prepared initially because the first objective was to determine if there were detectable metabolites present in the incubated samples. Initial analyses with HLMs were characterized by poor metabolism for phenacetin and tolbutamide, and totally absent for fluoxetine. After looking further into it and input by the Department of Forensic Sciences (DFS) laboratory which has experienced with HLM studies, a suggestion was that there might be too much organic solvent in the incubation solution, in addition to a low concentration of HML. An organic composition above 1 % is known to inhibit the activity of the microsomes, and acetonitrile is often used to terminate the metabolism. The preferred amount of organic solvent in the incubation is below 0.2 %, and preferably none at all. The NADPH regeneration system was also investigated by comparing it to the NADPH system the DFS lab routinely used, but the difference was negligible **(Section A.3)**. A

higher concentration of the stock solutions would result in a lower concentration of organic solvent in the incubation solutions. Hence, new stock solutions of 5 mg/mL (which would result in an organic contribution of less than 0.7 %) of the three drug analytes where made, and used for the rest of the study with HLM on LC-UV. In addition, the HLM concentration in the incubation solutions was increased to 1 mg/mL. The two first time periods for fluoxetine (40, 75 min), and tolbutamide (60, 90 min), and the first time period (20 min) for phenacetin were based on previous reports [99, 101, 115–117], but because of the low detectable initial metabolism, the incubation was also expanded to a total of 4 time periods for each drug. The last time point at 240 minutes was chosen because microsomes are known to have acceptable metabolizing activity for 4 hours incubation. The 4 hour incubations with all time points are shown in **Figure 24, 25, and 26**. Negative control samples without HLMs were prepared and measured in parallel for each analyte at each time period.

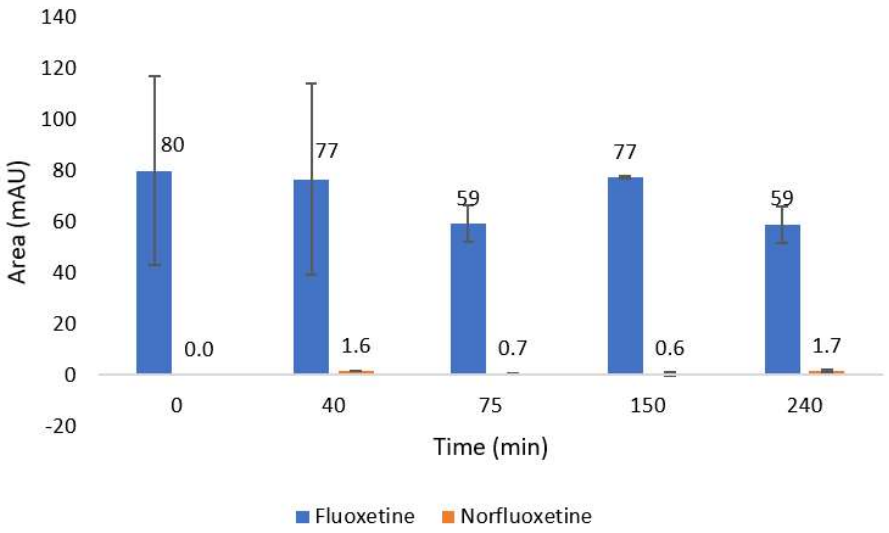

Figure 24: The signal area of fluoxetine and its metabolite norfluoxetine after 35 µg/mL fluoxetine incubation for 0, 40, 75, 150, and 240 min in 1 mg/mL HLM. The areas are shown as mean (n_s=3, n_i=1), with the error bars showing the positive and negative standard deviation. The areas were measured under the same conditions as in **Figure 21**.

After the initial corrections of amount of organic solvent, metabolism of fluoxetine into norfluoxetine was detected. **Figure 24** shows a small increase in signal area for norfluoxetine over time, but the signal area was below the LOQ for the LC-UV system. The negative controls prepared alongside the incubated solutions with HLMs showed no detectable signal for norfluoxetine.

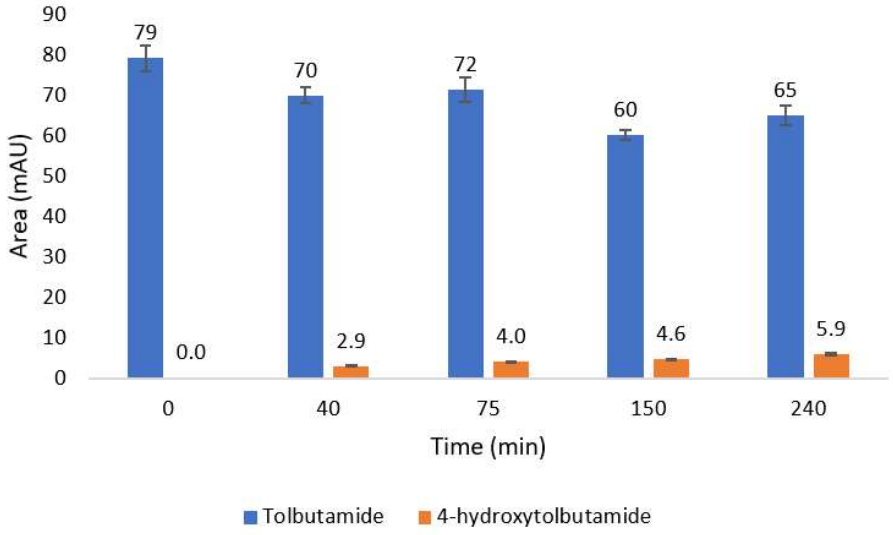

Figure 25: The signal area of tolbutamide and its metabolite 4-hydroxytolbutamide after 0, 40, 75, 150, and 240 min of 25 µg/mL tolbutamide incubation in 1 mg/mL HLM. The areas are shown as mean (n_s=3, n_i=1), with the error bars showing the positive and negative standard deviation. The areas were measured under the same conditions as in **Figure 21**.

Tolbutamide showed metabolism to a greater extent than fluoxetine, but **Figure 25** shows a slow increase for the last 3 hours of incubation compared to the first hour. Previous reports only describe incubation at one single time point (60 or 90 minutes [116, 117]) for in vitro studies with HLMs, hence it was uncertain if this was the norm for HLM metabolism of tolbutamide or if the NADPH system loose activity earlier than the commonly known 4 hours.

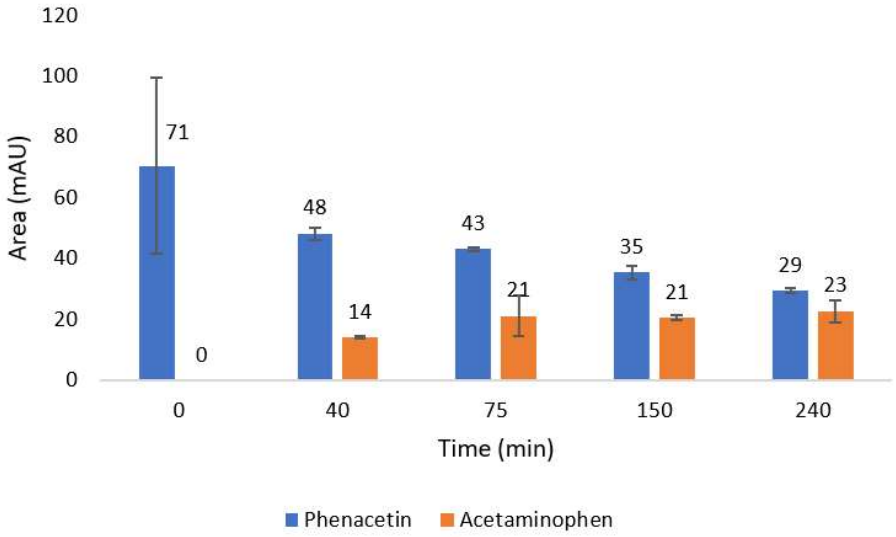

Figure 26: The signal area of phenacetin and its metabolite acetaminophen after 0, 40, 75, 150, and 240 min of 15 μg/mL phenacetin incubation in 1 mg/mL HLM. The areas are shown as mean (n_s=3, n_i=1), with the error bars showing the positive and negative standard deviation. The areas were measured under the same conditions as in **Figure 21**.

Figure 26 shows the metabolism of phenacetin to acetaminophen with an increase through the whole time period, however with the same flattening trend for the last three hours as tolbutamide showed. The signals are well within the signal area for LOQ and could be used to quantify CYP activity during incubation with HLMs with the LC-UV method.

The analysis of the HLM incubation with fluoxetine, tolbutamide, and phenacetin for 4 hours with HLMs show that the LC-UV method lack the sensitivity that is needed to detect and quantify all three metabolites. In regards to the column, there was room for an increase in injection volume to 5% of the column volume, but it was decided to keep it to 1 μL to have the possibility to try a column with a smaller ID with the same system. No ISTDs were used with the LC-UV, thus concentrations of the metabolite were not calculated. With a more sensitive detector, a more miniaturized system, or a larger injection volume, it might be possible to

get quantifiable detection of the metabolites after drug incubation on the LC-UV system.

In summary

The HLM metabolized both fluoxetine, tolbutamide and phenacetin into detectable amounts of their main metabolites norfluoxetine, 4-hydroxytolbutamide, and acetaminophen, however, the signal area for norfluoxetine were below that of the LOQ of the LC-UV method.

4.2. Development of a liquid chromatography-mass spectrometry method for determination of the analytes

Seeing that the LC-UV method did not have good enough sensitivity for detecting the metabolites after drug incubation with HLM, an LC-MS method was developed using the same column as the LC-UV method. The use of an MS as a detection method also provides the opportunity to use deuterated ISTDs for improved quantification. Fluoxetine d5, tolbutamide d9, and phenacetin d5 were chosen as ISTD, and the MS was to be used in MRM mode.

4.2.1. Direct injection to determine fragment ions

To detect the drugs, metabolites, and ISTDs with the MS in MRM mode, a direct injection to determine their two most abundant fragment ions (seen in **Section 3.13.2, Table 12**) were performed. The fragment ion with the highest abundance was to be used as a quantifier, and the second best as a qualifier. Norfluoxetine, however, proved to be a challenge. Although presenting as one of the two strongest signals with the LC-UV, no fragment ions were detected at acceptable levels at the MS. Even when trying direct injection with 100% MP B (0.1% FA in 90% ACN) as

solvent, no signal could be detected in sufficient abundance. Hence it was decided to try to change to MeOH as anorganic modifier. With 0.1% FA in 100% MeOH, one fragment ion at m/z 134 was detected at an acceptable level and confirmed by literature [53]. The norfluoxetine was also very old, so there was the possibility of degradation. A new standard was ordered, and MeOH was chosen to be the MP B on the LC-MS system. Due to the change of MP B, the method from the LC-UV system was not easily transferred to the LC-MS, as the two solvents are well known to have different chromatographic properties [118]. The solvent gradient program from the LC-UV system was set as a starting point and adjustments in flow rate and MP composition were done to achieve separation. However, with the use of an MS, it is not necessary to have baseline separation of the analytes as long as the masses are different, which was the case for all the analytes in this thesis. The objective was a balance between separation, interferences, and the possibility of a shorter analysis time than for the LC-UV system (seen in **Section 3.13.1, Table 7**).

In summary

To summarize, two fragment ions were determined for each analyte and ISTD, except for norfluoxetine which required a change in MP B from ACN to MeOH to detect a single fragment ion of accepted abundance. The change from ACN to MeOH required a new optimization of the elution conditions.

4.2.2. Adjusting solvent gradient

The 9 min chromatographic separation with the gradient program (described in **Section 3.13.2, Table 9**) is shown, with the new norfluoxetine, in **Figure 27**. The difference in the signal area obtained for the new and old norfluoxetine with

the same assumed concentration of 0.5 µg/mL are shown in **Figure 28**.

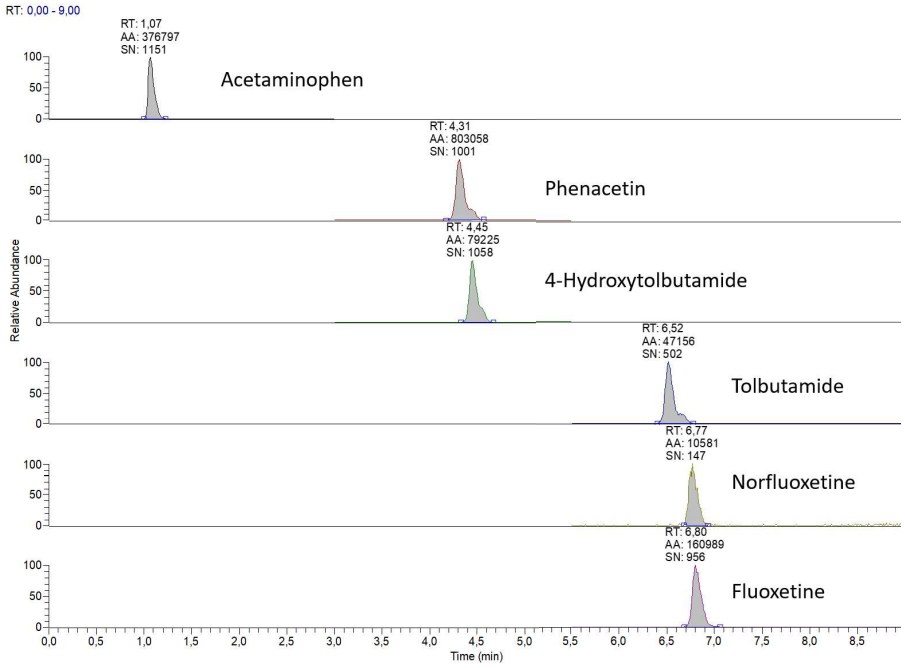

Figure 27: MRM-chromatogram of all the analytes. The elution order from the first to last where acetaminophen, phenacetin, 4-hydroxytolbutamide, tolbutamide, norfluoxetine, and fluoxetine with overlapping signal for phenacetin, and 4-hydroxytolbutamide, and tolbutamide, norfluoxetine, and fluoxetine, were aquired with a gradient ranging from 15-80% MP B in 6.5 min. The same 50 x 2.1 mm Polar Luna® C18 1.6 µm fully porous 100Å column as for the LC-UV method was used, the column oven temperature was 40 °C, and the flow rate was 0.3 mL/min.

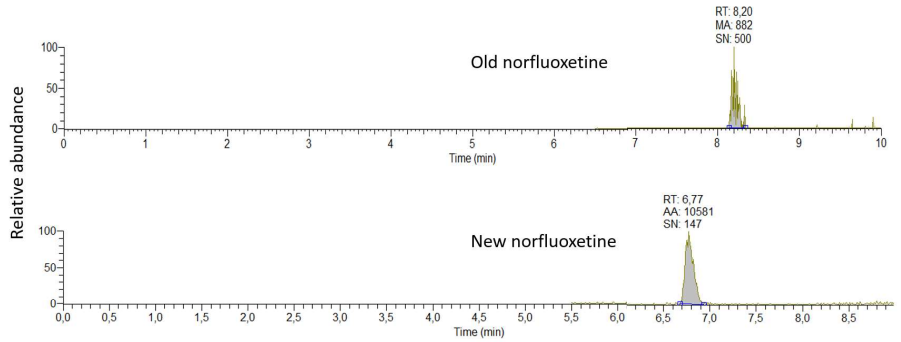

Figure 28: MRM-chromatogram of 0.5 µg/mL of the old and new norfluoxetine. The elution of the old norfluoxetine were aquired with a gradient ranging from 15-80% MP B in 8.5 min. The column oven temperature, the column, and flow rate were the same as for **Figure 27**. The elution of the new norfluoxetine were achieved with the same conditions as for **Figure 27**.

Figure 27 shows, as expected from experience with the LC-UV method, that there were two groups of analytes that co-eluted; phenacetin and 4-hydroxytolbutamide at 4.31 and 4.45 minutes, as well as tolbutamide, norfluoxetine, and fluoxetine at 6.52, 6.77, and 6.80 minutes, respectively. To evaluate if the co-eluting analytes interfered with each other, the co-eluting analytes were analysed separately and in-cocktail. The signal area for the analytes separately was generally lower than for that in-cocktail, but the RSD of the signal intensity between separate and in-cocktail analysis was below 5% for all analytes, which were considered to be acceptable due to the future plan of using ISTD. The RSD of the retention time were below 1.5% **(Section A.4, Table A.6-A.7)**, which were considered to be acceptable due to the future plan of using ISTD. **Figure 28** shows more than 90% increase in signal intensity for the new norfluoxetine compared to the old norfluoxetine but compared to the signal area of the other analytes, the signal area intensity was still low for norfluoxetine.

In summary

A solvent gradient program with a duration of 9 min, which included all the analytes for in-cocktail analyses, were established for the LC-MS system.

4.2.3. Optimization of signal area by adjusting the mass spectrometer vaporizer temperature and emitter voltage

An increase in vaporizer temperature and emitter voltage was previously reported to lower the signal for norfluoxetine, thus, vaporizer temperature and emitter voltage were adjusted to optimize the signal intensity for all the analytes [104]. The vaporizer temperature and emitter voltage in positive and negative ESI mode were altered to achieve an optimized signal intensity for each analyte and **Figure 29**

shows the signal intensity for each tested vaporizer temperature and positive voltage value, and **Figure 30** shows the signal intensity for alterations of the negative voltage.

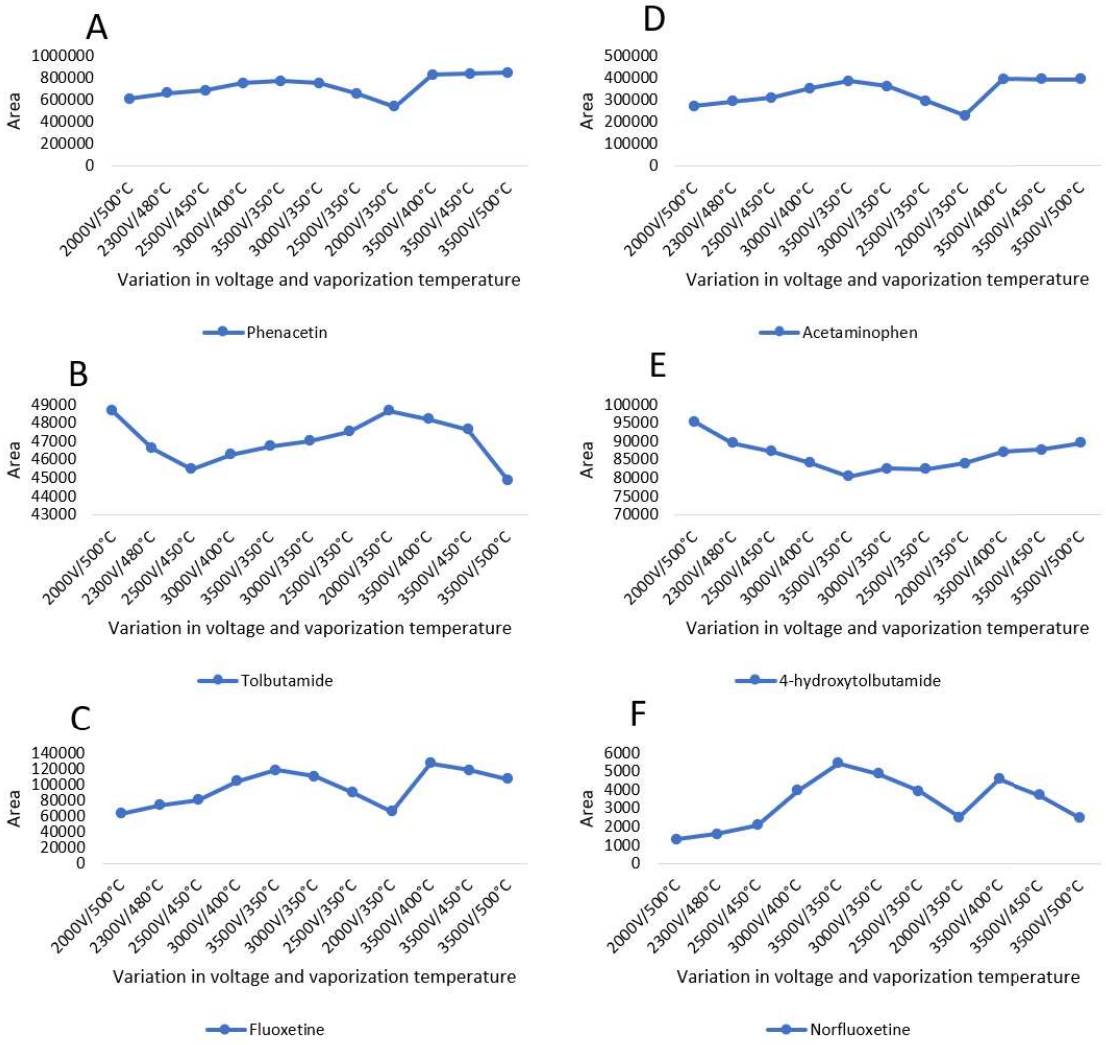

Figure 29: Area as a function of vaporizer temperature and voltage with ESI in positive mode for optimizing the analytes signal area. The vaporizer temperature ranged from 350-500 °C, and the voltage ranged from 2000-3500 V for phenacetin (A), tolbutamide (B), fluoxetine (C), acetaminophen (D), 4-hydroxytolbutamide (E), and norfluoxetine (E). The areas are shown as mean (n_s=1, n_i=3).

The graphs in **Figure 29** show that norfluoxetine has an overall lower signal inten-

sity than the other five analytes and the highest signal intensity for norfluoxetine was at 350 °C and 3500 V. A vaporizer temperature of 350 °C and emitter voltage of 3500 V give a signal intensity in the upper range for the other 5 analytes, thus it was decided to use the values that gave the highest signal intensity for norfluoxetine. Tolbutamide and 4 hydroxytolbutamide had a noticeable deviation for the two measurements of 500 °C, which could indicate instability at higher temperature as they were detected in negative mode, and should not be affected by the change in positive voltage. The RSD at 2000V/500 °C and 3500V/500 °C was calculated to be 6.3% for tolbutamide, and 5.1% for 4-hydroxytolbutamide which confirms that to be within the accepted limit for RSD of ±15%, and could be considered standard deviation within a single solution of analytes.

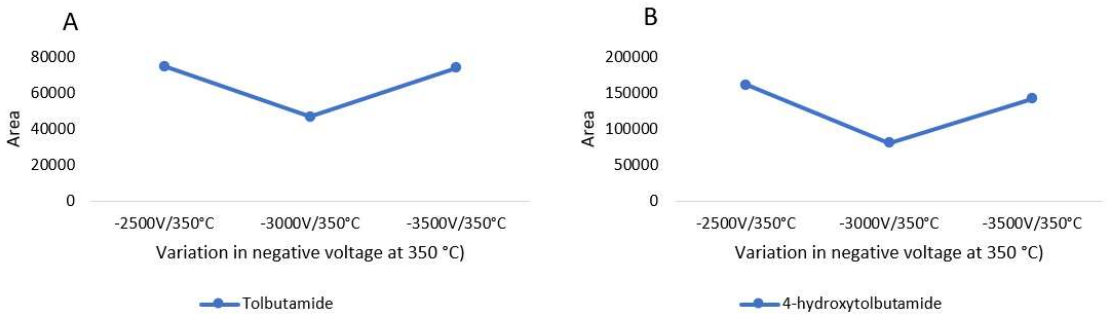

Figure 30: Area as a function of vaporizer temperature and voltage with ESI in negative mode for optimizing the analytes signal area. The vaporizer temperature were kept at 350 °C, and the voltage ranged from negative 2500 to negative 3500 V for tolbutamide (A) and 4-hydroxytolbutamide (B). The areas are shown as mean (n_s=1, n_i=3).

Although **Figure 30** points to 2500 V in negative mode as the optimal value, a closer lock at the raw data (**Section B, Table B.29**) could indicate otherwise. The raw data show a much higher signal for the first replicate than the 2 next replicates, and an RSD at 13% for tolbutamide and 12% for 4-hydroxytolbutamide. Although within the acceptance criteria of ±15%, the RSD for these two drugs was usually below 1% for repeated injections within a single solution of analytes.

When considering these deviations, the optimal signal intensity for tolbutamide and 4-hydroxytolbutamide was determined to be with an emitter voltage of 3500 V in negative mode.

In summary

The signal area was optimized by adjusting vaporizer temperature and voltage in positive and negative mode to give a signal area that best suited all the analytes.

4.2.4. Determination of limit of quantification

An in-cocktail working solution with 250 ng/mL of all the analytes in phosphate buffer and 0.1% FA, were sequential diluted to validation solutions of 75, 50, 25, 10, 7.5, 2.5, 1, and 0.75 ng/mL to determine LOQ. The chromatogram with retention time (RT), signal area (AA), and S/N (SN) for all the analytes at LOQ concentration, together with the concentrations are shown in **Figure 31**.

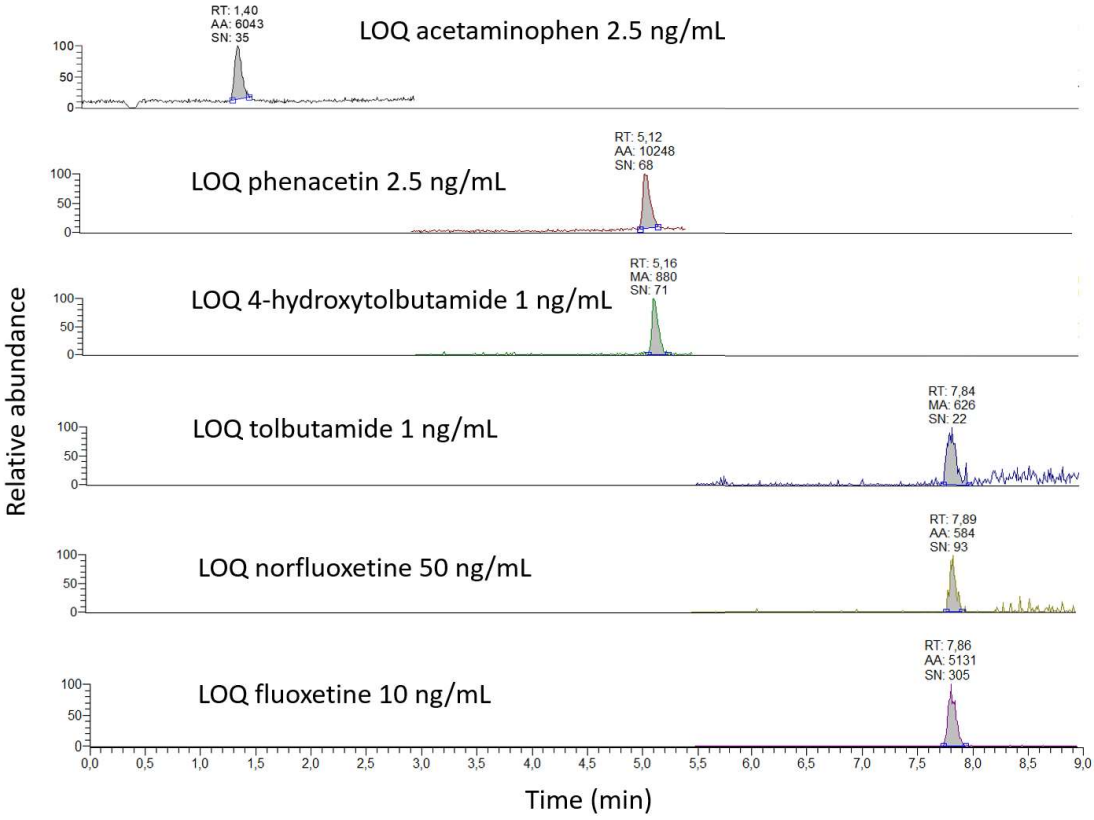

Figure 31: MRM-chromatograms for LOQ with visually accepted signal area for all the analytes. The concentration of LOQ, S/N ratio, retention time, and chromatogtraphic separation optained with the same conditions as **Figure 27** .

Figure 31 shows that the LOQs were determined to be 50 ng/mL for norfluoxetine, 10 ng/mL for fluoxetine, 2.5 ng/mL for acetaminophen and phenacetin, and 1 ng/mL for tolbutamide and 4-hydroxytolbutamide. LOQs were determined by visual evaluation and calculation of RSD (n_s=1, n_i=3) which had a range of 1 - 6% **(Section B.2.2, Table B.30)**, and thus were within the acceptance criteria of ±20%. The S/N given in the chromatograms were at ≥10, however, they were not in harmony with the visual evaluation of S/N most of the time, thus only used as a backup indicator to the visual evaluation.

In summary

The LOQ was determined to be 1 ng/mL for tolbutamide and 4-hydroxytolbutamide, 2.5 ng/mL for phenacetin and acetaminophen, 10 ng/mL for fluoxetine, and 50 ng/mL for norfluoxetine with the LC-MS system in aqueous phosphate buffer (negligible ammount of ACN).

4.2.5. Investigation of matrix effects to determine sample matrix for validation of calibration curve

Matrices used for the incubation of drugs with microsomes and organoids could give rise to matrix effects such as ion suppression or enhancement which needed to be investigated. Investigation of matrix effects was performed with analyses of all the analytes in-cocktail (unless otherwise stated) in the matrices cell medium with and without FBS, and HLM relative to a standard solvent matrix (0.1% FA in 50/50 MeOH/Water). Matrix effects calculated to 100% (analyte in cell medium or HLM/analyte in standard solvent matrix*100%) would indicate that no matrix effects occurred, above 100% indicates ion enhancement, and below 100% indicates ion suppression. Although the acceptance criteria for matrix effects are ±15%, matrix effects up to ±20% would be acceptable for this study. The linearity curve were set to have concentrations ranging from LOQ for the LC-MS system, to approximately the LOQ concentrations (0.5 µg/mL) from the LC-UV system.

Matrix effects were initially investigated for all the analytes in the HLM matrix for three concentration levels (low, middle, and high concentrations of the linearity curve) (described in **Section A.5, Table 4**) (n_S=1, n_i=3) for the three drugs, and (n_S=1, n_i=3) for the three metabolites). The initial investigation of matrix effects showed that there were ion enhancement for both phenacetin, tolbutamide, and their metabolites acetaminophen and 4-hydroxytolbutamide (32, 19, 207, and

125% respectively), while fluoxetine and its metabolite norfluoxetine were subjected to ion suppression (45, and 33% respectively) **(Section A.5, Figure A.2, and Figure A.3)**. After a closer look at the chromatograms, it was suspected that some of the matrix effects might be caused by the elution of the analytes too close to the injection solvent signal in the beginning, and the cleanup and re-equilibration at the end of the solvent gradient. This was investigated with analyses of only the highest concentration level by lowering the amount of MP B at the beginning from 15% to 10%, and expanding the gradient to 12.5 minutes by delaying the cleaning (80% MP B) and re-equilibration (15% MP B) step of the solvent gradient at the end of the solvent gradient program (seen in **Table 10**). The matrix effects were improved to be within ±20% (n_s=1, n_i=3) **(Section A.5, Table A.8)** for all the analytes in the HLM matrix, and the new and final gradient (seen in **Section 3.13.2 Table 10**) was used for the rest of the work within this study. Further, matrix effects in cell medium with and without FBS were also investigated with the same three concentration levels that were used to investigate matrix effects for HLM matrix (described in **Section A.5, Figure A.4, A.5, and A.6**). The matrix effects were within ±15% for all the analytes in both cell medium with and without FBS, except for acetaminophen which had 24% ion enhancement in cell medium with FBS **(Section A.5, Table A.9)**. Next, the linearity of the linearity curve were investigated with six concentration levels for each analyte (seen in **Table 5**), including LOQ, and with ISTD for the analyte/ISTD ratio were investigated in the matrices cell medium with and without serum, and HLMs relative to a standard solvent matrix (0.1% FA in 50/50 MeOH/Water) (**Figure 32 - 34**). The matrix with the linearity curve with the most accepted linearity (R^2 preferably ≥0.98 for all analytes) would be used as the matrix for a validation of the linearity curve, and then used for the calibration curve in the organoid metabolism study.

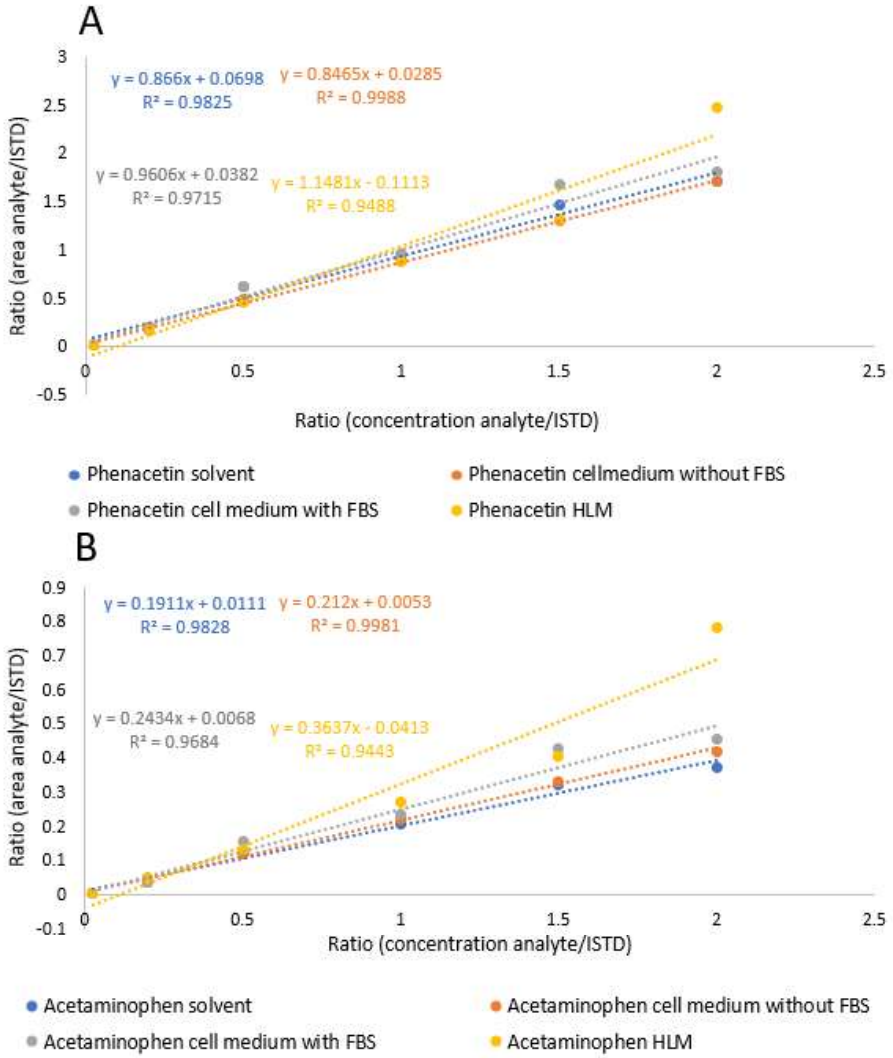

Figure 32: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for the drug phenacetin (A) and the metabolite acetaminophen (B) with isotope labeled phenacetin d5 as ISTD in various matrices. The analyte and ISTD areas were acquired with a gradient ranging from 10-80% MP B in 8.51 min (**Table 10**), and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

Figure 32 shows good linearity with an R^2 $\geq$0.98 for both phenacetin and acetaminophen in solvent and cell medium without FBS, but for cell medium with FBS the R^2 range is from 0.97 to 0.94, and not within the preferred R^2 value of

≥0.98. The linearity curves for both phenacetin and acetaminophen have similar slopes, except for acetaminophen in HLM which could indicate matrix effects.

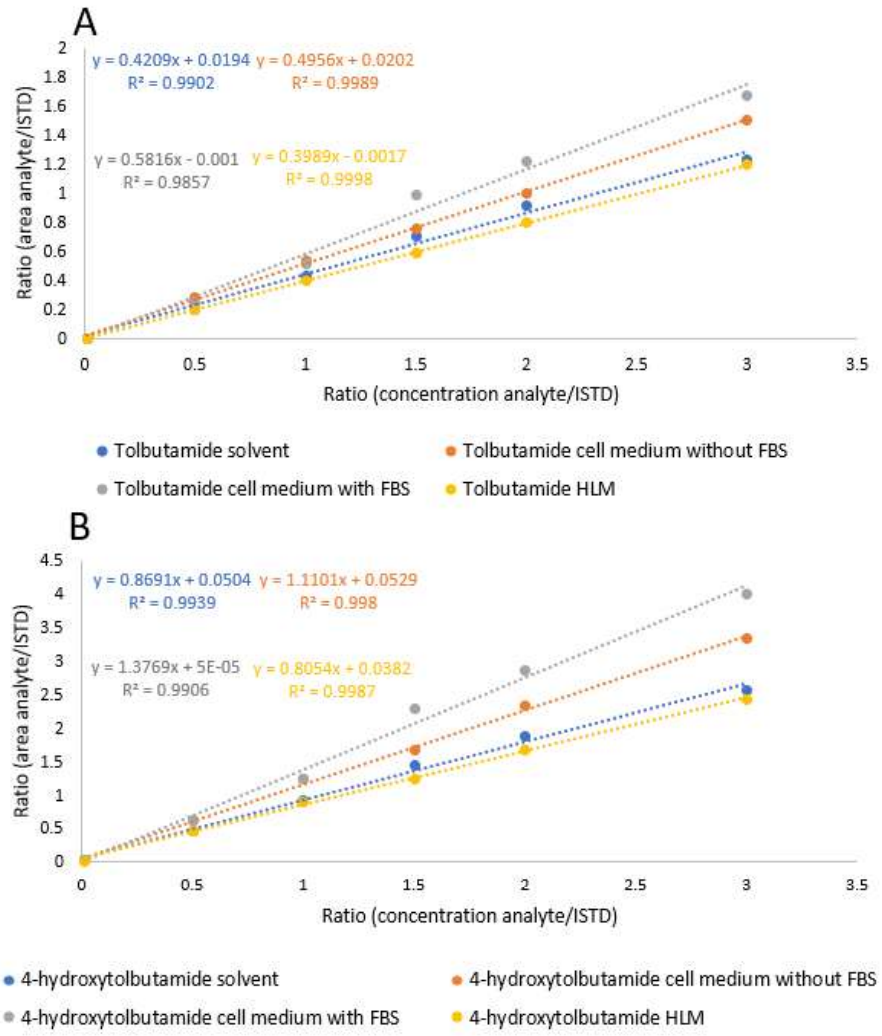

Figure 33: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for the drug tolbutamide (A) and the metabolite 4-hydroxytolbutamide (B) with isotope labeled tolbutamide d9 as ISTD in various matrices. The analyte and ISTD areas were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

In **Figure 33**, both the linearity curve of tolbutamide and 4-hydroxytolbutamide shows an R^2 >0.98 in all four matrices, and with that within the preferred value

of $R^2 \geq 0.98$. The similarity in slopes however are not optimal and the difference could indicate matrix effects.

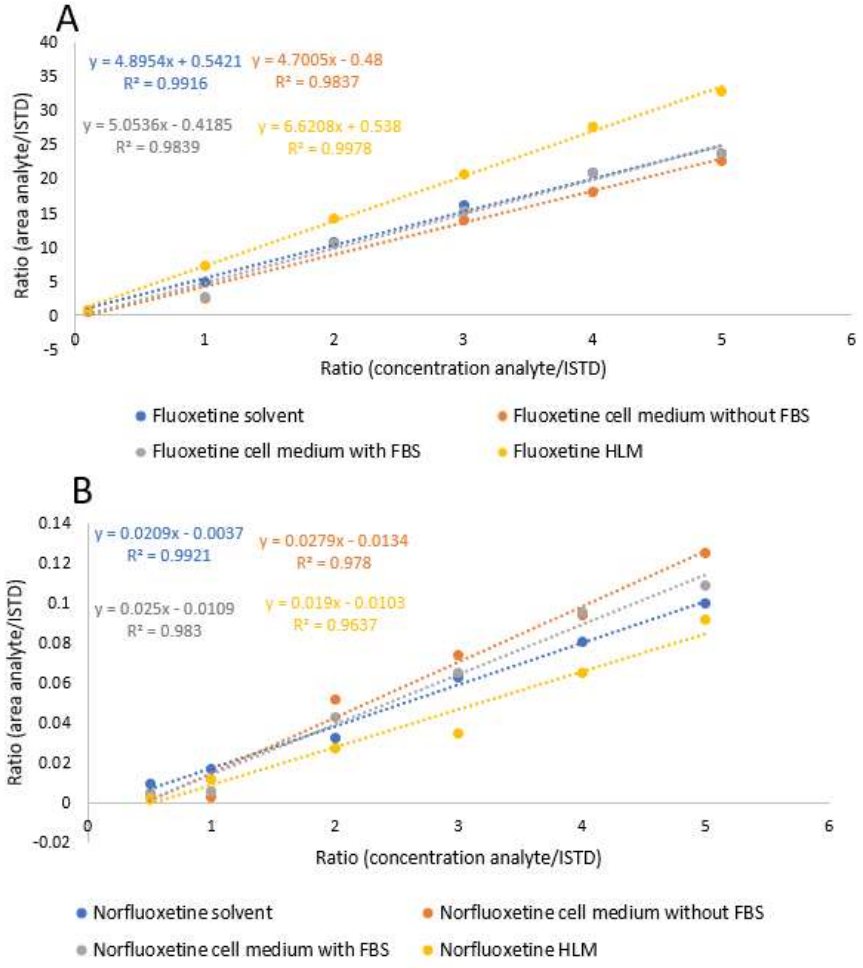

Figure 34: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for the drug fluoxetine (A) and the metabolite norfluoxetine (B) with isotope labeled fluoxetine d5 as ISTD in various matrices. The analyte and ISTD areas were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

Figure 34 shows that the linearity curves of fluoxetine have a similar slope for solvent and cell medium, but the slope for HLM deviate more which could indicate matrix effect for fluoxetine in HLM as well. The R^2 are ≥0.98 in all

matrices for fluoxetine, which shows god linearity through the whole concentration range in all four matrices. The R^2 values <0.98 could be caused by inaccuracy during the solution preparation. Due to the difference in slope for some of the calibration curves in the different matrices, the matrix effects were calculated for all the calibration curves relative to the solvent matrix by using the slope numbers, and are shown in **Table 16**.

Table 16: Matrix effects (ME) for all the analytes in various matrices. The calculated matrix effects for phenacetin (P), acetaminophen (A), tolbutmaide (T), 4-hydroxytolbutamide (4HT), fluoxetine (F), and norfluoxetine (N) for cell medium with and without FBS, and HLM matrix relative to the standard solvent matrix.

Matrix	P	A	T	4HT	F	N
Cell medium without FBS (ME %)	97.7	110.9	117.7	127.7	96.0	133.5
Cell medium with FBS (ME %)	110.9	127.4	138.2	158.4	103.2	119.6
HLM (ME %)	132.6	190.3	94.8	92.7	135.2	90.9

Table 16 shows that ion enhancement most likely cause by matrix effects was higher than the preferred value of ±20% for 4-hydroxytolbutamide, and norfluoxetine in cell medium without FBS. In cell medium with FBS, both acetaminophen, tolbutamide, and 4-hydroxytolbutamide show indication of ion enhancement with a range of 7.4-38.4% above the preferred value. In the HLM matrix the ion enhancement of phenacetin, acetaminophen, and fluoxetine, ranged from 12.6-15.2% above the preferred value for phenacetin and fluoxetine, and all the way to 70.3% above for acetaminophen. Although analytes in both cell medium with and without FBS, showed signs of matrix effects (see **Table 16** in figure **Figure 32 - 34**, only two analytes (4-hydroxytolbutamide and fluoxetine) in cell medium without FBS had values above ±20%. Thus, the matrix chosen for a validation of the method was the cell medium without FBS.

In summary

Matrix effects were investigated in cell medium with and without FBS, and HML relative to solvent only. The cell medium without FBS had the least matrix effects, and was chosen to be used as matrix for validation of the method.

4.2.6. Validating the method in cell medium without fetal bovine serum

The method was validated over 3 consecutive days according to the description in the guidelines from FDA and EMA [108, 109]. The linearity, accuracy and precision, analyte stability, and repeatability were investigated in the concentration range. As for the selectivity, there were no related molecules other than the cell medium matrix and matrix effects that already were investigated in the previous section, and determined to be acceptable. Hence, matrix effects were not re-investigated with comparison to a solvent matrix during the three days, together with selectivity which would be of greater importance for a biological matrix such as plasma or urine. Dilution integrity was investigated by calculating the concentrations of the negative control in the organoid **(Section 4.3)** studies, and the HLM metabolism study **(Section 4.2.7)** (even though the method was not being validated with HLM matrix), as they were diluted 10 times before analysis to fit the range of the linearity curve.

The average validation curves over the three days for phenacetin and acetaminophen are shown in **Figure 35**, tolbutamide and 4-hydroxytolbutamide in **Figure 36**, and fluoxetine and norfluoxetine in **Figure 37**. Rep 1 is the first analysis, and rep 2 are the repeated analysis after the solutions were being stored at 5 °C for 24 hours to investigate analyte stability in the aqueous cell medium. Day 1 has two sets of validation solutions, one set without 0.1% FA, and one where the last set

of working solutions (**Table A.5**) were frozen overnight (-20 °C), and a new set of validation solutions were prepared from them with FA. The lack of FA was a mistake but exploited as it could indicate the stability of the analytes for the freezing thawing cycle in aqueous cell medium. A similar slope of the validation curves indicate little variations in the signal area between the two replicates separated by 24 hours. No carry-over was detected in any of the blank solution analyzed following the highest validation solution.

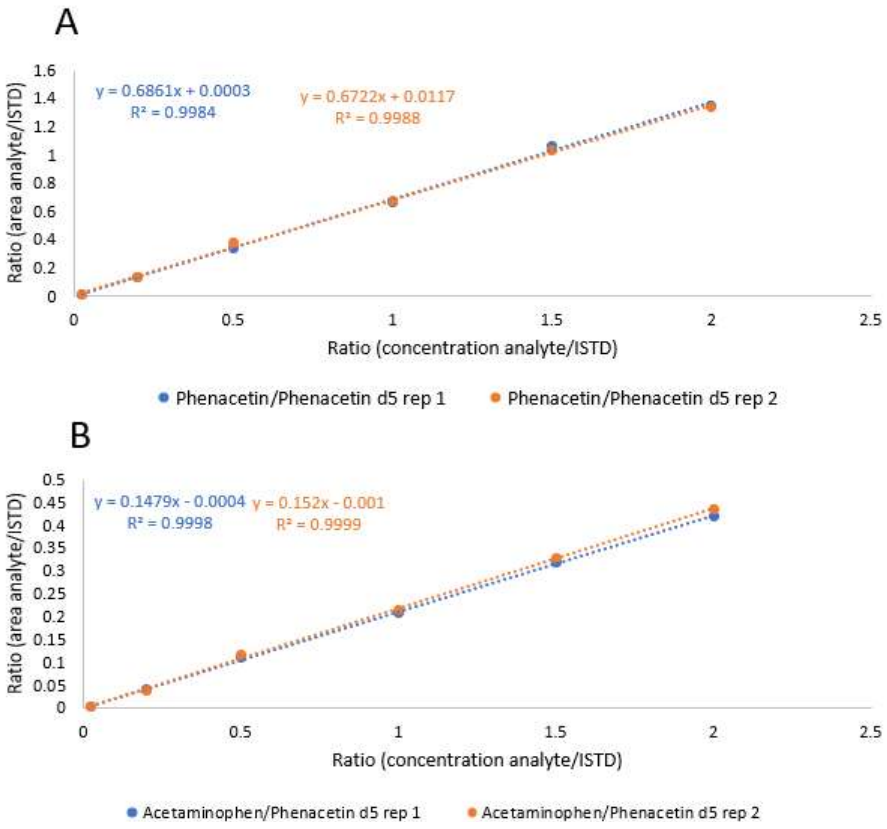

Figure 35: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for phenacetin (A) and acetaminophen (B). The curves are based on data from 2 analyses separated by 24 hours of 4 sets of validation solutions. Rep 1 is the first analysis of each calibration set, and rep 2 are the repeated analysis after 24 hours. The analyte and ISTD areas were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The areas are presented as mean of all 4 sets for both rep 1 and rep 2 with n_s=1, n_i=3.

Figure 35 shows close similarity in slope and good linearity for the analyte/ISTD ratio of phenacetin/phenacetin d5 and acetaminophen/phenacetin d5 through the whole range for the four sets of analyzed validation solutions with a R^2 >0.99 for all four validation curves. The RSDs of the signal area both inter and intraday for phenacetin, acetaminophen, and the ISTD phenacetin d5, were within the acceptance criteria of ±20% for the LOQ validation solution, and ±15% for the other five non-zero validation solutions for all analyses with only one exception. Acetaminophen had an RSD of 26% for the LOQ validation solution of rep 2 of day one without FA, which could question the analyte stability preceding 24 hours in lower concentrations. Acetaminophen was the first eluting analyte (t_R = 1.88 min), thus there could also be an issue with the ionization when FA was absent in the validation solution. With a later elution time, the acetaminophen would be exposed to the FA in the MP for a longer period of time before ionization. The RSD of the retention times were below 1% for all analyses, thus considered to be acceptable. The overall evaluation of the validation curves for phenacetin, acetaminophen, and the ISTD phenacetin d5, indicate that no significant impact on the analyses was caused by not adding FA to the calibration solutions, or add an extra freeze/thaw cycle to the working solutions. Thus, the method was considered within acceptance criteria for a validated method for phenacetin, and acetaminophen in the M10 bioanalytical method validation guidelines [108, 109].

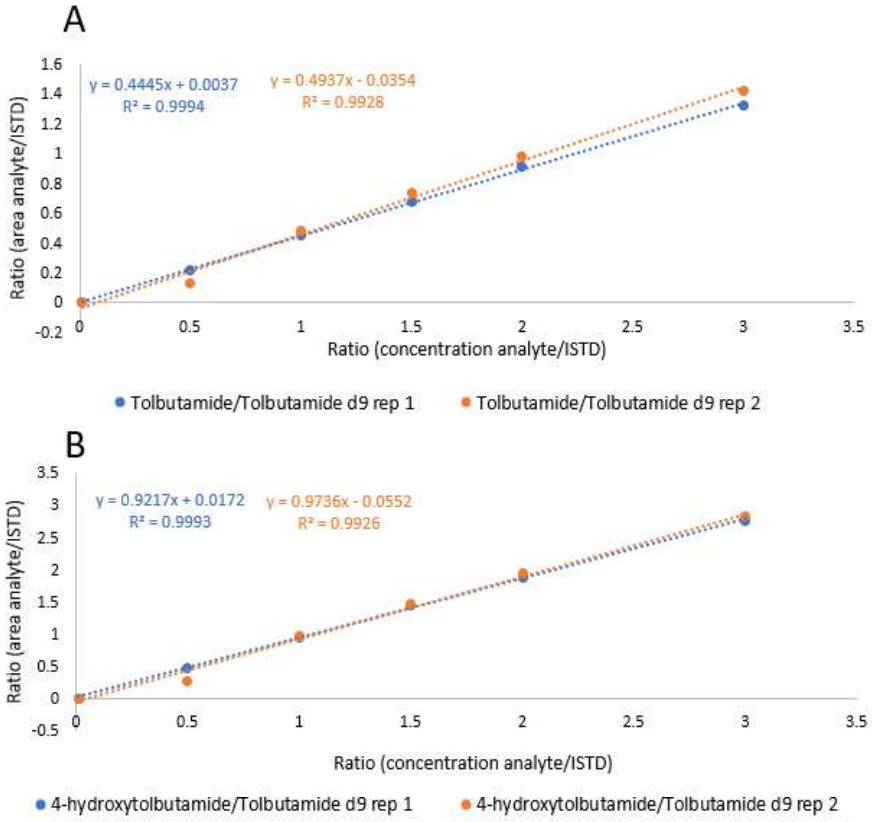

Figure 36: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for tolbutamide (A) and 4-hydroxytolbutamide (B). The curves are based on data from 2 analyses separated by 24 hours of 4 sets of validation solutions. Rep 1 is the first analysis of each calibration set, and rep 2 are the repeated analysis after 24 hours. The analyte and ISTD areas were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The areas are presented as mean of all 4 sets for both rep 1 and rep 2 with n_s=1, n_i=3.

As **Figure 36** shows, the validation curves for the two repeated analyses show a more similar slope for 4-hydroxytolbutamide than tolbutamide, but good linearity through the whole range with an R^2 >0.99 for all four validation curves. However, the inter, and intraday RSD for the signal area for five of the total of 8 validation curves, had an RSD for the LOQ solution of tolbutamide that exceeded the acceptable ±20% (ranged from 25% to 49%), which consequently also gave an RSD for the LOQ validation solution of 30% and 42%. However, the unaccepted values

were limited to the LOQ validation solution, and by taking a look at the signal at the chromatogram, a re-evaluation, and an increase in the concentration to 2.5 ng/mL instead of 1 ng/mL could be performed for the LOQ concentration for tolbutamide. The analyte stability for LOQ concentrations of tolbutamide in aqueous cell medium solutions could also be questioned. 4-Hydroxytolbutamide also had one RSD value slightly above 20% (22%). Except for the previously mentioned LOQ values, the remaining RSD values were within acceptance level of ±20% for LOQ and ±15% for the rest of the non-zero validation solutions both inter and intra day. The retention times had an RSD below 1% for both tolbutamide, 4-hydroxytolbutamide, and the ISTD tolbutamide d9, which just as for phenacetin, acetaminophen, and the ISTD phenacetin d5, were considered acceptable. Even though there is a need for re-evaluation of the LOQ concentration for tolbutamide, the overall evaluation of the bioanalytical method was within the acceptance criteria for a validated method for tolbutamide, and 4-hydroxytolbutamide in the M10 bioanalytical method validation guidelines [108, 109].

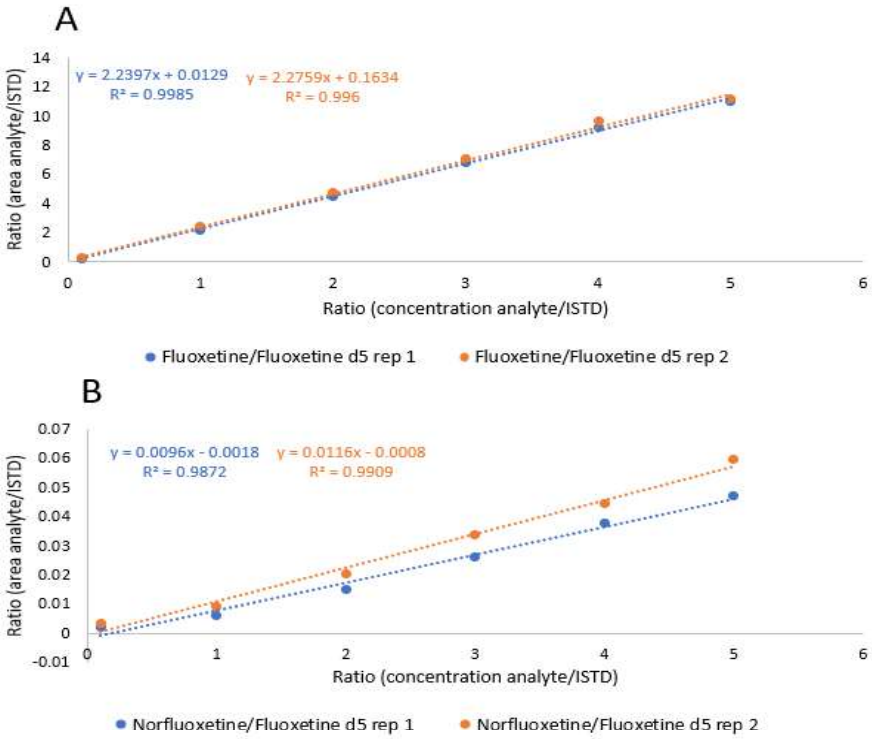

Figure 37: Ratio of area analyte/area ISTD versus concentration analyte/concentration ISTD for fluoxetine (A) and norfluoxetine (B). The curves are based on data from 2 analyses separated by 24 hours of 4 sets of validation solutions. Rep 1 is the first analysis of each calibration set, and rep 2 are the repeated analysis after 24 hours. The analyte and ISTD areas were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The areas are presented as mean of all 4 sets for both rep 1 and rep 2 with n_s=1, n_i=3.

Figure 37 shows a closer similarity in slope for the validation curves for fluoxetine than norfluoxetine, but both show good linearity through the whole concentration range with an R^2 >0.98 for both repetitions. The RSD for the signal area of norfluoxetine, however, demanded a closer look. For both replicates from day 1 with and without FA, there were more values above the acceptance criteria of 20% for the LOQ and 15% for the other non-zero validation solutions than below for norfluoxetine. Thus, there was a growing suspicion towards norfluoxetine beeing sensitive to the freezing thawing cycle. The RSD for the signal area for

days 2 and 3 however, were within the acceptance criteria for all the validation solutions for rep 1, except for the LOQ concentration. Norfluoxetine also had RSD values for the signal area intensity above acceptance criteria for 6 of the twelve values for rep 2 for days 2 and 3. However, the datasheet for both fluoxetine and norfluoxetine from the manufacturer reports analyte instability in aqueous solution for more than a day, thus, poor RSD values for rep 2 did not come as a surprise. All the RSDs for signal area for fluoxetine are within acceptance criteria for intraday for both repetitions separately. However, the RSD of the average validation curve are above the acceptance criteria on practically all calculations (only two of the twelve are within for rep 1), which indicate poor repeatability. The retention times were acceptable with an RSD of less than 1% for fluoxetine, norfluoxetine, and fluoxetine d5 as well. In general, method could not be repeated within the validation acceptance criteria for fluoxetine or norfluoxetine. Fluoxetine could be used for individual analyses for drug metabolism studies, when the main focus is to determine CYP activity qualitatively, but the stability of norfluoxetine through freezing thawing cycles, and ionization compatibility with ESI should be investigated. The validation solutions were to be further used as calibration solutions for drug metabolism studies in HLM, iPSC, and PHS.

In summary

The calibration curve for phenacetin, acetaminophen tolbutamide were validated within the acceptance criteria, with the exception of a need to re-evaluate the concentration of LOQ for tolbutamide in cell medium. Fluoxetine and norfluoxetine were not repeatable within the acceptance criterias.

4.2.7. In-coctail versus single drug metabolism study using human liver microsomes combined with investigation of the validation element dilution integrity

Previous experiments in-house with heroin metabolism in organoids and spheroids using LC-MS, had been performed with 10 μM heroin [119]. The experiments succeeded in detecting phase 1 metabolites of heroin, thus 10 μM of the drugs from this study were chosen to be incubated with the iPSC organoids and PHS. A concentration of 10 μM was equal to 1.8, 2.7, and 3.5 μg/mL phenacetin, tolbutamide, and fluoxetine respectively, which were approximately 10 times higher than the concentration of the highest non-zero calibrator. The linearity of the calibration curve was not validated above the concentration of the highest non-zero calibrator, thus, the incubated organoid samples would have to be diluted 10 times to fit the concentration range of the validated calibration curve. The dilution integrity was therefore investigated with the use of HLM. An HLM incubation experiment for all the drugs separate for their respective time points (seen in **Section 3.14.1, Table 13**) was performed with 10 μM of the three drugs. In addition, an in-cocktail experiment with the 6 different time points was performed to examine if the metabolism was the same for the analytes separately and in-cocktail. The CYP enzymes mainly involved in the phase 1 metabolism of the three drugs in this study are distinct, which mean the metabolism should not be influenced by the presence of several drugs metabolized by separately distinctive CYP enzymes. The enzymatic activity could however be influenced by the amount of NADPH available. However, the analysis of the samples containing the drugs separately could not be performed in time for this thesis due to instrumental errors. Calibration curves were made for all the analytes in the HLM matrix instead of the cell medium without FBS. The concentration of the drugs and their metabolites after in-cocktail incubation with 10 μM for 8 time periods between 0-240 minutes are

shown in **Figure 38**. Negative control samples without HLM were prepared and measured in parallel for each time period.

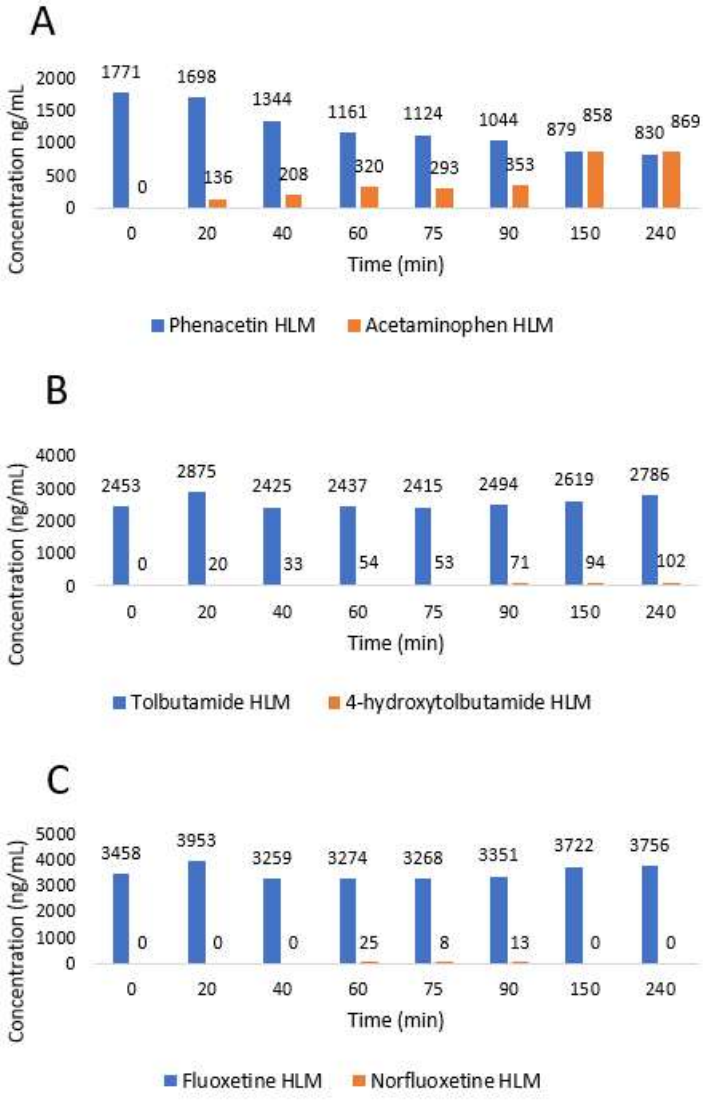

Figure 38: The concentration of all the analytes after 10 μM drug incubation with 1 mg/mL HLM for 8 time periods ranging from 0-240 min. Phenacetin and its metabolite acetaminophen (A). Tolbutamide and its metabolite 4-hydroxytolbutamide (B). Fluoxetine and its metabolite norfluoxetine (C). The concentrations were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**. The concentrations are shown as mean (n_s=3, n_i=1).

Figure 38 shows the concentrations of the drugs and metabolites after 10 µM of the drugs were incubated with HLMs. A concentration of 10 µM was a much lower concentration than the drug concentrations used in the HLM study with LC-UV detection (15, 25, and 35 µg/mL for phenacetin, tolbutamide, and fluoxetine, respectively). However, both phenacetin and tolbutamide metabolize into quantifiable amounts of the metabolites acetaminophen, and 4-hydroxytolbutamide already from the first time point of 20 min, which was within the range of previously reported time periods used for HLM metabolism studies with phenacetin and tolbutamide [64, 116]. The metabolite norfluoxetine on the other hand was not detected in quantifiable amounts for any of the incubation time periods. Nevertheless, no detectable amounts of norfluoxetine did not confirm no metabolism in HLM, because norfluoxetine was embossed by low signal detection. The concentrations were corrected for negative concentration for zero signal area.

A back calculation of the three drugs in the negative control samples was used to investigate the dilution integrity of the calibration curve **(Table 17)**.

Table 17: Drug concentration in negative control samples after 0, 20, 40, 60, 75, 90, 150, 240 min. Calculated concentrations of phenacetin, tolbutamide, and fluoxetine in the negative control samples from the experiment with HLMs where the original concentrations were 10 µM. The concentrations are presented as mean (n_s=3, n_i=1).

Drug (µM)	0 min	20 min	40 min	60 min	75 min	90 min	150 min	240 min
Phenacetin	8.80	9.28	8.90	8.93	8.68	8.92	8.65	9.07
Tolbutamide	9.51	9.99	9.56	9.25	9.24	9.66	9.36	9.50
Fluoxetine	13.6	14.3	14.9	14.9	13.9	13.8	14.4	15.5

Table 17 shows a range in concentration of 8.65-9.28 µM for phenacetin, 9.25-9.99 µM for tolbutamide, and 13.6-15.5 µM for fluoxetine from back calculation of the negative control samples. The concentrations of phenacetin and tolbutamide were within the acceptance criteria of ±15%, but fluoxetine was closer to 30%, thus not

within the acceptance criteria. However, the RSD of the negative control samples calculated for all time points were 2.3, 2.6, and 4.5% for phenacetin, tolbutamide, and fluoxetine, respectively. An RSD below ±15% are within the acceptance criteria for back calculation of a calibration standard, hence also considered acceptable for back calculation of the negative control samples. The acceptable RSD for the negative control samples indicate not only no spontaneous metabolism, but that the increased concentration of fluoxetine could be a result of a higher concentration in the incubated sample than the intended 10 µM. An additional consideration was that the matrix of the negative control samples was without HLM, while the calibration curve and hence the Y equation were from analysis with HLM matrix, which could also influence the ionization and consequently the signal area intensity and calculated concentration.

In summary

After 4 hour incubation of the three drugs with HLM, the metabolites acetaminophen and 4-hydroxytolbutamide were detected in quantifiable concentrations from the first time period exceeding zero, while norfluoxetine was only detected below LOQ. Back calculation from the calibration curves shows that the dilution integrity was within the acceptance criteria for phenacetin and tolbutamide, but too high for fluoxetine. Additionally it was found that no spontaneous metabolism occurred.

4.3. Application of the developed method for investigation of cytochrome P450 activity in liver organoids and spheroids

The bioanalytical method which was developed was to be used for evaluating CYP activity in organoids. Hence, CYP metabolizing properties of 20-30 iPSC-derived organoids and 20-30 PHS were investigated after 6, and 24 hours incubation at 37 °C with 10 μM of each drug separately in 96-well plates. Although protein LoBind tubes are the preferred tubes when working with protein bound analytes, such as drugs and metabolites, there was a global shortage of LoBind towards the end of this study. However, a small side experiment showed that the RSD between analyses performed with LoBind and Safe Lock tubes were acceptable for this study **(Section A.6)**. Thus, the incubated solutions from the 96 well, were prepared in Safe Lock tubes before the transfer to autosample vials. The iPSC-derived organoids showed no detectable metabolism but could be investigated by repeating the experiment with more than 20-30 organoids. The concentration of drugs and metabolites after incubation with PHS, and after FA and ISTD were added (total volume of 120 μL) are shown in **Figure 39**. Negative control samples without PHS were prepared and measured in parallel for each time period.

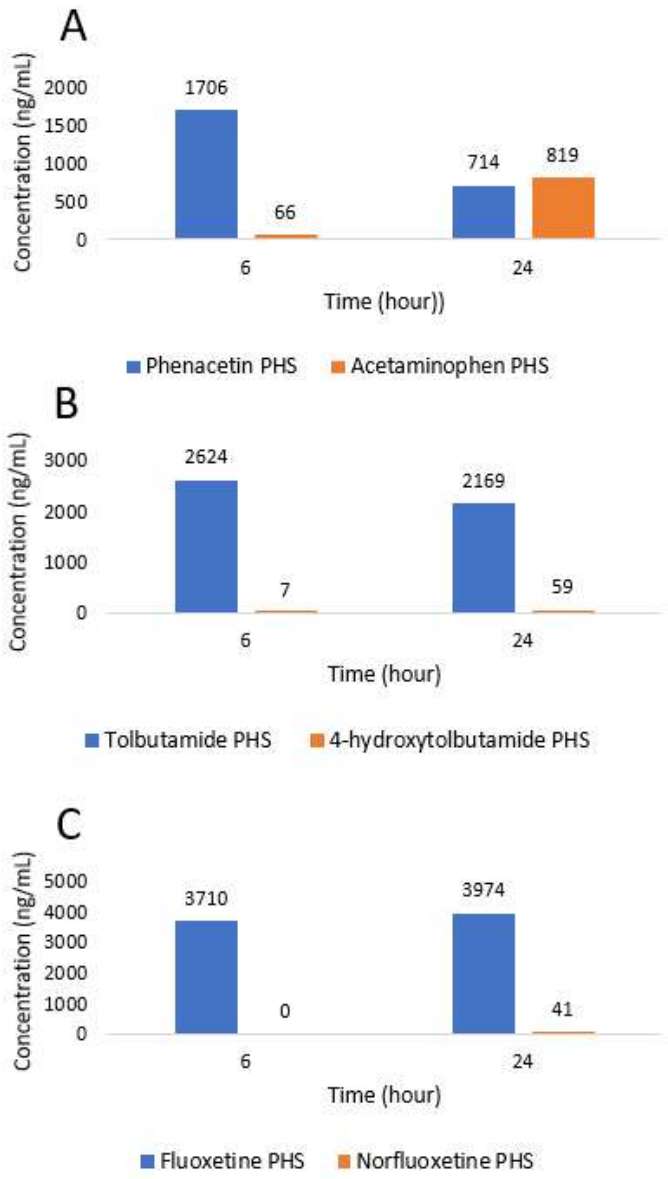

Figure 39: The concentration of all the analytes after 10 μM drug incubation with 20 PHS for 6 and 24 hours at 37 °C. Phenacetin and its metabolite acetaminophen (A). Tolbutamide and its metabolite 4-hydroxytolbutamide (B). Fluoxetine and its metabolite norfluoxetine (C).The concentrations are presented as mean (n_s=3, n_i=1), and were aquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**.

Figure 39 shows that the PHS had CYP metabolizing properties for all three drugs. However, only acetaminophen and 4-hydroxytolbutamide were quantifiable

after both 6 and 24 hours. Norfluoxetine was detected, but the concentration was below LOQ and hence not quantifiable with this method. To fit the calibration curve, the incubated solutions were diluted ten times, thus there was the possibility to analyze the incubated solution without the dilution, but due to instrumental errors, further analyses and a possible extending of the calibration range of the method could not be performed within the time frame of this thesis. The concentrations were corrected for negative concentration for zero signal area. The concentration of drugs in the negative control was calculated to check the possibility of spontaneous metabolism and investigate dilution integrity in cell medium without FBS. The concentrations of each drug are shown in **Table 18**.

Table 18: Drug concentration in the negative control samples after 6 and 24 hours. The concentrations of phenacetin, tolbutamide, and fluoxetine in the negative control samples were acquired with the same gradient as in (**Figure 32**), and the same columns, and column temperature as **Figure 27**.

Drug (μM)	6 h	24h
Phenacetin	10.2	10.2
Tolbutamide	10.4	8.5
Fluoxetine	11.7	12.5

Table 18 indicate no spontaneous metabolism for phenacetin with concentrations of 10.2 μM calculated in the negative control samples in the experiment with PHS for both 6 and 12 hours. Fluoxetine had a higher concentration than the assumed 10 μM which also indicates that no spontaneous metabolism occur. The most likely reasons for the higher concentration are evaporation of the solvent, inaccuracy during sample preparation, or matrix effects. Tolbutamide, however, had a lower calculated concentration after 24 hours and that could indicate some spontaneous metabolism, but there were no detection of 4-hydroxytolbutamide in the negative control samples. All the calculated concentrations were within ±

20% of the original concentration of 10 μM which are the acceptance criteria for back calculation from the calibration curve, thus, also indicate that the dilution integrity is acceptable. A closer look at the calibration curve used for calculating the concentrations revealed a higher signal area of norfluoxetine throughout the whole calibration range (average 1213-8510) than was the case for all the validation calibration curves (average 58 - 1337). The norfluoxetine used for preparing the calibration curve in this experiment with PHS was prepared from a stock solution that only had been frozen once, confirming the increasing suspicion throughout the work with this thesis that norfluoxetine are sensitive to the freezing-thawing cycle, and should be restricted to one cycle, two at the most.

In summary

The PHS showed metabolizing properties as metabolites of all three drugs were detected after 24 hours incubation with the drugs. Acetaminophen, and 4-hydroxytolbutamide were present in quantifiable concentrations for both 6 and 24 hours incubation of the drugs with 20-30 PHS. Norfluoxetine, however, was not detected in quantifiable concentrations. No metabolites were detected from drug incubation with 20-30 iPSC.

5. Concluding remarks

An LC-MS method for the determination of selected drugs and their metabolites to study CYP activity in organoids was developed and validated in cell medium without FBS. The validation elements for the drugs phenacetin and tolbutamide, and their metabolites acetaminophen and 4-hydroxytolbutamide were within the acceptance criteria for all the evaluated elements, with an exception of the LOQ for tolbutamide which should be reevaluated in cell medium without FBS, and most likely increased from 1 ng/mL to 2.5 ng/mL. The concentration of fluoxetine, however, was not repeatable interday, but was within all acceptance criteria intraday. The metabolite norfluoxetine needs a further investigation of its stability. The validated method was used to investigate CYP activity in 1 mg/mL HLMs and 20-30 of the 3D liver tissue models PHS, and iPSC derived organoids.

The HLMs metabolized both phenacetin, tolbutamide, and fluoxetine, into their respective metabolites, acetaminophen, 4-hydroxytolbutamide, and norfluoxetine, which confirmed CYP activity. However, only acetaminophen, and 4-hydroxytolbutamide were detected in quantifiable concentrations. Norfluoxetine was detected, but below LOQ, thus could not be quantified.

The PHS also showed metabolizing properties and CYP activity by providing detectable amounts of all three metabolites after incubation for 24 hours with 20-30 PHS. Acetaminophen and 4-hydroxytolbutamide were detected in quantifiable concentrations, but once again, norfluoxetine was detected, but below LOQ. Acetaminophen was also detected in quantifiable concentrations after 6 hours, and although 4-hydroxytolbutamide also was detected after 6 hours, the sensitivity of the method was not sufficient to measure quantifiable amounts of 4-hydroxytolbutamide.

The incubation of drugs with 20-30 iPSC derived organoids provided no detectable

metabolites, indicating none or too little CYP activity. Taken together, using LC-MS to study drug metabolism in organ representations like spheroids and organoids is a viable approach. A graphical overview of the work conducted in this study are shown in **Figure 40**.

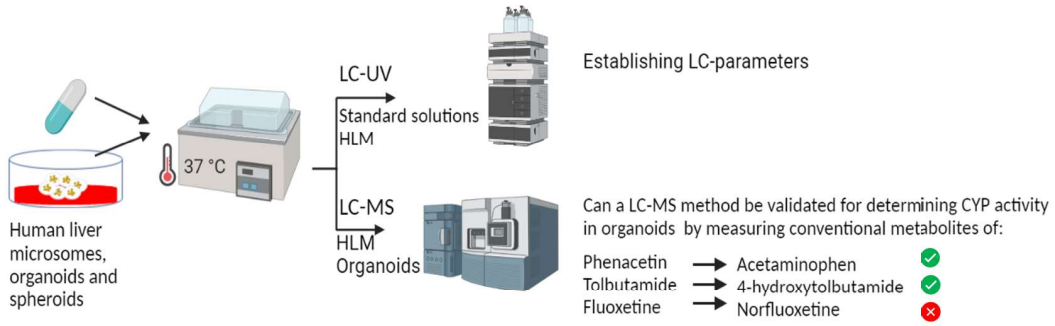

Figure 40: A graphical overview of the work conducted in this study. A LC-MS bioanalythical method was established and validated for determining CYP activity in HLM, PHS, and iPSC induced organoids. Phenacetin and tolbutamide were metabolised into metabolites in quantifiable concentrations.

5.1. Future work

Continued work to determine CYP activity in the iPSC organoids should be conducted. Fluoxetine and norfluoxetine can be considered exchanged for another telltale drug with a metabolite that has a stronger ESI-MS response analysis, or conduct an investigation of the stability of norfluoxetine through the freezing thawing cycle. The range of the calibration curve can be expanded if possible to include the drug concentration in the incubated solutions. Another possibility is the use of a column with a more narrow ID to increase sensitivity or an increased injection volume on the present system. The PHS experiment should be repeated for in-cocktail drug incubations to evaluate possible drug-drug interactions as well. In addition, both PHS and iPSC organoid experiments can be expanded to other drugs for further evaluation of metabolizing properties.

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6. Appendix

A. Additional theory, descriptions, and experiments

This section includes additional theory, a more thorough description of the preparing of the stock, validation, and calibration solutions used in this thesis. There are also additional side experiments with two NADPH regenerating systems, overlapping analytes, initial investigation of matrix effect, and a comparison of solutions prepared in cell medium without FBS, and HLM matrix with Safe Lock and LoBind tubes.

A.1. Additional theory on ultraviolet detection

A molecule can absorb energy from light, and the wavelength of the light determines the consequence of the absorption. The absorption can cause a vibration of the molecule, excite electrons, or cause it to lose electrons altogether. Light in the UV (200-400 nm)-Vis (400-700 nm) range causes excited electrons in many molecules, meaning an electron jumps from a lower level energy to a higher level energy molecular orbital. When the electron falls back down to a lower energy orbital, it releases the excess energy as a photon. The molecule or the part of the molecule that absorbs strongly in the UV-Vis region is known as a chromophore, and based on the extent of interaction and conjugation in the chromophore, a spectrophotometric detector like UV can be used as a detection method [120].

A.2. Preparing the stock solutions

All the stock solutions were vortexed for 1 minute before divided into aliquots.

Fluoxetine solution of 0.5 mg/mL was prepared by adding 500 µL ACN to a vial holding 10 mg fluoxetine, then pipetting the solution into a 50 mL centrifuge tube holding 9.5 mL ACN. This step was repeated once more, giving a total volume of 20 mL. Fluoxetine 1 mg/mL solution was prepared by evaporating 4 mL of the 0.5 mg/mL in a speed-vac, and then re-solving the dried powder in 2 mL ACN.

Norfluoxetine from two vials was used, one purchased 20 years ago and one newly purchased for this thesis. The first norfluoxetine (old norfluoxetine) solution of 1 mg/mL was prepared by adding 500 µL ACN to a vial holding 2 mg norfluoxetine, pipetting the solution into a 15 mL centrifuge tube containing 500 mL ACN. This step was repeated once more. 1 mL ACN was then added to the pipetted solution, giving a total volume of 2 mL. The second norfluoxetine (new norfluoxetine) solution of 1 mg/mL was prepared in argon degassed ACN. 1 mL degassed ACN was added to a vial holding 5 mg norfluoxetine before pipetted into 1 mL ACN in a 5 mL volumetric flask, this step was repeated once more and then diluted to the mark with degassed ACN. New argon was added before vortexing, and again laid on top of the aliquoted solutions.

Tolbutamide solution of 1 mg/mL was prepared by dissolving 15.5 mg in 1 mL ACN, then vortexing the solution for 1 min before pipetting out 0.645 mL into ACN in a 10 mL volumetric flask and diluting to the mark with ACN.

4-Hydroxytolbutamide solution of 1 mg/mL was made by adding 500 µL ACN two times to a vial holding 5 mg, before pipetting the solution into 3 mL ACN in a 5 mL volumetric flask and diluting to the mark with ACN.

Phenacetin solution of 1 mg/mL was prepared by dissolving 12.1 mg in 1 mL ACN, then vortexing the solution for 1 min before pipetting out 0.826 mL into ACN in a 10 mL volumetric flask and diluting to the mark with ACN.

Acetaminophen solution of 1 mg/mL was prepared by dissolving 9.6 mg in 1 mL ACN, then vortexing the solution for 1 min before pipetting out 0.645 mL into

ACN in a 10 mL volumetric flask and diluting to the mark with ACN.

After initial analyzes, new 5 mg/mL stock solutions of fluoxetine, tolbutamide, and phenacetin were prepared. A concentration of 5mg/mL tolbutamide and phenacetin were prepared by dissolving 50.4 mg tolbutamide and 50.8 mg phenacetin in two separate 10 mL volumetric flasks and fill to the mark with ACN. A 5 mg/mL fluoxetine stock solution was made by evaporating 4 mL of the 0.5 mg/mL solution in a speed vac and re-solving the dried powder in a total of 0.4 mL ACN.

Fluoxetine d5 solution of 1 mg/mL was prepared in argon degassed ACN. 1 mL ACN was added to the vial holding 5 mg fluoxetine d5 before being pipetted into 1 mL ACN in a 5 mL volumetric flask, this step was repeated twice more and then diluted to the mark with ACN. New argon was added before vortexing and again on top of the aliquoted solutions.

Tolbutamide d9 solution of 1 mg/mL was prepared in argon degassed ACN. 1 mL ACN was added to the 5 mg purchased vial before being extracted into ACN in a 5 mL volumetric flask, this step was repeated once more and then diluted to the mark with ACN. New argon was added before vortexing, and again on top of the aliquoted solutions.

Phenacetin d5 solution of 1 mg/mL was made by adding 1 mL ACN to a purchased vial of 5 mg, before being extracted into ACN in a 5 mL volumetric flask, this step was repeated twice more and then diluted to the mark with ACN.

Thiourea was prepared by diluting 100.3 mg in 2 mL water, and then furter diluting the resulting 50 mg/mL solution to 1 mg/mL by mixing 20 µL with 980 mL water.

All the stock solutions were stored in the dark at -20 °C, except for tolbutamide-d9, fluoxetine-d5, and the newest norfluoxetine solution which was stored in -80 °C.

A.2.1. Preparation of validation and calibration solutions for liquid chromatography-ultra violet detection

The validation solutions made for validation of the linearity curve could also be used as calibration solutions. Therefore, in this description, the validation solutions are referred to as calibration solutions (blank, zero, and non-zero calibrators).

Three working solutions (W1-W3) were prepared according to **Table A.1**, where W1 consisted of 100 µg/mL fluoxetine (F), tolbutamide (T), and acetaminophen (A), and W2 consisted of 100 µg/mL norfluoxetine (N), 4-hydroxytolbutamide (4HT), and phenacetin (P). W3 consisted of 250 µg/mL thiourea which was used as t_M with a final concentration of 25 µg/mL in all the calibration solutions.

Table A.1: Working solutions used to prepare the non-zero calibrators for LC-UV. The three working solutions that were used to make the non-zero calibrators were prepared by diluting stock solutions of 0.5 and 1 mg/mL in phosphate buffer.

Working solution	F (µL) 0.5 mg/mL	N (µL) 1 mg/mL	T (µL) 1 mg/mL	4HT(µL) 1 mg/mL	P (µL) 1 mg/mL	A (µL) 1 mg/mL	Thio (µL) 1 mg/mL	Buffer (µL)
W1	100	0	50	0	0	50	0	300
W2	0	50	0	50	50	0	0	350
W3	0	0	0	0	0	0	12.5	375

The non-zero calibrator with the highest concentration, 50 µg/mL was prepared straight from the 0.5 and 1 mg/mL stock solutions and W3, by mixing 25 µL fluoxetine 0.5 mg/mL, with 12.5 µL 1 mg/mL of norfluoxetine, tolbutamide, 4-hydroxytolbutamide, phenacetin, and acetaminophen, in addition to 25 µL 250 µg/mL thiourea (W3) and phosphate buffer to a total volume of 250 µL. The non-zero calibrators from 40 µg/mL to LOQ, were prepared from W1-W3 according to **Table A.2** were each had a total volume of 250 µL. A blank was prepared by adding 0.1 % FA to phosphate buffer.

Table A.2: Calibration solutions for LC-UV The non-zero calibrators ranged from LOQ to 50 µg/mL for all the analytes, and all but the 50 µg/mL where prepared from W1-W3 by dilution with phosphate buffer according to this table for total volumes of 250 µL.

Concentration	W1 (µL)	W2 (µL)	W3 (µL)	Water (µL)
40 µg/mL	100	100	25	25
30 µg/mL	75	75	25	75
20 µg/mL	50	50	25	125
10 µg/mL	25	25	25	175
LOQ	1.875	1.25	25	221.875

A.2.2. Preparation of validation and calibration solutions for liquid chromatography-mass spectrometry

The validation solutions for LC-MS consisted of a blank (matrix), and a zero (blank plus ISTD), in addition to six non-zero concentration levels. The validation solutions were prepared in the same way for all four different matrices used in this thesis, solvent (0.1% FA in 50/50 water/MeOH (v/v)), cell medium with and without 2% FBS, and HLM matrix. The non-zero validation solutions had a range of LOQ to 500 ng/mL (10/50, 100, 200, 300, 400, 500) for fluoxetine and norfluoxetine, LOQ to 300 ng/mL (1, 50, 100, 150, 200, 300) for tolbutamide and 4-hydroxytolbutamide, and LOQ to 200 ng/ml (2.5, 20, 50, 100, 150, 200) for phenacetin and acetaminophen **(Table 5)**. The validation and calibration solutions were prepared by thawing the 1 mg/mL stock solutions of analytes and dilute them to a first set of working solutions (WF1, WN1, WT+4HT1, WP+A1) as shown in **Table A.3**. WF1, WN1, WT+4HT1, and WP+A1 were then used to prepare a second set of working solutions (WF2, WN2, WT+4HT2, WP+A2) which are shown in **Table A.4**. The third and last set of working solutions (WA-WF) were prepared from WF1, WN1, WT+4HT1, WP+A1, WF2, WN2,

WT+4HT2, and WP+A2 according to **Table A.5**.

Table A.3: The first set of working solutions; WF1, WN1, WT+4HT1, and WP+A1. The first set of working solutions for the calibration solutions on the LC-MS was prepared by diluting stock solutions of 1 mg/mL.

Working solution	F (µL) 1 mg/mL	N (µL) 1 mg/mL	T (µL) 1 mg/mL	4HT(µL) 1 mg/mL	P (µL) 1 mg/mL	A (µL) 1 mg/mL	Matrix (µL)
WF1	50						950
WN1		50					950
WT+4HT1			20	20			960
WP+A1					20	20	960

Table A.4: The second set of working solutions; F2, N2, T+4HT2, and P+A2. The second set of working solutions was prepared by diluting the first set.

Working solution	F1 (µL)	N1 (µL)	T+4HT1 (µL)	P+A1 (µL)	Matrix
WF2	100				900
WN2		100			900
WT+4HT2			50		950
WP+A2				100	900

Table A.5: The third and last set of working solutions; WA, WB, WC, WD, WE, and WF. The third and last set of working solutions was made by dilutions from the first and second set.

Working solution	F1 (µL)	N1 (µL)	T+4HT1 (µL)	P+A1 (µL)	F2 (µL)	N2 (µL)	T+4HT2 (µL)	P+A2 (µL)	Matrix
WF	100	100	150	100					550
WE	80	80	100	75					665
WD	60	60	75	50					755
WC	40	40	50	25					845
WB					200	200	500	100	0
WA					20	100	10	12.5	857.5

A working solution of 50 µg/mL ISTDs (WISTD1) was prepared by thawing the stock solutions of 1 mg/mL phenacetin d5. tolbutamide d9, and fluoxetine d5, and dilute 50 µL of each in 850 µL matrix for a total volume of 1 mL. A furter dilution of WISTD1to 1 µg/mL (WISTD2) was prepared by adding 20 µL WISTD1 to 980 µL of the current matrix.

The non-zero calibrators (1-6) were prepared by adding 20 µL WA-WF in 1-6 in addition to 20 µL ISTD and 20 µL 1.1 M FA in 140 µL matrix for a total volume of 200 µL. The blank was prepared by adding 20 µL 1.1 M FA to 180 µL matrix, and the zero-calibrator by adding 20 µL 1.1 M FA and 20 µL WISTD2 to 160 µL matrix.

A.3. The difference between NADPH regenerating systems are negligible

As the initial experiments of incubating the three drugs with HLM showed little or no detectable metabolites, there was arranged an extern experiment at the DFS. A second NADPH regenerating system (Corning) was to be tested and compared

to the in-house NADPH system (Tebu-Bio). Assistance regarding possible improvements to the protocol (.e.g. lower percent organic solvent in incubation) and technique were also provided at the DFS. Only phenacetin (15 µg/mL) was investigated as it had shown detectable concentrations of the metabolite acetaminophen after incubation with HLM, thus it was considered certain that a comparable absorbance signal would be detected. In addition, the time periods of incubation were increased from 20 min to 40, 75, and 120 min. The measured signal area for the negative controls, and the samples with HLMs, for both NADPH regenerating systems at each time period are shown in **Figure A.1**.

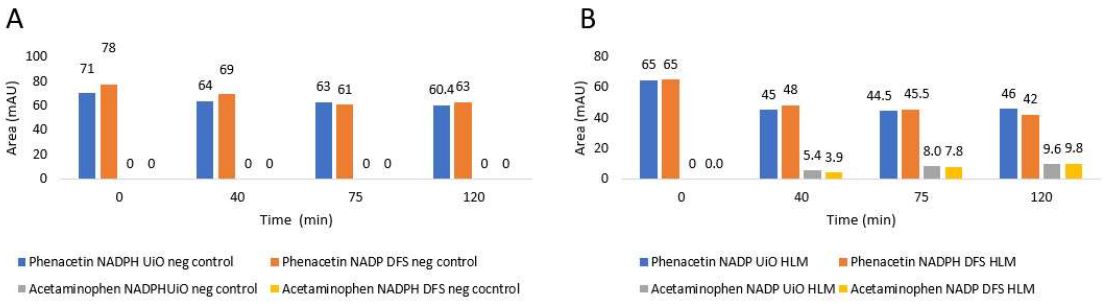


Figure A.1: Signal area for phenacetin and acetaminophen after 15 µg/mL phenactin incubation with 1 mg/mL HLM for 0-120 min. The signal area for the negative control samples (A) for both NADPH regenerating systems, and the signal area for the samples incubated with HLM (B). The signal areas were obtained with the same conditions as **Figure 21**

Figure A.1 shows that there was no detectable metabolism of phenacetin to acetaminophen in the negative control samples. The RSDs between the analyses performed with the two NADPH regenerating systems were within the acceptance criteria for a single solution variation of ± 15% which together with no detected metabolites, indicates no spontaneous metabolism of phenacetin. However, the signal areas were decreasing along with increasing incubation time, which did seem suspicious towards the possibility of spontaneous metabolism or degradation over time. The metabolism of phenacetin into acetaminophen, were almost identical for the two regenerating systems and confirmed by calculation of RSD to be 5.2, 2.1,

and 2.0 % for 40, 75, and 120 min, respectively. Thus considered to be negligible in relation to this study.

A.4. Investigation of co-eluting analytes with the liquid chromatography-mass spectrometry system

There were analytes with overlapping retention time in the chromatogram from the MS. Tolbutamide, norfluoxetine, and fluoxetine overlapped, and so did phenacetin and 4-hydroxytolbutamide. Potential interference in the signal area or retention time for the overlapping analytes were investigated and shown in **Table A.6 and A.7**.

Table A.6: Signal area comparison for the co-eluting analytes The signal area for the overlapping analytes tolbutamide, norfluoxetine, and fluoxetine, as well as phenacetin and 4-hydroxytolbutamide, separately and in-cocktail. The signal areas were obtained with the same conditions as in Figure 27.

Parameter	T	N	F	P	4HT
In-coctail (area)	33021	711	14330	914292	68536
Separately (area)	35321	715	14847	978177	67561
Average	34171	713	14588.5	946234.5	68048.5
St.dev.s	1626.3	2.8	365.6	45173.52	689.4
RSD (%)	4.8	0.4	2.5	4.8	1

Table A.7: Retention time comparison for the co-eluting analytes. Retention time for the overlapping analytes tolbutamide, norfluoxetine, and fluoxetine, as well as phenacetin and 4-hydroxytolbutamide, separately and in-cocktail. Retention times were obtained with the same conditions as **Figure 27**, and are presented as mean (n_s=1, n_i=3).

Parameter	T	N	F	P	4HT
In-coctail (min)	7.65	8.20	8.25	5.23	5.23
Separately (min)	7.63	8.26	8.24	5.13	5.12
Average	7.64	8.23	8.245	5.18	5.175
St.dev.s	0.01	0.04	0.007	0.07	0.08
RSD (%)	0.19	0.52	0.086	1.37	1.50

Table A.6 and A.7 shows that the signal area in-cocktail and separately have an RSD below 5%, and below 2% RSD for the retention time for all the measured analytes. In general, the signal area was slightly lower in-cocktail, but as the RSD shows, the deviations are not outside the acceptance criteria of ± 15% for variations within a single solution above LOQ.

A.5. Initial investigation of matrix effects

The signal area in solvent matrix (0.1% FA in 50/50 MeOH/water) compared to HLM matrix was investigated first and are shown in **Figure A.2, and A.3**. Although no metabolism had been seen in the 0 minutes or negative control samples previously, due to the higher sensitivity in the MS as opposed to the UV, the three drugs and the three metabolites were prepared in two separate working solutions with a low, middle, and high concentration **Table 4**. That way it could also be controlled that the drugs were not metabolized in the HLM matrix after FA was added.

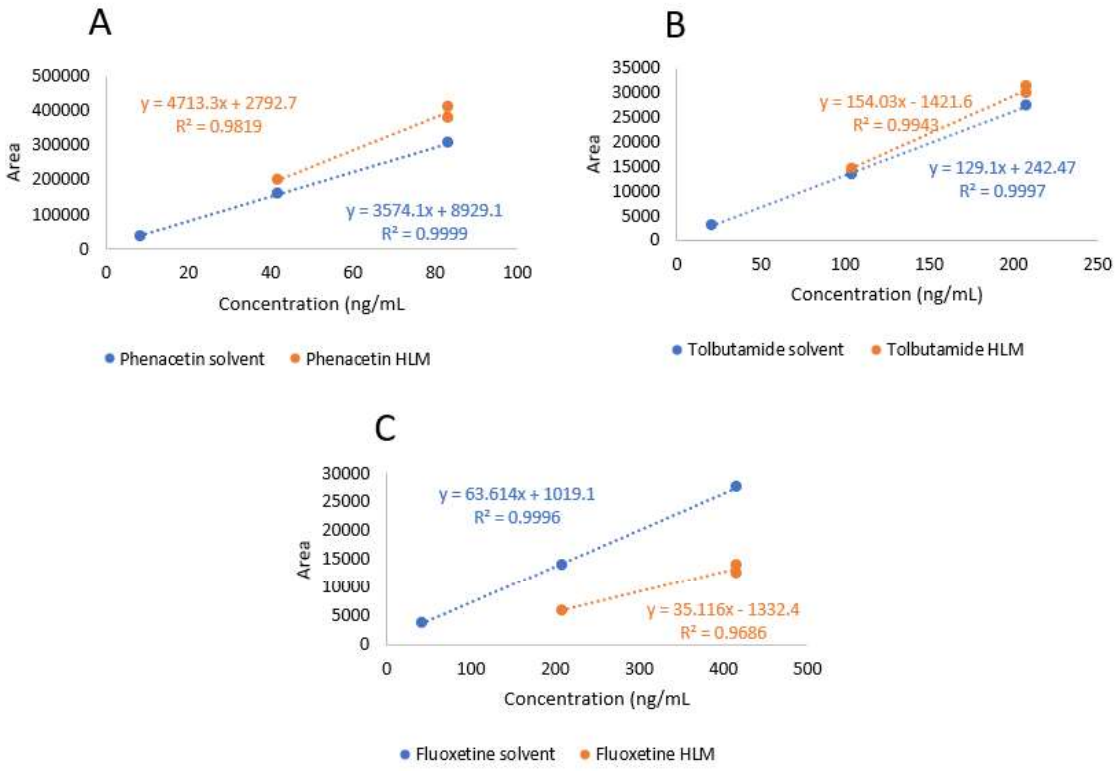


Figure A.2: Area as a function of concentration for the three drugs. The signal areas for the three drugs phenacetin (A), tolbutamide (B), and fluoxetine (C), were optained with the same conditions as **Figure 27**. The areas are presented as mean (n_s=1, n_i=3).

Figure A.2 shows that an error had occurred during sample preparation of the drugs, and the replicate with the low concentration had been prepared with the working solution for the high concentration instead. As the slope of the curve was the point of interest, which could be determined with only two data points, the sample preparation, and analysis were not redone at this point.

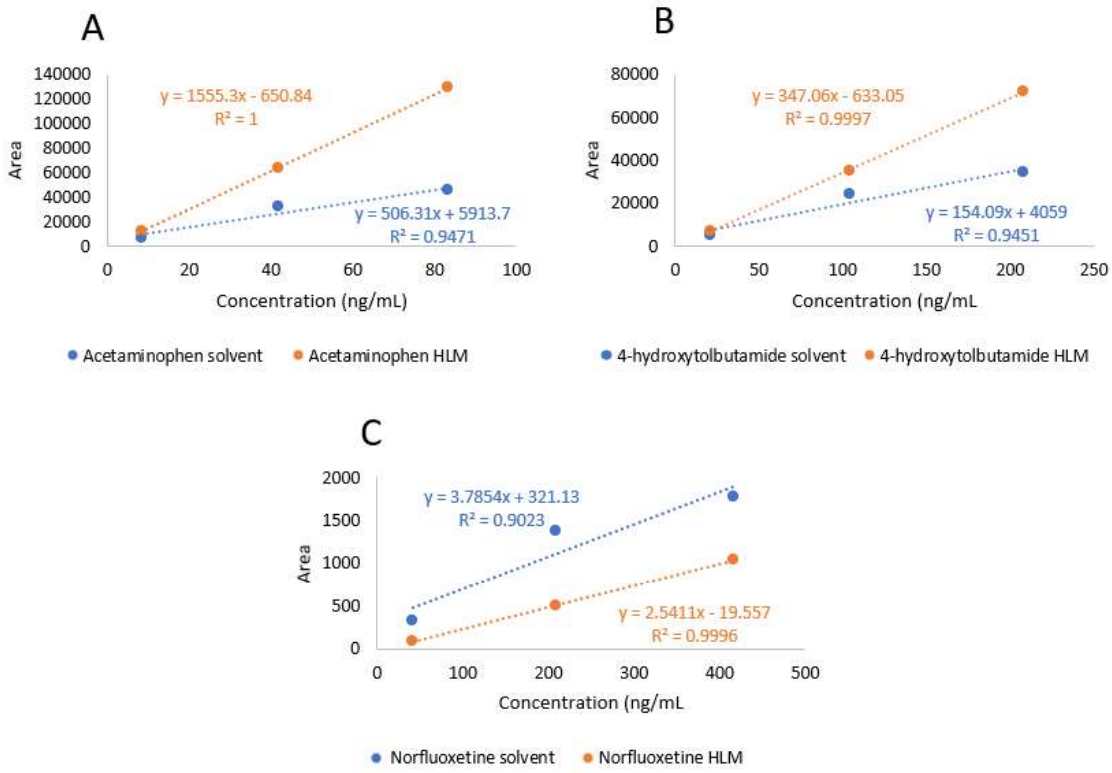


Figure A.3: Area as a function of concentration for the three metabolites. The signal areas for the three metabolites acetaminophen (A), 4-hydroxytolbutamide (B), and norfluoxetine (C), were optained with the same conditions as **Figure 27**. The areas are presented as mean (n_s=1, n_i=3).

Figure A.2 and A.3 shows that there was ion enhancement for both phenacetin, tolbutamide, and their metabolites acetaminophen, and 4-hydroxytolbutamide, while fluoxetine and its metabolite norfluoxetine, were subjected to ion suppression. The calculation of matrix effects was done with the slope numbers, and showed ion enhancement of 32% for phenacetin, 19% for tolbutamide, 207% for acetaminophen, and 125% for 4-hydroxytolbutamide. Fluoxetine and norfluoxetine had an ion suppression of 45%, and 33% respectively. As matrix effects tend to not be repeatable, the preferred value would be within ±20% for this method even though the acceptance criteria were ± 15%. The middle concentration level for all the metabolites appeared to have a higher concentration than intended,

indicating a problem with the sample preparation, and not matrix effects. The higher concentration in addition to the double analysis with the highest non-zero calibrator for the drugs, did so the linearity could not be properly evaluated. As described in **(Section 4.2.5)**, the gradient was adjusted to improve the matrix effects. The new matrix effects were calculated with the use of the average of the signal area, and were within ±20% for the three drugs and metabolites **(Table A.8)**. Hence, the new gradient were used to investigate matrix effects in cell medium. The cell medium was deprived of organoids, hence all the analytes were prepared and analyzed in-cocktail. The solvent matrix compared to cell medium matrix with and without FBS are shown in **Figures A.4, A.5, and A.6**.

Table A.8: Matrix effects in HLMs analysed with the high concentration from Table 4in solvent and HLM matrix. The matrix effects are presented as mean (n_s=1, n_i=3), and were obtained with a gradient ranging from 10-80% MP B in 8.51 min (**Table 10**), and the same columns, and column temperature as **Figure 27**.

Matrix	P	A	T	4HT	F	N
HLMs (ME %)	100.1	116.1	85.4	93.9	81.5	86.6

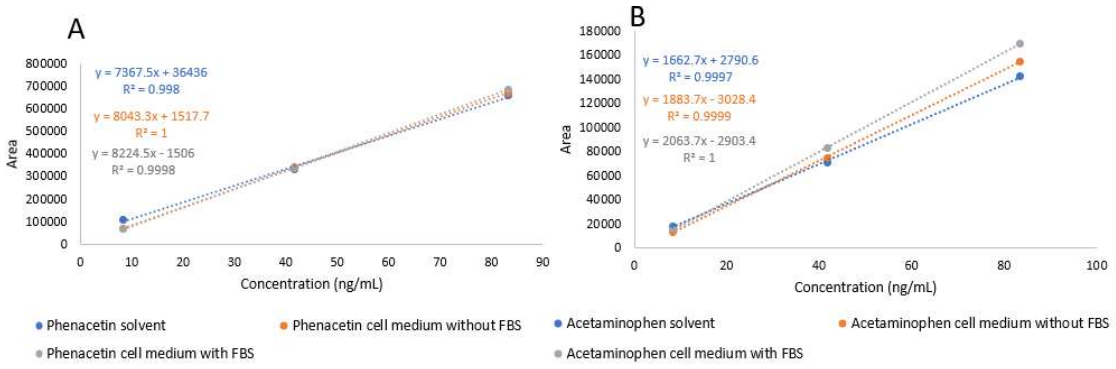


Figure A.4: Area as a function of concentration for phenacetin (A) and acetaminophen (B) in cell medium with and without FBS. The areas were acquired with the same gradient as in **Figure 32, and Table A.8**, and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

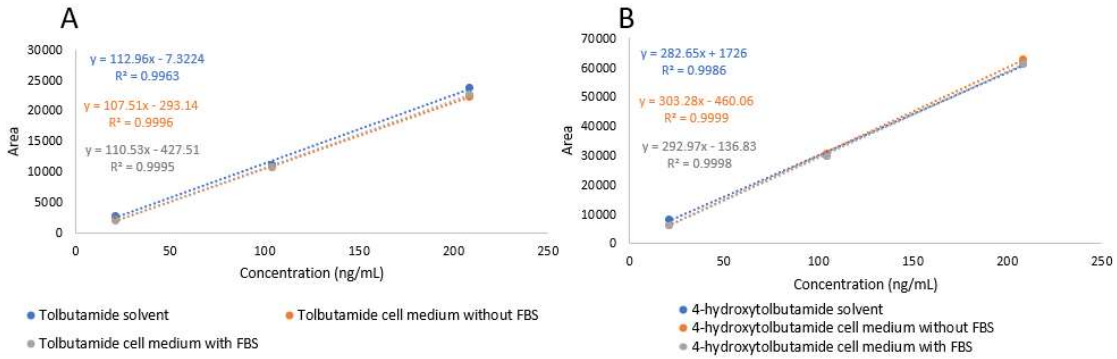


Figure A.5: Area as a function of concentration for tolbutamide (A) and 4-hydroxytolbutamide (B) in cell medium with and without FBS. The areas were acquired with the same gradient as in **Figure 32, and Table A.8**, and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

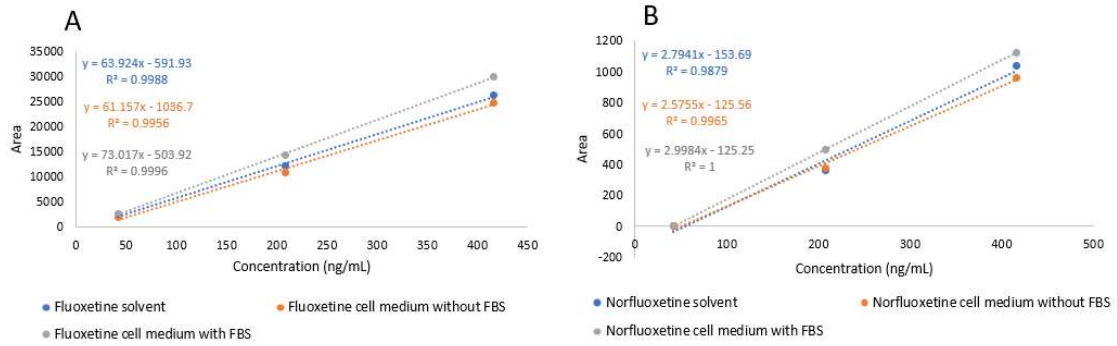


Figure A.6: Area as a function of concentration for fluoxetine (A) and norfluoxetine (B) in cell medium with and without FBS. The areas were acquired with the same gradient as in **Figure 32, and Table A.8**, and the same columns, and column temperature as **Figure 27**. The analyte/ISTD areas are shown as mean (n_s=1, n_i=3).

There were no signs of spontaneous metabolism in the chromatograms and **Figure A.4 - A.6** shows that it seemed to be little matrix effects. The matrix effects were calculated to be within ± 15% for all analytes except for acetaminophen in cell medium with FBS, which was calculated to 24% ion enhancement **(Table A.9)**. Norfluoxetine could not be detected for the lowest concentration in any of the three matrices, hence was set as zero. Thus, the matrix that gave the least ion enhancement and suppression was the cell medium without FBS.

Table A.9: Matrix effects in cell medium with and without FBS relative to solvent matrix. The calculated matrix effects for phenacetin (P), acetaminophen (A), tolbutamide (T), 4-hydroxytolbutamide (4HT), fluoxetine (F), and norfluoxetine (N) in cell medium with and without FBS. The calculations were obtained from signal areas in **Table A.4-A.6**.

Matrix	P	A	T	4HT	F	N
Cell medium without FBS (ME%)	109.2	113.3	95.2	107.3	95.7	92.2
Cell medium with FBS (ME%)	111.6	124.1	97.8	103.7	114.2	107.3

A.6. Global shortage of protein LoBind tubes, can Safe Lock tubes be an alternative in a crisis

Due to the global shortage of protein LoBind tubes towards the end of this thesis, a small side experiment was performed to see if Safe Lock could be used for the work in this study and perhaps in other situations in a crisis without to much loss of analyte. Both the matrix with HLM and the matrix with cell medium contain proteins that bind to the analyte and potentially the tubes, hence, an experiment with both matrices was performed.

A.6.1. Safe Lock replacing LoBind tubes in human liver microsome metabolism studies can be an alternative in a crisis

The analyte concentrations in the incubation solution were equal to that of the highest non-zero calibrator (200 ng/mL phenacetin, 300 ng/mL tolbutamide, and 500 ng/mL fluoxetine), and all three ISTD had a concentration of 100 ng/mL. Two sets of incubation solutions with HLM were prepared according to the description in **Section 3.14.1**, with all three drugs in-cocktail. One in LoBind tubes, and one in safe lock tubes, with three time periods (1, 2.5, and 4 hours) and n_S=3 for each time period. To save LoBind tubes, negative control solutions were not prepared as they had shown no metabolism in earlier experiments. The RSD between the

analyses performed with the LoBind and Safe Lock tubes for each analyte, are given in **Table A.10** together with the analyte/ISTD ratio.

Table A.10: LoBind versus Safe Lock in HLM matrix. The calculated RSD values from the analyses with LoBind and Safe Lock tubes for all the analyte areas, and analyte/ISTD ratio. The areas used for calculating the RSD were obtained with the same conditions as **Figure 32**, and the same columns, and column temperature as **Figure 27**, with n_s=1, and n_i=3.

LoBind versus Safe Lock (RSD %)	1 hour	2.5 hours	4 hours
Phenacetin	2.6	6.8	3.5
Phenacetin/Phenacetin d	7.5	4.2	1.3
Acetaminophen	9.2	5.6	1.9
Acetaminophen/Phenacetin d5	14.1	3.0	0.3
Tolbutamide	5.4	4.4	0.9
Tolbutamide/Tolbutamide d9	8.4	3.1	1.6
4-hydroxytolbutamide	8.4	5.7	3.9
4-hydroxytolbutamide/Tolbutamide d9	11.4	4.4	4.7
Fluoxetine	2.0	8.1	5.1
Fluoxetine/Fluoxetine d5	11.3	3.8	0.4
Norfluoxetine	-	-	-
Norfluoxetine/Fluoxetine d5	-	-	-

The trends in the signal area intensity were as expected a lower signal for the Safe Lock tubes relative to the LoBind tubes. However, except for norfluoxetine which could not be evaluated due to poor metabolism and consequently low detection, **Table A.10** shows an RSD below ±15% for all the analytes. Thus, indicating that the loss of protein bound analyte was acceptable in Safe Lock in a crisis with the global shortage of LoBind tubes.

A.6.2. Safe lock tubes with cell medium matrix can be an alternative to LoBind in a crisis

The experiment with cell medium without FBS as a matrix in LoBind versus Safe Lock tubes were performed by making a set of calibration solutions (1-6) as described in **section 3.8.4** from the same working solutions as validation solutions of the 3rd day of validating the LC-MS method. They were prepared simultaneously, but the one prepared in LoBind was analyzed straight upon completion, while the other set was stored in the safe lock tubes overnight (20 hours) at 4-8 °C. The known instability of fluoxetine and norfluoxetine in aqueous solution for more than a day was kept in mind as possible interferences that could occur during this investigation.

Table A.11: LoBind versus Safe Lock in cell medium without FBS. The calculated RSD values from the analyses with LoBind and Safe Lock tubes for all the analyte areas, and analyte/ISTD ratio. The areas used for calculating the RSD were obtained with the same conditions as **Figure 32**, and the same columns, and column temperature as **Figure 27**, with n_s=1, and n_i=3.

LoBind versus Safe Lock (RSD %)	1	2	3	4	5	6
Phenacetin	0.6	5.1	3.7	0.2	.01	1.0
Phenacetin/Phenacetin d	0.7	5.0	2.8	0.4	0.0	0.6
Acetaminophen	7.7	2.9	6.9	1.0	2.9	0.0
Acetaminophen/Phenacetin d5	6.4	2.8	6.0	0.4	3.0	0.3
Tolbutamide	7.3	5.8	1.3	3.8	2.5	4.9
Tolbutamide/Tolbutamide d9	4.6	1.2	1.5	0.9	2.5	2.0
4-hydroxytolbutamide	0.3	2.5	2.5	2.8	3.5	1.0
4-hydroxytolbutamide/Tolbutamide d9	3.1	2.1	0.3	1.8	1.6	1.9
Fluoxetine	3.5	1.0	1.1	1.0	3.7	2.5
Fluoxetine/Fluoxetine d5	15.4	10.2	7.5	4.3	0.8	0.3
Norfluoxetine	33.6	26.3	8.6	0.2	0.3	8.4
Norfluoxetine/Fluoxetine d5	44.6	17.3	0.0	5.5	4.8	6.2

Except for an RSD above 20% for the validation solution 1 (LOQ), and above 15% for non-zero calibrator 2 for norfluoxetine and norfluoxetine/fluoxetine d5, **Table A.11** shows that the RSD between LoBind and Safe Lock kept validation solutions were acceptable for all the analytes and analyte/ISTD. Norfluoxetine could not be properly evaluated for HLM matrix in LoBind versus Safe Lock either and was, in general, providing challenges with detection with LS-MS. Nevertheless, the deviation between LoBind and Safe Lock tubes were considered to be acceptable for this study during a global shortage of LoBind tubes.

B. Raw data

This section contains all the raw data with average, standard deviation, and RSD from analyses used for calculations, and making graphs. The section is divided into two subsections, where the first subsection (**B.1**) contains raw data for the LC-UV analyses, and the second subsection (**B.2**) subsection contains raw data from the analyses from the LC-MS system. The rawdata also includes calculated average, standard deviation and RSD (%) for each individual analysis.

B.1. Raw data for the work with liquid chromatography-ultraviolet detection

B.1.1. Raw data for determining retention time for each analyte separately and in cocktail

Table B.1 shows the raw data for t_M, t_R, with the calculated t'_R used for evaluating the precicion of the retention times.

Table B.1: Raw data for the determination of retention time for each analyte both separately and in-cocktail.

Separate	Phenacetin			Acetaminophen		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.510	5.615	5.105	0.508	0.966	0.458
Rep 2	0.509	5.617	5.108	0.508	0.961	0.453
Rep 3	0.508	5.610	5.102	0.510	0.964	0.454
Rep 4	0.510	5.607	5.097	0.510	0.965	0.455
Rep 5	0.509	5.591	5.082	0.510	0.965	0.455
Average	0.509	5.608	5.099	0.509	0.964	0.455
St.dev.	0.001	0.010	0.010	0.001	0.002	0.002
RSD	0.16	0.18	0.20	0.22	0.20	0.41
Separate	Tolbutamide			4-hydroxytolbutamide		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.509	12.000	11.491	0.508	5.899	5.391
Rep 2	0.510	11.998	11.488	0.508	5.902	5.394
Rep 3	0.509	11.991	11.482	0.508	5.905	5.397
Rep 4	0.509	11.991	11.482	0.509	5.908	5.399
Rep 5	0.509	11.991	11.482	0.508	5.900	5.392
Average	0.509	11.994	11.485	0.508	5.903	5.395
St.dev.	0.000	0.004	0.004	0.000	0.004	0.003
RSD(%)	0.09	0.04	0.04	0.09	0.06	0.06
Separate	Fluoxetine			Norfluoxetine		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.513	12.742	12.229	0.510	12.341	11.831
Rep 2	0.509	12.795	12.286	0.509	12.346	11.837
Rep 3	0.509	12.781	12.272	0.509	12.345	11.836
Rep 4	0.508	12.805	12.297	0.509	12.354	11.845
Rep 5	0.509	12.828	12.319	0.509	12.362	11.853
Average	0.510	12.790	12.281	0.509	12.350	11.840
St.dev.	0.002	0.032	0.034	0.000	0.008	0.009
RSD(%)	0.38	0.25	0.27	0.09	0.07	0.07

In-cocktail	Phenacetin			Acetaminophen		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.507	5.610	5.103	0.507	0.989	0.482
Rep 2	0.507	5.579	5.072	0.507	0.957	0.450
Rep 3	0.507	5.581	5.074	0.507	0.957	0.450
Rep 4	0.507	5.571	5.064	0.507	0.958	0.451
Rep 5	0.508	5.570	5.062	0.508	0.959	0.451
Average	0.507	5.582	5.075	0.507	0.964	0.457
St.dev.	0.000	0.016	0.016	0.000	0.014	0.014
RSD	0.09	0.29	0.32	0.09	1.45	3.09
In-cocktail	Tolbutamide			4-hydroxytolbutamide		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.507	11.943	11.436	0.507	5.912	5.405
Rep 2	0.507	11.944	11.437	0.507	5.885	5.378
Rep 3	0.507	11.963	11.456	0.507	5.890	5.383
Rep 4	0.507	11.955	11.448	0.507	5.878	5.371
Rep 5	0.508	11.958	11.450	0.508	5.877	5.369
Average	0.507	11.953	11.445	0.507	5.888	5.381
St.dev.	0.000	0.009	0.009	0.000	0.014	0.014
RSD(%)	0.09	0.07	0.08	0.09	0.24	0.27
In-cocktail	Fluoxetine			Norfluoxetine		
	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.507	12.747	12.240	0.507	12.211	11.704
Rep 2	0.507	12.747	12.240	0.507	12.211	11.704
Rep 3	0.507	12.765	12.258	0.507	12.229	11.722
Rep 4	0.507	12.764	12.257	0.507	12.226	11.719
Rep 5	0.508	12.765	12.257	0.508	12.226	11.718
Average	0.507	12.760	12.253	0.507	12.221	11.713
St.dev.	0.001	0.009	0.009	0.000	0.009	0.009
RSD(%)	0.10	0.07	0.07	0.09	0.07	0.07

B.1.2. Raw data for re-evaluation of lambda max

Table B.2 shows the raw data signal area from LC-UV analyses over various wavelengths for all the analytes.

Table B.2: Raw data for the re-evaluation of λ max.

	Phenacetin (mAU)			Acetaminophen (mAU)			
nm	225	245	250	225	242	243	249
Set 1 rep 1	338.9172	197.1657		413.90341	238.74944	232.835	
Set 1 rep 2	337.0371	196.8629		417.80829	238.30049	232.077	
Set 1 rep 3	337.863	196.202		414.555	237.242	231.743	
Set 2 rep 1	334.602		185.925	412.588			213.424
Set 2 rep 2	333.169		185.456	406.753			210.991
Set 2 rep 3	332.313		185.240	404.890			210.784
Gj.snitt(x)	334.997	196.532	185.540	411.319	237.771	231.910	211.733
St.dev(s)	2.402	0.468	0.350	5.394	0.748	0.236	1.468
RSD(%)	0.72	0.24	0.19	1.31	0.31	0.10	0.69

	Tolbutamide (mAU)			4-hydroxytolbutamide(mAU)		
nm	225	227	229	225	227	229
Set 1 rep 1	132.1337	122.992	109.751	198.61739	181.56271	164.784
Set 1 rep 2	131.8793	122.230	111.039	195.80594	180.89224	163.730
Set 1 rep 3	131.770	121.105	110.196	196.165	181.291	162.581
Set 2 rep 1	129.392			197.100		
Set 2 rep 2	129.184			192.946		
Set 2 rep 3	129.144			192.118		
Average	130.274	121.667	110.618	194.827	181.092	163.155
St.dev(s)	1.419	0.796	0.596	2.168	0.282	0.813
RSD(%)	1.09	0.65	0.54	1.11	0.16	0.50

	Fluoxetine (mAU)				Norfluoxetine (mAU)				
nm	225	226	268	276	225	226	227	264	276
Set 1 rep 1	96.86867	91.95959			332.2374	312.72458	294.420		
Set 1 rep 2	96.88856	92.74908			330.95877	313.6618	296.094		
Set 1 rep 3	97.889	90.939			328.414	311.809	294.429		
Set 2 rep 1	95.185		6.489	2.359	322.698			35.946	8.22815
Set 2 rep 2	94.608		6.252	2.203	323.053			36.059	8.44683
Set 2 rep 3	95.859		6.403	2.399	323.612			35.766	8.36348
Average	96.086	91.844	6.381	2.321	325.747	312.735	295.262	35.924	8.346
St.dev(s)	1.317	1.280	0.120	0.104	3.721	1.310	1.177	0.148	0.110
RSD(%)	1.37	1.39	1.88	4.47	1.14	0.42	0.40	0.41	1.32

B.1.3. Raw data for determining limit of quantitation for liquid chromatography-ultra violet detection

Table B.3 shows the raw data for the signal area used for determining the LOQ (±20%)for LC-UV in addition to the visual evaluation.

Table B.3: Raw data for determining limit of quantitation for liquid chromatography-ultra violet detection

Analyte	A	P	4HT	T	N	F
LOQ concentration (µg/mL)	0.75	0.5	0.5	0.75	0.5	0.75
Rep 1 (mAU)	2.604	2.182	1.387	1.318	2.233	1.275
Rep 2 (mAU)	2.487	2.186	1.370	1.249	2.292	1.385
Rep 3 (mAU)	2.329	2.146	1.351	1.296	2.444	1.460
Rep 4 (mAU)	2.501	2.166	1.348	1.266	2.463	1.448
Rep 5 (mAU)	2.642	2.177	1.330	1.165	2.478	1.527
Rep 6 (mAU)	2.724	2.153	1.324	1.165	2.431	1.489
Average (area)	2.548	2.168	1.243	1.243	2.390	1.431
Standard deviation (area)	0.139	0.016	0.024	0.065	0.102	0.090
RSD (%)	5.47	0.75	1.76	5.22	4.26	6.26

B.1.4. Raw data for the partial validation over 5 days for the calibration curve for liquid chromatography-ultraviolet detection

Table B.4-B.23 shows the raw data the signal area and retention times for the validation of the linearity curve for the LC-UV over 5 concecutive days.

Table B.4: Raw data for the calibration curve for day 1 of the partial validation for LC-UV.

Day 1	Phenacetin							Acetaminophen						
Conc. (ng/mL)	0	0.5	10	20	30	40	50	0	0.75	10	20	30	40	50
1 (area)	n.d.	1.806	43.961	92.471	136.132	189.303	247.290	n.d.	1.041	49.461	107.471	156.838	221.513	274.41306
2 (area)	n.d.	1.713	43.885	91.339	135.652	172.263	222.649	n.d.	1.017	49.286	105.702	152.603	205.976	246.91861
3 (area)	n.d.	1.710	43.878	91.100	128.240	169.805	231.416	n.d.	0.987	45.182	104.464	145.314	196.482	258.46259
4 (area)	n.d.	1.752	43.939	91.456	126.645	171.856	232.366	n.d.	0.946	48.458	103.592	144.866	201.475	262.9556
5 (area)	n.d.	1.742	43.708	91.162	126.090	171.129	233.650	n.d.	0.997	47.932	105.119	150.885	200.859	262.97064
6 (area)	n.d.	1.659	44.125	89.273	126.165	175.447	232.166	n.d.	1.038	47.295	103.308	150.627	203.012	263.2847
Average	-	1.730408	43.91594	91.13357	129.820547	174.967265	233.2563117	-	1.0040523	47.935565	104.94266	150.188868	204.886178	261.500867
St.dev.	-	0.049209	0.135592	1.03905489	4.76857332	7.268125325	7.937050789	-	0.0358004	1.5757284	1.53152276	4.53472239	8.71371613	8.90007433
RSD (%)	-	2.84	0.31	1.14	3.67	4.15	3.40	-	3.57	3.29	1.46	3.02	4.25	3.40
Day 1	Tolbutamide							4-hydroxytolbutamide						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	0.754	17.351	37.489	55.531	77.406	98.063	n.d.	1.033	25.481	53.674	78.987	110.188	146.18707
2 (area)	n.d.	0.706	17.769	37.095	55.537	71.242	87.329	n.d.	1.168	25.603	53.384	78.930	101.016	131.02591
3 (area)	n.d.	0.753	17.361	37.012	52.161	70.487	91.663	n.d.	1.023	25.496	53.177	74.240	99.363	135.81575
4 (area)	n.d.	0.747	17.475	36.572	51.877	70.731	90.770	n.d.	1.032	25.368	52.847	73.818	100.267	137.06081
5 (area)	n.d.	0.781	17.407	36.844	51.676	70.665	92.078	n.d.	1.011	25.320	53.480	72.867	99.621	137.86075
6 (area)	n.d.	0.744	17.411	35.793	52.033	72.155	91.591	n.d.	0.976	25.208	52.090	73.466	102.313	137.99341
Average	-	0.74741	17.46241	36.80085	53.1356117	72.114275	91.91571333	-	1.0402433	25.412878	53.1086417	75.3845717	102.128245	137.657283
St.dev.	-	0.024267	0.156307	0.5785839	1.86466479	2.661650811	3.473660876	-	0.0659376	0.1420788	0.57360877	2.80451988	4.08980975	4.91189939
RSD (%)	-	3.25	0.90	1.57	3.51	3.69	3.78	-	6.34	0.56	1.08	3.72	4.00	3.57
Day 1	Fluoxetine							Norfluoxetine						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	0.92788	27.13517	59.18058	86.78779	122.78536	156.65141	n.d.	1.95551	51.79182	109.87184	161.17523	226.89157	290.80008
2 (area)	n.d.	0.93790	27.57066	58.32255	87.10489	111.01760	140.14130	n.d.	1.92030	52.06705	108.83809	159.72034	205.35524	259.48026
3 (area)	n.d.	0.91775	27.39174	57.63127	80.88528	108.56353	144.70605	n.d.	1.95865	51.79624	107.39257	150.50739	200.04796	271.54086
4 (area)	n.d.	0.95868	27.53202	57.17578	80.38785	111.34038	145.93376	n.d.	1.92064	51.85862	106.22228	149.36754	203.30588	272.02002
5 (area)	n.d.	0.90760	27.24866	57.77236	80.27653	109.99459	147.36456	n.d.	1.94385	51.67528	106.94803	148.08861	202.83940	274.29742
6 (area)	n.d.	0.93197	27.15081	56.42252	80.44587	113.70743	146.02000	n.d.	1.93767	51.05802	104.43327	149.70702	208.14413	273.08121
Average	-	0.930297	27.33918	57.7508433	82.648035	112.9114817	146.9028467	-	1.9394367	51.707838	107.284347	153.094355	207.76403	273.536642
St.dev.	-	0.017577	0.187939	0.94666751	3.33737729	5.132138147	5.418694057	-	0.0165507	0.3434996	1.92305408	5.76744792	9.75165986	10.0267408
RSD (%)	-	1.89	0.69	1.64	4.04	4.55	3.69	-	0.85	0.66	1.79	3.77	4.69	3.67

Table B.5: Raw data for the retention time for phenacetin and acetaminophen for day 1 of the validation of the linearity curve for LC-UV.

Dag 1	Phenacetin																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.482	5.472	4.99	0.483	5.486	5.003	0.482	5.475	4.993	0.482	5.491	5.009	0.481	5.46	4.979	0.481	5.462	4.981
Rep 2	0.482	5.48	4.998	0.482	5.478	4.996	0.482	5.469	4.987	0.483	5.494	5.011	0.482	5.491	5.009	0.482	5.473	4.991
Rep 3	0.482	5.479	4.997	0.482	5.492	5.01	0.482	5.484	5.002	0.481	5.463	4.982	0.481	5.475	4.994	0.482	5.478	4.996
Rep 4	0.481	5.454	4.973	0.482	5.478	4.996	0.482	5.485	5.003	0.481	5.467	4.986	0.483	5.468	4.985	0.482	5.48	4.998
Rep 5	0.481	5.466	4.985	0.482	5.476	4.994	0.483	5.486	5.003	0.482	5.474	4.992	0.483	5.471	4.988	0.481	5.467	4.986
Rep 6	0.482	5.479	4.997	0.482	5.482	5	0.483	5.475	4.992	0.481	5.468	4.987	0.483	5.475	4.992	0.482	5.489	5.007
Gj.snitt(x)	0.482	5.472	4.990	0.482	5.482	5.000	0.482	5.479	4.997	0.482	5.476	4.995	0.482	5.473	4.991	0.482	5.475	4.993
St.dev(s)	0.001	0.010	0.010	0.000	0.006	0.006	0.001	0.007	0.007	0.001	0.013	0.012	0.001	0.010	0.010	0.001	0.010	0.009
RSD (%)	0.107	0.187	0.196	0.085	0.111	0.119	0.107	0.127	0.138	0.170	0.240	0.249	0.204	0.188	0.205	0.107	0.176	0.185
Dag 1	Acetaminophen																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	0.881	0.399	0.483	0.878	0.395	0.482	0.874	0.392	0.482	0.875	0.393	0.481	0.87	0.389	0.481	0.866	0.385
2 (area)	0.482	0.883	0.401	0.482	0.879	0.397	0.482	0.874	0.392	0.483	0.874	0.391	0.482	0.871	0.389	0.482	0.87	0.388
3 (area)	0.482	0.882	0.4	0.482	0.878	0.396	0.482	0.877	0.395	0.481	0.875	0.394	0.481	0.869	0.388	0.482	0.868	0.386
4 (area)	0.481	0.88	0.399	0.482	0.878	0.396	0.482	0.875	0.393	0.481	0.871	0.39	0.483	0.872	0.389	0.482	0.87	0.388
5 (area)	0.481	0.876	0.395	0.482	0.876	0.394	0.483	0.874	0.391	0.482	0.873	0.391	0.483	0.871	0.388	0.481	0.868	0.387
6 (area)	0.482	0.881	0.399	0.482	0.876	0.394	0.483	0.879	0.396	0.481	0.869	0.388	0.483	0.87	0.387	0.482	0.871	0.389
Average	0.482	0.881	0.399	0.482	0.878	0.395	0.482	0.876	0.393	0.482	0.873	0.391	0.482	0.871	0.388	0.482	0.869	0.387
St.dev.	0.001	0.002	0.002	0.000	0.001	0.001	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.002	0.001
RSD (%)	0.107	0.276	0.512	0.085	0.140	0.306	0.107	0.237	0.494	0.170	0.275	0.546	0.204	0.120	0.210	0.107	0.211	0.380

Table B.6: Raw data for the retention time for tolbutamide and 4-hydroxytolbutamide for day 1 of the validation of the linearity curve for LC-UV.

Dag 1	Tolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	11.957	11.475	0.483	11.949	11.466	0.482	11.93	11.44	0.482	11.95	11.465	0.481	11.923	11.44	0.481	11.94	11.46
2 (area)	0.482	11.957	11.475	0.482	11.948	11.466	0.482	11.94	11.46	0.483	11.97	11.484	0.482	11.953	11.47	0.482	11.93	11.44
3 (area)	0.482	11.943	11.461	0.482	11.953	11.471	0.482	11.95	11.46	0.481	11.9	11.415	0.481	11.935	11.45	0.482	11.95	11.46
4 (area)	0.481	11.934	11.453	0.482	11.944	11.462	0.482	11.94	11.46	0.481	11.92	11.44	0.483	11.926	11.44	0.482	11.93	11.45
5 (area)	0.481	11.927	11.446	0.482	11.936	11.454	0.483	11.95	11.47	0.482	11.92	11.441	0.483	11.931	11.45	0.481	11.93	11.45
6 (area)	0.482	11.943	11.461	0.482	11.937	11.455	0.483	11.93	11.45	0.481	11.94	11.457	0.483	11.934	11.45	0.482	11.96	11.48
Average	0.482	11.944	11.462	0.482	11.945	11.462	0.482	11.938	11.456	0.482	11.932	11.450	0.482	11.934	11.452	0.482	11.938	11.456
St.dev.	0.001	0.012	0.012	0.000	0.007	0.007	0.001	0.010	0.010	0.001	0.024	0.024	0.001	0.011	0.011	0.001	0.013	0.013
RSD (%)	0.107	0.101	0.102	0.085	0.057	0.059	0.107	0.087	0.090	0.170	0.205	0.208	0.204	0.088	0.093	0.107	0.107	0.111
Dag 1	4-hydroxytolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	5.772	5.29	0.483	5.786	5.303	0.482	5.776	5.294	0.482	5.788	5.306	0.481	5.763	5.282	0.481	5.764	5.283
2 (area)	0.482	5.794	5.312	0.482	5.777	5.295	0.482	5.768	5.286	0.483	5.793	5.31	0.482	5.79	5.308	0.482	5.772	5.29
3 (area)	0.482	5.779	5.297	0.482	5.792	5.31	0.482	5.782	5.3	0.481	5.76	5.279	0.481	5.777	5.296	0.482	5.777	5.295
4 (area)	0.481	5.76	5.279	0.482	5.779	5.297	0.482	5.784	5.302	0.481	5.769	5.288	0.483	5.766	5.283	0.482	5.78	5.298
5 (area)	0.481	5.766	5.285	0.482	5.776	5.294	0.483	5.785	5.302	0.482	5.777	5.295	0.483	5.77	5.287	0.481	5.765	5.284
6 (area)	0.482	5.779	5.297	0.482	5.782	5.3	0.483	5.774	5.291	0.481	5.772	5.291	0.483	5.776	5.293	0.482	5.783	5.301
Average	0.482	5.775	5.293	0.482	5.782	5.300	0.482	5.778	5.296	0.482	5.777	5.295	0.482	5.774	5.292	0.482	5.774	5.292
St.dev.	0.001	0.012	0.012	0.000	0.006	0.006	0.001	0.007	0.007	0.001	0.012	0.012	0.001	0.010	0.010	0.001	0.008	0.007
RSD (%)	0.107	0.206	0.217	0.085	0.105	0.113	0.107	0.115	0.124	0.170	0.213	0.218	0.204	0.168	0.185	0.107	0.136	0.140

Table B.7: Raw data for the retention time for fluoxetine and norfluoxetine for day 1 of the validation of the linearity curve for LC-UV.

Dag 1	Fluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	12.968	12.486	0.483	12.918	12.435	0.482	12.85	12.37	0.482	12.84	12.36	0.481	12.792	12.31	0.481	12.78	12.3
2 (area)	0.482	12.967	12.485	0.482	12.913	12.431	0.482	12.87	12.38	0.483	12.86	12.381	0.482	12.824	12.34	0.482	12.78	12.3
3 (area)	0.482	12.956	12.474	0.482	12.912	12.43	0.482	12.87	12.39	0.481	12.81	12.327	0.481	12.806	12.33	0.482	12.79	12.31
4 (area)	0.481	12.947	12.466	0.482	12.908	12.426	0.482	12.87	12.38	0.481	12.83	12.344	0.483	12.803	12.32	0.482	12.78	12.3
5 (area)	0.481	12.949	12.468	0.482	12.9	12.418	0.483	12.88	12.39	0.482	12.83	12.344	0.483	12.806	12.32	0.481	12.79	12.31
6 (area)	0.482	12.961	12.479	0.482	12.901	12.419	0.483	12.86	12.38	0.481	12.84	12.36	0.483	12.807	12.32	0.482	12.81	12.33
Average	0.482	12.958	12.476	0.482	12.909	12.427	0.482	12.865	12.383	0.482	12.834	12.353	0.482	12.806	12.324	0.482	12.789	12.308
St.dev.	0.001	0.009	0.008	0.000	0.007	0.007	0.001	0.009	0.009	0.001	0.019	0.019	0.001	0.010	0.010	0.001	0.010	0.010
RSD (%)	0.107	0.069	0.068	0.085	0.055	0.055	0.107	0.074	0.075	0.170	0.149	0.150	0.204	0.080	0.082	0.107	0.081	0.083
Dag 1	Norfluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	12.525	12.043	0.483	12.451	11.968	0.482	12.37	11.89	0.482	12.35	11.866	0.481	12.283	11.8	0.481	12.26	11.78
2 (area)	0.482	12.521	12.039	0.482	12.448	11.966	0.482	12.38	11.9	0.483	12.37	11.888	0.482	12.32	11.84	0.482	12.27	11.79
3 (area)	0.482	12.511	12.029	0.482	12.445	11.963	0.482	12.39	11.91	0.481	12.31	11.833	0.481	12.306	11.83	0.482	12.28	11.8
4 (area)	0.481	12.501	12.02	0.482	12.44	11.958	0.482	12.38	11.9	0.481	12.33	11.851	0.483	12.298	11.82	0.482	12.27	11.78
5 (area)	0.481	12.501	12.02	0.482	12.434	11.952	0.483	12.39	11.91	0.482	12.33	11.851	0.483	12.302	11.82	0.481	12.27	11.79
6 (area)	0.482	12.514	12.032	0.482	12.434	11.952	0.483	12.38	11.9	0.481	12.35	11.867	0.483	12.302	11.82	0.482	12.29	11.81
Average	0.482	12.512	12.031	0.482	12.442	11.960	0.482	12.383	11.901	0.482	12.341	11.859	0.482	12.302	11.820	0.482	12.273	11.791
St.dev.	0.001	0.010	0.010	0.000	0.007	0.007	0.001	0.009	0.009	0.001	0.019	0.019	0.001	0.012	0.012	0.001	0.011	0.010
RSD (%)	0.107	0.080	0.079	0.085	0.058	0.058	0.107	0.073	0.074	0.170	0.157	0.158	0.204	0.097	0.100	0.107	0.087	0.089

Table B.8: Raw data for the calibration curve for day 2 of the partial validation for LC-UV.

Day 2	Phenacetin							Acetaminophen						
Conc. (ng/mL)	0	0.5	10	20	30	40	50	0	0.75	10	20	30	40	50
1 (area)	n.d.	2.148	47.383	95.873	144.972	203.098	11.11670*	n.d.	2.419	55.338	106.672	169.138	238.266	9.78925*
2 (area)	n.d.	2.162	46.873	93.235	135.154	178.066	231.784	n.d.	2.431	53.288	104.878	161.031	208.329	283.3692
3 (area)	n.d.	2.093	45.774	92.930	131.837	180.280	229.832	n.d.	2.266	50.388	109.300	154.241	212.758	282.2899
4 (area)	n.d.	2.209	45.449	95.572	132.884	183.187	231.553	n.d.	2.192	50.917	111.491	152.792	214.865	280.5809
5 (area)	n.d.	2.001	47.072	95.622	135.314	182.785	230.943	n.d.	2.076	52.135	111.001	158.453	214.804	282.4795
6 (area)	n.d.	2.020	46.138	92.800	133.883	185.599	228.152	n.d.	2.307	50.089	108.289	159.561	214.108	280.0983
Average	-	2.105467	46.4481	94.33868	135.6738	185.5024	230.4528	-	2.281728	52.02586	108.605	159.2026	217.1882	281.7636
St.dev.	-	0.08268	0.773686	1.489254	4.745082	8.999357	1.492035	-	0.13614	2.012062	2.540842	5.804549	10.6109	1.372903
RSD (%)	-	3.93	1.67	1.58	3.50	4.85	0.65	-	5.97	3.87	2.34	3.65	4.89	0.49
Day 2	Tolbutamide							4-hydroxytolbutamide						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	1.090	18.828	37.807	56.982	78.861	4.22270*	n.d.	1.324	28.481	57.204	86.768	121.813	6.98147*
2 (area)	n.d.	1.203	18.605	37.421	53.138	69.795	93.131	n.d.	1.295	28.067	56.304	81.231	106.725	142.5356
3 (area)	n.d.	1.119	17.759	36.774	52.560	71.302	91.657	n.d.	1.284	27.069	55.644	79.774	108.023	141.6096
4 (area)	n.d.	1.085	17.733	36.838	51.716	72.285	92.744	n.d.	1.356	27.105	57.187	79.740	110.406	142.4711
5 (area)	n.d.	1.097	17.968	36.725	53.527	72.395	91.698	n.d.	1.269	27.814	57.232	82.069	110.006	142.3375
6 (area)	n.d.	1.099	18.136	35.476	53.874	73.445	91.157	n.d.	1.230	27.338	55.977	81.282	112.291	140.909
Average	-	1.115567	18.17163	36.84019	53.63257	73.01388	92.0774	-	1.292887	27.6455	56.5914	81.81065	111.544	141.9726
St.dev.	-	0.044547	0.452645	0.793788	1.808592	3.117358	0.824958	-	0.043791	0.570003	0.706654	2.595807	5.390348	0.700303
RSD (%)	-	3.99	2.49	2.15	3.37	4.27	0.90	-	3.39	2.06	1.25	3.17	4.83	0.49
Day 2	Fluoxetine							Norfluoxetine						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	1.437	30.723	62.232	93.516	130.881	5.73144*	n.d.	2.200	54.615	109.767	167.258	234.298	12.23035*
2 (area)	n.d.	1.470	30.011	60.250	86.893	116.227	128.151	n.d.	2.288	53.348	108.125	153.842	206.495	259.8546
3 (area)	n.d.	1.468	29.274	59.141	84.535	117.196	128.572	n.d.	2.222	51.523	106.425	151.221	208.739	258.3271
4 (area)	n.d.	1.444	29.930	60.745	85.265	119.573	127.691	n.d.	2.183	52.492	108.622	152.114	212.860	258.6311
5 (area)	n.d.	1.711	29.921	59.930	87.428	118.043	126.219	n.d.	2.538	53.598	107.385	156.561	211.401	257.0063
6 (area)	n.d.	1.534	29.700	57.595	86.724	121.262	126.178	n.d.	2.255	52.551	104.107	154.200	216.445	256.2318
Average	-	1.510705	29.92637	59.98219	87.39357	120.5303	127.3625	-	2.280885	53.021	107.4051	155.8661	215.0396	258.0102
St.dev.	-	0.104062	0.472943	1.557223	3.190117	5.374208	1.10728	-	0.131504	1.070039	1.970654	5.878608	10.03566	1.419365
RSD (%)	-	6.89	1.58	2.60	3.65	4.46	0.87	-	5.77	2.02	1.83	3.77	4.67	0.55

Table B.9: Raw data for the retention time for phenacetin and acetaminophen for day 2 of the validation of the linearity curve for LC-UV.

Dag 2	Phenacetin																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.485	5.509	5.024	0.483	n.d.	-	0.482	5.468	4.986	0.481	5.509	5.028	0.485	5.54	5.055	0.507	5.445	4.938
Rep 2	0.482	5.49	5.008	0.483	n.d.	-	0.482	5.474	4.992	0.484	5.487	5.003	0.484	5.533	5.049	0.485	5.508	5.023
Rep 3	0.482	5.467	4.985	0.482	n.d.	-	0.483	5.505	5.022	0.483	5.492	5.009	0.486	5.551	5.065	0.482	5.496	5.014
Rep 4	0.482	5.478	4.996	0.483	n.d.	-	0.483	5.495	5.012	0.484	5.495	5.011	0.487	5.552	5.065	0.483	5.522	5.039
Rep 5	0.482	5.491	5.009	0.482	n.d.	-	0.481	5.472	4.991	0.484	5.49	5.006	0.487	5.573	5.086	0.483	5.498	5.015
Rep 6	0.483	5.491	5.008	0.481	n.d.	-	0.485	5.519	5.034	0.483	5.51	5.027	0.489	5.567	5.078	0.482	5.482	5
Gj.snitt(x)	0.483	5.488	5.005	0.482	-	-	0.483	5.489	5.006	0.483	5.497	5.014	0.486	5.553	5.066	0.487	5.492	5.005
St.dev(s)	0.001	0.014	0.013	0.001	-	-	0.001	0.021	0.019	0.001	0.010	0.011	0.002	0.015	0.014	0.010	0.027	0.035
RSD (%)	0.251	0.258	0.264	0.169	-	-	0.283	0.378	0.389	0.242	0.180	0.216	0.360	0.275	0.273	2.024	0.483	0.702
Dag 2	Acetaminophen																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.485	0.89	0.405	0.483	0.877	0.394	0.482	0.875	0.393	0.481	0.876	0.395	0.485	0.881	0.396	0.507	0.896	0.389
2 (area)	0.482	0.881	0.399	0.483	0.878	0.395	0.482	0.876	0.394	0.484	0.878	0.394	0.484	0.879	0.395	0.485	0.878	0.393
3 (area)	0.482	0.878	0.396	0.482	0.876	0.394	0.483	0.878	0.395	0.483	0.875	0.392	0.486	0.883	0.397	0.482	0.871	0.389
4 (area)	0.482	0.88	0.398	0.483	0.88	0.397	0.483	0.88	0.397	0.484	0.875	0.391	0.487	0.887	0.4	0.483	0.873	0.39
5 (area)	0.482	0.883	0.401	0.482	0.876	0.394	0.481	0.877	0.396	0.484	0.876	0.392	0.487	0.886	0.399	0.483	0.874	0.391
6 (area)	0.483	0.885	0.402	0.481	0.876	0.395	0.485	0.882	0.397	0.483	0.886	0.403	0.489	0.886	0.397	0.482	0.87	0.388
Average	0.483	0.883	0.400	0.482	0.877	0.395	0.483	0.878	0.395	0.483	0.878	0.395	0.486	0.884	0.397	0.487	0.877	0.390
St.dev.	0.001	0.004	0.003	0.001	0.002	0.001	0.001	0.003	0.002	0.001	0.004	0.004	0.002	0.003	0.002	0.010	0.010	0.002
RSD (%)	0.251	0.483	0.797	0.169	0.183	0.296	0.283	0.297	0.413	0.242	0.482	1.119	0.360	0.363	0.469	2.024	1.108	0.459

Table B.10: Raw data for the retention time for tolbutamide and 4-hydroxytolbutamide for day 2 of the validation of the linearity curve for LC-UV.

Dag 2	Tolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.485	11.979	11.494	0.483	11.946	11.463	0.482	11.94	11.46	0.481	11.96	11.481	0.485	12.023	11.54	0.507	12.03	11.52
2 (area)	0.482	11.942	11.46	0.483	11.954	11.471	0.482	11.93	11.45	0.484	11.96	11.475	0.484	12.021	11.54	0.485	11.98	11.49
3 (area)	0.482	11.929	11.447	0.482	11.956	11.474	0.483	11.96	11.48	0.483	11.96	11.477	0.486	12.049	11.56	0.482	11.97	11.49
4 (area)	0.482	11.954	11.472	0.483	11.947	11.464	0.483	11.96	11.47	0.484	11.96	11.472	0.487	12.049	11.56	0.483	11.98	11.49
5 (area)	0.482	11.961	11.479	0.482	11.938	11.456	0.481	11.94	11.46	0.484	11.97	11.487	0.487	12.074	11.59	0.483	11.98	11.5
6 (area)	0.483	11.963	11.48	0.481	11.953	11.472	0.485	11.99	11.5	0.483	12	11.516	0.489	12.07	11.58	0.482	11.95	11.46
Average	0.483	11.955	11.472	0.482	11.949	11.467	0.483	11.952	11.470	0.483	11.968	11.485	0.486	12.048	11.561	0.487	11.979	11.492
St.dev.	0.001	0.017	0.017	0.001	0.007	0.007	0.001	0.021	0.020	0.001	0.016	0.016	0.002	0.022	0.021	0.010	0.028	0.019
RSD (%)	0.251	0.146	0.144	0.169	0.056	0.060	0.283	0.179	0.175	0.242	0.134	0.141	0.360	0.186	0.181	2.024	0.230	0.166
Dag 2	4-hydroxytolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.485	5.802	5.317	0.483	5.775	5.292	0.482	5.768	5.286	0.481	5.809	5.328	0.485	5.837	5.352	0.507	5.845	5.338
2 (area)	0.482	5.784	5.302	0.483	5.79	5.307	0.482	5.773	5.291	0.484	5.787	5.303	0.484	5.828	5.344	0.485	5.804	5.319
3 (area)	0.482	5.767	5.285	0.482	5.788	5.306	0.483	5.804	5.321	0.483	5.792	5.309	0.486	5.844	5.358	0.482	5.799	5.317
4 (area)	0.482	5.778	5.296	0.483	5.78	5.297	0.483	5.793	5.31	0.484	5.796	5.312	0.487	5.846	5.359	0.483	5.819	5.336
5 (area)	0.482	5.786	5.304	0.482	5.777	5.295	0.481	5.773	5.292	0.484	5.789	5.305	0.487	5.864	5.377	0.483	5.795	5.312
6 (area)	0.483	5.791	5.308	0.481	5.79	5.309	0.485	5.816	5.331	0.483	5.81	5.327	0.489	5.86	5.371	0.482	5.777	5.295
Average	0.483	5.785	5.302	0.482	5.783	5.301	0.483	5.788	5.305	0.483	5.797	5.314	0.486	5.847	5.360	0.487	5.807	5.320
St.dev.	0.001	0.012	0.011	0.001	0.007	0.007	0.001	0.020	0.018	0.001	0.010	0.011	0.002	0.014	0.012	0.010	0.023	0.016
RSD (%)	0.251	0.204	0.205	0.169	0.118	0.136	0.283	0.338	0.346	0.242	0.173	0.205	0.360	0.233	0.226	2.024	0.400	0.300

Table B.11: Raw data for the retention time for fluoxetine and norfluoxetine for day 2 of the validation of the linearity curve for LC-UV.

Dag 2	Fluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.485	12.972	12.487	0.483	12.899	12.416	0.482	12.86	12.37	0.481	12.84	12.355	0.485	12.86	12.38	0.507	13	12.49
2 (area)	0.482	12.94	12.458	0.483	12.906	12.423	0.482	12.86	12.37	0.484	12.85	12.361	0.484	12.873	12.39	0.485	12.82	12.33
3 (area)	0.482	12.946	12.464	0.482	12.909	12.427	0.483	12.88	12.39	0.483	12.85	12.365	0.486	12.893	12.41	0.482	12.83	12.35
4 (area)	0.482	12.957	12.475	0.483	12.899	12.416	0.483	12.86	12.38	0.484	12.84	12.356	0.487	12.896	12.41	0.483	12.82	12.34
5 (area)	0.482	12.961	12.479	0.482	12.891	12.409	0.481	12.85	12.37	0.484	12.86	12.372	0.487	12.913	12.43	0.483	12.83	12.35
6 (area)	0.483	12.966	12.483	0.481	12.899	12.418	0.485	12.89	12.4	0.483	12.88	12.396	0.489	12.905	12.42	0.482	12.8	12.32
Average	0.483	12.957	12.474	0.482	12.901	12.418	0.483	12.865	12.383	0.483	12.851	12.368	0.486	12.890	12.404	0.487	12.850	12.363
St.dev.	0.001	0.012	0.011	0.001	0.006	0.006	0.001	0.014	0.012	0.001	0.015	0.015	0.002	0.020	0.019	0.010	0.073	0.063
RSD (%)	0.251	0.093	0.090	0.169	0.049	0.050	0.283	0.106	0.100	0.242	0.121	0.124	0.360	0.155	0.150	2.024	0.565	0.509
Dag 2	Norfluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.485	12.533	12.048	0.483	12.433	11.95	0.482	12.38	11.89	0.481	12.34	11.862	0.485	12.356	11.87	0.507	12.56	12.05
2 (area)	0.482	12.501	12.019	0.483	12.441	11.958	0.482	12.37	11.89	0.484	12.35	11.869	0.484	12.377	11.89	0.485	12.3	11.81
3 (area)	0.482	12.502	12.02	0.482	12.447	11.965	0.483	12.4	11.91	0.483	12.36	11.877	0.486	12.392	11.91	0.482	12.31	11.83
4 (area)	0.482	12.518	12.036	0.483	12.437	11.954	0.483	12.39	11.9	0.484	12.35	11.869	0.487	12.395	11.91	0.483	12.31	11.82
5 (area)	0.482	12.522	12.04	0.482	12.427	11.945	0.481	12.37	11.89	0.484	12.36	11.879	0.487	12.415	11.93	0.483	12.32	11.83
6 (area)	0.483	12.525	12.042	0.481	12.436	11.955	0.485	12.41	11.93	0.483	12.39	11.907	0.489	12.406	11.92	0.482	12.28	11.8
Average	0.483	12.517	12.034	0.482	12.437	11.955	0.483	12.386	11.903	0.483	12.360	11.877	0.486	12.390	11.904	0.487	12.345	11.858
St.dev.	0.001	0.013	0.012	0.001	0.007	0.007	0.001	0.015	0.014	0.001	0.016	0.016	0.002	0.021	0.020	0.010	0.104	0.094
RSD (%)	0.251	0.103	0.100	0.169	0.055	0.057	0.283	0.122	0.116	0.242	0.130	0.133	0.360	0.171	0.167	2.024	0.841	0.794

Table B.12: Raw data for the calibration curve for day 3 of the validation of the linearity curve for LC-UV.

Day 3	Phenacetin							Acetaminophen						
Conc. (ng/mL)	0	0.5	10	20	30	40	50	0	0.75	10	20	30	40	50
1 (area)	n.d.	2.001	46.922	93.783	141.256	194.847	261.596	n.d.	3.486	53.605	111.704	168.848	237.106	313.7583
2 (area)	n.d.	1.954	46.072	91.904	128.524	177.406	238.051	n.d.	3.232	52.965	109.694	154.141	216.427	288.9979
3 (area)	n.d.	2.043	45.971	88.436	130.675	176.555	243.668	n.d.	3.354	53.971	104.996	156.577	219.106	298.6917
4 (area)	n.d.	1.951	46.277	90.692	128.599	176.438	245.342	n.d.	3.252	53.733	108.994	159.390	216.149	304.4382
5 (area)	n.d.	1.968	46.615	91.821	131.290	177.065	246.414	n.d.	3.102	54.041	107.767	160.647	216.566	310.2452
6 (area)	n.d.	1.939	47.692	90.551	134.908	178.557	241.512	n.d.	3.300	52.990	106.403	168.015	217.214	300.2581
Average	-	1.976012	46.59133	91.1978	132.542	180.1445	246.097	-	3.287722	53.55086	108.2595	161.2698	220.428	302.7316
St.dev.	-	0.03914	0.644074	1.781841	4.863955	7.242854	8.1554	-	0.128794	0.471271	2.400052	5.994781	8.23993	8.854478
RSD (%)	-	1.98	1.38	1.95	3.67	4.02	3.31	-	3.92	0.88	2.22	3.72	3.74	2.92
Day 3	Tolbutamide							4-hydroxytolbutamide						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	1.425	18.624	37.374	56.868	77.804	102.996	n.d.	1.164	27.970	55.739	84.061	115.329	156.4637
2 (area)	n.d.	1.381	17.689	36.567	52.124	71.870	94.548	n.d.	1.238	27.137	55.015	76.570	105.458	142.9509
3 (area)	n.d.	1.448	17.905	34.686	51.416	71.756	94.698	n.d.	1.202	27.469	52.997	76.955	105.806	145.9894
4 (area)	n.d.	1.409	17.907	35.680	52.267	71.417	95.225	n.d.	1.170	27.798	53.765	77.022	105.062	145.5428
5 (area)	n.d.	1.317	17.849	35.726	52.581	71.366	94.454	n.d.	1.186	27.796	53.859	77.943	105.122	145.8552
6 (area)	n.d.	1.310	19.079	35.212	55.340	73.155	94.801	n.d.	1.173	28.845	53.389	80.243	105.931	144.7639
Average	-	1.381572	18.17544	35.87404	53.43268	72.89479	96.12034	-	1.189025	27.83579	54.12721	78.79894	107.1179	146.9277
St.dev.	-	0.057244	0.548651	0.963244	2.159128	2.491548	3.379239	-	0.027803	0.576693	1.040412	2.899309	4.037937	4.803738
RSD (%)	-	4.14	3.02	2.69	4.04	3.42	3.52	-	2.34	2.07	1.92	3.68	3.77	3.27
Day 3	Fluoxetine							Norfluoxetine						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	1.462	25.923	53.826	82.215	115.804	150.747	n.d.	1.429	43.978	88.457	134.911	184.795	239.3775
2 (area)	n.d.	1.364	25.017	52.873	74.393	103.712	137.384	n.d.	1.444	42.226	86.620	121.697	168.064	219.9606
3 (area)	n.d.	1.402	25.795	49.902	74.864	104.567	140.705	n.d.	1.450	42.979	83.299	123.846	170.004	225.2939
4 (area)	n.d.	1.439	25.514	50.898	74.485	105.337	141.794	n.d.	1.121	42.154	85.004	120.399	168.844	225.9436
5 (area)	n.d.	1.411	25.095	51.384	75.957	104.376	141.675	n.d.	1.407	42.390	85.097	123.720	168.905	226.585
6 (area)	n.d.	1.453	26.152	50.300	78.341	105.938	139.175	n.d.	1.376	44.300	82.034	125.657	171.924	223.2857
Average	-	1.421955	25.58268	51.53052	76.70925	106.6223	141.9131	-	1.371255	43.00439	85.0853	125.0383	172.0893	226.741
St.dev.	-	0.036523	0.45783	1.527078	3.074576	4.564362	4.638364	-	0.125444	0.931098	2.290459	5.17117	6.366881	6.638505
RSD (%)	-	2.57	1.79	2.96	4.01	4.28	3.27	-	9.15	2.17	2.69	4.14	3.70	2.93

Table B.13: Raw data for the retention time for phenacetin and acetaminophen for day 3 of the validation of the linearity curve for LC-UV.

Dag 3	Phenacetin																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.488	5.55	5.062	0.486	5.527	5.041	0.488	5.541	5.053	0.488	5.566	5.078	0.49	5.58	5.09	0.49	5.563	5.073
Rep 2	0.487	5.543	5.056	0.483	5.507	5.024	0.486	5.538	5.052	0.49	5.575	5.085	0.49	5.576	5.086	0.492	5.577	5.085
Rep 3	0.487	5.571	5.084	0.485	5.492	5.007	0.487	5.533	5.046	0.491	5.586	5.095	0.49	5.588	5.098	0.493	5.593	5.1
Rep 4	0.487	5.549	5.062	0.487	5.535	5.048	0.486	5.549	5.063	0.488	5.598	5.11	0.489	5.589	5.1	0.493	5.58	5.087
Rep 5	0.488	5.54	5.052	0.484	5.528	5.044	0.487	5.547	5.06	0.49	5.573	5.083	0.489	5.593	5.104	0.493	5.591	5.098
Rep 6	0.485	5.523	5.038	0.487	5.554	5.067	0.488	5.549	5.061	0.491	5.588	5.097	0.491	5.558	5.067	0.492	5.572	5.08
Gj.snitt(x)	0.487	5.546	5.059	0.485	5.524	5.039	0.487	5.543	5.056	0.490	5.581	5.091	0.490	5.581	5.091	0.492	5.579	5.087
St.dev(s)	0.001	0.016	0.015	0.002	0.022	0.021	0.001	0.007	0.007	0.001	0.012	0.012	0.001	0.013	0.013	0.001	0.011	0.010
RSD (%)	0.225	0.282	0.299	0.336	0.393	0.411	0.184	0.119	0.130	0.279	0.210	0.229	0.154	0.228	0.264	0.238	0.204	0.204
Dag 3	Acetaminophen																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.488	0.892	0.404	0.486	0.886	0.4	0.488	0.889	0.401	0.488	0.886	0.398	0.49	0.89	0.4	0.49	0.859	0.369
2 (area)	0.487	0.894	0.407	0.483	0.88	0.397	0.486	0.884	0.398	0.49	0.891	0.401	0.49	0.891	0.401	0.492	0.864	0.372
3 (area)	0.487	0.895	0.408	0.485	0.88	0.395	0.487	0.885	0.398	0.491	0.895	0.404	0.49	0.892	0.402	0.493	0.872	0.379
4 (area)	0.487	0.894	0.407	0.487	0.887	0.4	0.486	0.883	0.397	0.488	0.892	0.404	0.489	0.892	0.403	0.493	0.867	0.374
5 (area)	0.488	0.894	0.406	0.484	0.884	0.4	0.487	0.884	0.397	0.49	0.893	0.403	0.489	0.891	0.402	0.493	0.869	0.376
6 (area)	0.485	0.888	0.403	0.487	0.889	0.402	0.488	0.89	0.402	0.491	0.895	0.404	0.491	0.863	0.372	0.492	0.867	0.375
Average	0.487	0.893	0.406	0.485	0.884	0.399	0.487	0.886	0.399	0.490	0.892	0.402	0.490	0.887	0.397	0.492	0.866	0.374
St.dev.	0.001	0.003	0.002	0.002	0.004	0.003	0.001	0.003	0.002	0.001	0.003	0.002	0.001	0.012	0.012	0.001	0.004	0.003
RSD (%)	0.225	0.287	0.478	0.336	0.421	0.634	0.184	0.330	0.536	0.279	0.375	0.602	0.154	1.301	3.057	0.238	0.514	0.917

Table B.14: Raw data for the retention time for tolbutamide and 4-hydroxytolbutamide for day 3 of the validation of the linearity curve for LC-UV.

Dag 3	Tolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.488	12.039	11.551	0.486	12.014	11.528	0.488	12.03	11.54	0.488	12.07	11.578	0.49	12.08	11.59	0.49	12.13	11.64
2 (area)	0.487	12.048	11.561	0.483	11.965	11.482	0.486	12.03	11.55	0.49	12.1	11.608	0.49	12.084	11.59	0.492	12.14	11.64
3 (area)	0.487	12.053	11.566	0.485	12.006	11.521	0.487	12.04	11.55	0.491	12.11	11.614	0.49	12.082	11.59	0.493	12.14	11.65
4 (area)	0.487	12.046	11.559	0.487	12.03	11.543	0.486	12.03	11.54	0.488	12.1	11.61	0.489	12.081	11.59	0.493	12.13	11.64
5 (area)	0.488	12.035	11.547	0.484	12.013	11.529	0.487	12.05	11.56	0.49	12.08	11.592	0.489	12.105	11.62	0.493	12.13	11.63
6 (area)	0.485	12.002	11.517	0.487	12.072	11.585	0.488	12.03	11.54	0.491	12.09	11.601	0.491	12.087	11.6	0.492	12.11	11.62
Average	0.487	12.037	11.550	0.485	12.017	11.531	0.487	12.034	11.547	0.490	12.090	11.601	0.490	12.087	11.597	0.492	12.128	11.635
St.dev.	0.001	0.018	0.018	0.002	0.035	0.033	0.001	0.007	0.007	0.001	0.014	0.013	0.001	0.009	0.010	0.001	0.009	0.009
RSD (%)	0.225	0.153	0.153	0.336	0.289	0.290	0.184	0.061	0.063	0.279	0.117	0.116	0.154	0.078	0.084	0.238	0.078	0.079
Dag 3	4-hydroxytolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.488	5.837	5.349	0.486	5.824	5.338	0.488	5.837	5.349	0.488	5.859	5.371	0.49	5.874	5.384	0.49	5.853	5.363
2 (area)	0.487	5.836	5.349	0.483	5.804	5.321	0.486	5.835	5.349	0.49	5.871	5.381	0.49	5.869	5.379	0.492	5.865	5.373
3 (area)	0.487	5.858	5.371	0.485	5.793	5.308	0.487	5.83	5.343	0.491	5.878	5.387	0.49	5.879	5.389	0.493	5.879	5.386
4 (area)	0.487	5.843	5.356	0.487	5.833	5.346	0.486	5.847	5.361	0.488	5.89	5.402	0.489	5.878	5.389	0.493	5.868	5.375
5 (area)	0.488	5.834	5.346	0.484	5.827	5.343	0.487	5.845	5.358	0.49	5.866	5.376	0.489	5.882	5.393	0.493	5.88	5.387
6 (area)	0.485	5.816	5.331	0.487	5.852	5.365	0.488	5.844	5.356	0.491	5.881	5.39	0.491	5.849	5.358	0.492	5.858	5.366
Average	0.487	5.837	5.350	0.485	5.822	5.337	0.487	5.840	5.353	0.490	5.874	5.385	0.490	5.872	5.382	0.492	5.867	5.375
St.dev.	0.001	0.014	0.013	0.002	0.021	0.020	0.001	0.007	0.007	0.001	0.011	0.011	0.001	0.012	0.013	0.001	0.011	0.010
RSD (%)	0.225	0.233	0.244	0.336	0.361	0.375	0.184	0.114	0.127	0.279	0.189	0.205	0.154	0.205	0.236	0.238	0.186	0.185

Table B.15: Raw data for the retention time for fluoxetine and norfluoxetine for day 3 of the validation of the linearity curve for LC-UV.

Dag 3	Fluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.488	13.024	12.536	0.486	12.958	12.472	0.488	12.93	12.45	0.488	12.93	12.441	0.49	12.914	12.42	0.49	12.93	12.44
2 (area)	0.487	13.027	12.54	0.483	12.917	12.434	0.486	12.94	12.45	0.49	12.96	12.473	0.49	12.929	12.44	0.492	12.95	12.46
3 (area)	0.487	13.028	12.541	0.485	12.967	12.482	0.487	12.95	12.46	0.491	12.97	12.479	0.49	12.925	12.44	0.493	12.94	12.45
4 (area)	0.487	13.029	12.542	0.487	12.97	12.483	0.486	12.94	12.45	0.488	12.96	12.47	0.489	12.927	12.44	0.493	12.94	12.45
5 (area)	0.488	13.021	12.533	0.484	12.961	12.477	0.487	12.95	12.46	0.49	12.95	12.459	0.489	12.947	12.46	0.493	12.93	12.44
6 (area)	0.485	12.999	12.514	0.487	13	12.513	0.488	12.94	12.45	0.491	12.96	12.464	0.491	12.933	12.44	0.492	12.93	12.43
Average	0.487	13.021	12.534	0.485	12.962	12.477	0.487	12.941	12.454	0.490	12.954	12.464	0.490	12.929	12.439	0.492	12.937	12.445
St.dev.	0.001	0.011	0.011	0.002	0.027	0.025	0.001	0.007	0.007	0.001	0.014	0.013	0.001	0.011	0.011	0.001	0.008	0.008
RSD (%)	0.225	0.087	0.084	0.336	0.206	0.204	0.184	0.052	0.054	0.279	0.109	0.107	0.154	0.084	0.089	0.238	0.063	0.063
Dag 3	Norfluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.488	12.584	12.096	0.486	12.501	12.015	0.488	12.46	11.98	0.488	12.45	11.963	0.49	12.425	11.94	0.49	12.44	11.95
2 (area)	0.487	12.59	12.103	0.483	12.458	11.975	0.486	12.47	11.98	0.49	12.49	11.996	0.49	12.445	11.96	0.492	12.46	11.96
3 (area)	0.487	12.59	12.103	0.485	12.504	12.019	0.487	12.48	11.99	0.491	12.49	12.003	0.49	12.441	11.95	0.493	12.45	11.96
4 (area)	0.487	12.59	12.103	0.487	12.512	12.025	0.486	12.46	11.98	0.488	12.48	11.995	0.489	12.442	11.95	0.493	12.45	11.95
5 (area)	0.488	12.583	12.095	0.484	12.501	12.017	0.487	12.48	12	0.49	12.47	11.984	0.489	12.462	11.97	0.493	12.44	11.95
6 (area)	0.485	12.561	12.076	0.487	12.542	12.055	0.488	12.47	11.98	0.491	12.48	11.985	0.491	12.446	11.96	0.492	12.44	11.95
Average	0.487	12.583	12.096	0.485	12.503	12.018	0.487	12.471	11.984	0.490	12.477	11.988	0.490	12.444	11.954	0.492	12.446	11.953
St.dev.	0.001	0.011	0.010	0.002	0.027	0.026	0.001	0.007	0.007	0.001	0.015	0.014	0.001	0.012	0.012	0.001	0.007	0.007
RSD (%)	0.225	0.089	0.087	0.336	0.216	0.213	0.184	0.057	0.059	0.279	0.118	0.117	0.154	0.095	0.101	0.238	0.058	0.057

Table B.16: Raw data for the calibration curve for day 4 of the validation of the linearity curve for LC-UV.

Day 4	Phenacetin							Acetaminophen						
Conc. (ng/mL)	0	0.5	10	20	30	40	50	0	0.75	10	20	30	40	50
1 (area)	n.d.	2.356	6.996	97.264	173.784	236.709	287.946	n.d.	2.861	4.777	105.204	191.152	266.856	348.8291
2 (area)	n.d.	2.407	0.673258*	97.044	139.748	200.530	254.040	n.d.	2.899	n.d.	106.731	153.814	225.633	305.6648
3 (area)	n.d.	2.136	0.878858*	97.504	135.623	198.486	252.774	n.d.	1.882	n.d.	106.708	148.086	225.221	303.1552
4 (area)	n.d.	2.112	0.671035*	98.409	138.185	198.190	246.295	n.d.	2.503	n.d.	107.680	152.391	224.563	295.0024
5 (area)	n.d.	2.080	n.d.	99.304	143.994	195.433	241.393	n.d.	2.581	n.d.	107.844	158.577	222.801	291.5496
6 (area)	n.d.	2.053	29.177	100.863	141.092	194.747	245.597	n.d.	2.529	28.792	111.863	155.587	222.409	293.9276
Average	-	2.190485	18.08644	98.39788	145.4042	204.0159	254.6741	-	2.54236	16.78483	107.6716	159.9346	231.2471	306.3548
St.dev.	-	0.151095	15.68393	1.470801	14.18282	16.15587	16.97059	-	0.36554	16.98116	2.258339	15.68442	17.49255	21.52804
RSD (%)	-	6.90	86.72	1.49	9.75	7.92	6.66	-	14.38	101.17	2.10	9.81	7.56	7.03
Day 4	Tolbutamide							4-hydroxytolbutamide						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	1.443	2.475	36.286	62.873	87.440	113.916	n.d.	1.469	3.826	54.733	96.838	131.722	171.8142
2 (area)	n.d.	1.438	n.d.	35.678	50.634	74.271	101.531	n.d.	1.425	0.418815*	53.872	77.526	111.831	151.6375
3 (area)	n.d.	1.284	n.d.	36.068	48.359	74.450	100.472	n.d.	1.281	0.498465*	54.138	74.387	111.990	150.1348
4 (area)	n.d.	1.255	n.d.	36.287	50.142	72.284	97.462	n.d.	1.202	0.416871*	54.916	76.939	110.181	146.3046
5 (area)	n.d.	1.172	n.d.	35.903	52.156	71.979	96.843	n.d.	1.186	n.d.	54.836	79.857	108.844	143.0346
6 (area)	n.d.	1.264	10.351	36.976	51.302	72.285	97.645	n.d.	1.183	16.309	55.718	78.288	109.128	144.8008
Average	-	1.309472	6.413195	36.19963	52.57762	75.45156	101.3116	-	1.290982	10.06729	54.70206	80.63931	113.9494	151.2877
St.dev.	-	0.108826	5.568911	0.446009	5.201856	5.970662	6.44778	-	0.126693	8.826559	0.648505	8.136685	8.805671	10.5623
RSD (%)	-	8.31	86.84	1.23	9.89	7.91	6.36	-	9.81	87.68	1.19	10.09	7.73	6.98
Day 4	Fluoxetine							Norfluoxetine						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	0.833	3.399	55.823	100.208	136.961	163.776	n.d.	1.277	5.641	84.841	152.053	209.252	261.8888
2 (area)	n.d.	0.988	n.d.	57.035	78.308	116.126	142.966	n.d.	1.506	0.527404*	85.884	121.563	175.309	232.3653
3 (area)	n.d.	0.707	n.d.	55.831	76.709	116.597	143.921	n.d.	0.922	0.568655*	84.127	115.505	176.437	230.1509
4 (area)	n.d.	0.764	n.d.	56.573	77.504	113.686	139.491	n.d.	1.018	n.d.	85.727	118.337	172.058	223.9959
5 (area)	n.d.	0.839	n.d.	56.989	80.904	111.849	136.000	n.d.	1.027	0.617	85.634	124.322	170.267	219.7529
6 (area)	n.d.	0.879	15.735	56.878	79.884	113.279	139.037	n.d.	1.134	24.971	86.750	122.141	172.362	222.4098
Average	-	0.835166	9.567295	56.52137	82.25282	118.083	144.1985	-	1.147359	10.40952	85.4939	125.6535	179.2807	231.7606
St.dev.	-	0.096532	8.722933	0.561451	8.92926	9.42034	10.00912	-	0.213477	12.85793	0.905325	13.29873	14.85529	15.50855
RSD (%)	-	11.56	91.17	0.99	10.86	7.98	6.94	-	18.61	123.52	1.06	10.58	8.29	6.69

Table B.17: Raw data for the retention time for phenacetin and acetaminophen for day 4 of the validation of the linearity curve for LC-UV.

Dag 4	Phenacetin																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	5.562	5.069	0.503	5.575	5.072	0.489	5.547	5.058	0.493	5.602	5.109	0.494	5.607	5.113	0.490	5.573	5.083
2 (area)	0.493	5.571	5.078	0.516	5.543	5.027	0.490	5.529	5.039	0.493	5.599	5.106	0.494	5.578	5.084	0.492	5.583	5.091
3 (area)	0.490	5.574	5.084	0.515	n.d.	-	0.490	5.539	5.049	0.495	5.603	5.108	0.493	5.598	5.105	0.493	5.613	5.120
4 (area)	0.492	5.571	5.079	0.514	5.553	5.039	0.492	5.590	5.098	0.493	5.594	5.101	0.493	5.575	5.082	0.493	5.608	5.115
5 (area)	0.494	5.586	5.092	0.515	n.d.	-	0.492	5.580	5.088	0.494	5.589	5.095	0.492	5.588	5.096	0.493	5.606	5.113
6 (area)	0.493	5.589	5.096	0.491	5.538	5.047	0.494	5.579	5.085	0.492	5.603	5.111	0.493	5.583	5.090	0.493	5.603	5.110
Average	0.493	5.576	5.083	0.509	5.552	5.046	0.491	5.561	5.070	0.493	5.598	5.105	0.493	5.588	5.095	0.492	5.598	5.105
St.dev.	0.001	0.010	0.010	0.010	0.016	0.019	0.002	0.025	0.024	0.001	0.006	0.006	0.001	0.012	0.012	0.001	0.016	0.015
RSD (%)	0.280	0.182	0.194	1.976	0.295	0.377	0.374	0.457	0.473	0.209	0.102	0.117	0.153	0.220	0.239	0.246	0.284	0.290
Dag 4	Acetaminophen																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	0.877	0.384	0.503	0.877	0.374	0.489	0.867	0.378	0.493	0.874	0.381	0.494	0.875	0.381	0.490	0.867	0.377
2 (area)	0.493	0.876	0.383	0.516	n.d.	-	0.490	0.864	0.374	0.493	0.877	0.384	0.494	0.875	0.381	0.492	0.870	0.378
3 (area)	0.490	0.877	0.387	0.515	n.d.	-	0.490	0.867	0.377	0.495	0.878	0.383	0.493	0.875	0.382	0.493	0.878	0.385
4 (area)	0.492	0.876	0.384	0.514	n.d.	-	0.492	0.871	0.379	0.493	0.876	0.383	0.493	0.874	0.381	0.493	0.876	0.383
5 (area)	0.494	0.881	0.387	0.515	n.d.	-	0.492	0.873	0.381	0.494	0.877	0.383	0.492	0.874	0.382	0.493	0.877	0.384
6 (area)	0.493	0.879	0.386	0.491	0.869	0.378	0.494	0.875	0.381	0.492	0.874	0.382	0.493	0.876	0.383	0.493	0.877	0.384
Average	0.493	0.878	0.385	0.509	0.873	0.376	0.491	0.870	0.378	0.493	0.876	0.383	0.493	0.875	0.382	0.492	0.874	0.382
St.dev.	0.001	0.002	0.002	0.010	0.006	0.003	0.002	0.004	0.003	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.005	0.003
RSD (%)	0.280	0.224	0.447	1.976	0.648	0.752	0.374	0.481	0.703	0.209	0.191	0.270	0.153	0.086	0.214	0.246	0.519	0.898

Table B.18: Raw data for the retention time for tolbutamide and 4-hydroxytolbutamide for day 4 of the validation of the linearity curve for LC-UV.

Dag 4	Tolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	12.116	11.623	0.503	12.115	11.612	0.489	12.083	11.594	0.493	12.155	11.662	0.494	12.152	11.658	0.490	12.115	11.625
2 (area)	0.493	12.134	11.641	0.516	n.d.	-	0.490	12.059	11.569	0.493	12.158	11.665	0.494	12.125	11.631	0.492	12.138	11.646
3 (area)	0.490	12.132	11.642	0.515	n.d.	-	0.490	12.089	11.599	0.495	12.561	12.066	0.493	12.140	11.647	0.493	12.152	11.659
4 (area)	0.492	12.158	11.666	0.514	n.d.	-	0.492	12.162	11.670	0.493	12.120	11.627	0.493	12.120	11.627	0.493	12.155	11.662
5 (area)	0.494	12.136	11.642	0.515	n.d.	-	0.492	12.127	11.635	0.494	12.145	11.651	0.492	12.146	11.654	0.493	12.151	11.658
6 (area)	0.493	12.144	11.651	0.491	12.088	11.597	0.494	12.139	11.645	0.492	12.143	11.651	0.493	12.119	11.626	0.493	12.158	11.665
Average	0.493	12.137	11.644	0.509	12.102	11.605	0.491	12.110	11.619	0.493	12.214	11.720	0.493	12.134	11.641	0.492	12.145	11.653
St.dev.	0.001	0.014	0.014	0.010	0.019	0.011	0.002	0.039	0.038	0.001	0.171	0.170	0.001	0.014	0.014	0.001	0.016	0.015
RSD (%)	0.280	0.114	0.121	1.976	0.158	0.091	0.374	0.322	0.323	0.209	1.397	1.449	0.153	0.117	0.122	0.246	0.133	0.128
Dag 4	4-hydroxytolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	5.855	5.362	0.503	5.868	5.365	0.489	5.843	5.354	0.493	5.896	5.403	0.494	5.899	5.405	0.490	5.863	5.373
2 (area)	0.493	5.864	5.371	0.516	5.836	5.320	0.490	5.827	5.337	0.493	5.890	5.397	0.494	5.871	5.377	0.492	5.874	5.382
3 (area)	0.490	5.867	5.377	0.515	n.d.	-	0.490	5.835	5.345	0.495	5.895	5.400	0.493	5.892	5.399	0.493	5.897	5.404
4 (area)	0.492	5.864	5.372	0.514	5.854	5.340	0.492	5.883	5.391	0.493	5.886	5.393	0.493	5.869	5.376	0.493	5.893	5.400
5 (area)	0.494	5.879	5.385	0.515	n.d.	-	0.492	5.872	5.380	0.494	5.882	5.388	0.492	5.880	5.388	0.493	5.889	5.396
6 (area)	0.493	5.883	5.390	0.491	5.834	5.343	0.494	5.872	5.378	0.492	5.894	5.402	0.493	5.874	5.381	0.493	5.885	5.392
Average	0.493	5.869	5.376	0.509	5.848	5.342	0.491	5.855	5.364	0.493	5.891	5.397	0.493	5.881	5.388	0.492	5.884	5.391
St.dev.	0.001	0.010	0.010	0.010	0.016	0.018	0.002	0.023	0.022	0.001	0.006	0.006	0.001	0.012	0.012	0.001	0.013	0.012
RSD (%)	0.280	0.178	0.189	1.976	0.275	0.345	0.374	0.396	0.406	0.209	0.095	0.107	0.153	0.207	0.223	0.246	0.217	0.216

Table B.19: Raw data for the retention time for fluoxetine and norfluoxetine for day 4 of the validation of the linearity curve for for LC-UV.

Dag 4	Fluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	13.093	12.600	0.503	13.077	12.574	0.489	12.981	12.492	0.493	12.995	12.502	0.494	12.959	12.465	0.490	12.917	12.427
2 (area)	0.493	13.094	12.601	0.516	n.d.	-	0.490	12.967	12.477	0.493	13.017	12.524	0.494	12.958	12.464	0.492	12.955	12.463
3 (area)	0.490	13.098	12.608	0.515	n.d.	-	0.490	12.985	12.495	0.495	13.031	12.536	0.493	12.968	12.475	0.493	12.966	12.473
4 (area)	0.492	13.126	12.634	0.514	n.d.	-	0.492	13.043	12.551	0.493	12.982	12.489	0.493	12.958	12.465	0.493	12.970	12.477
5 (area)	0.494	13.107	12.613	0.515	n.d.	-	0.492	13.012	12.520	0.494	13.005	12.511	0.492	12.981	12.489	0.493	12.970	12.477
6 (area)	0.493	13.109	12.616	0.491	13.043	12.552	0.494	13.023	12.529	0.492	13.001	12.509	0.493	12.956	12.463	0.493	12.974	12.481
Average	0.493	13.105	12.612	0.509	13.060	12.563	0.491	13.002	12.511	0.493	13.005	12.512	0.493	12.963	12.470	0.492	12.959	12.466
St.dev.	0.001	0.012	0.013	0.010	0.024	0.016	0.002	0.029	0.027	0.001	0.017	0.016	0.001	0.010	0.010	0.001	0.021	0.020
RSD (%)	0.280	0.095	0.099	1.976	0.184	0.124	0.374	0.222	0.220	0.209	0.132	0.132	0.153	0.074	0.082	0.246	0.165	0.162
Dag 4	Norfluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.493	12.650	12.157	0.503	12.638	12.135	0.489	12.517	12.028	0.493	12.515	12.022	0.494	12.472	11.978	0.490	12.420	11.930
2 (area)	0.493	12.655	12.162	0.516	12.642	12.126	0.490	12.500	12.010	0.493	12.544	12.051	0.494	12.474	11.980	0.492	12.457	11.965
3 (area)	0.490	12.657	12.167	0.515	n.d.	-	0.490	12.522	12.032	0.495	12.561	12.066	0.493	12.487	11.994	0.493	12.473	11.980
4 (area)	0.492	12.686	12.194	0.514	n.d.	-	0.492	12.580	12.088	0.493	12.510	12.017	0.493	12.477	11.984	0.493	12.477	11.984
5 (area)	0.494	12.666	12.172	0.515	12.661	12.146	0.492	12.549	12.057	0.494	12.532	12.038	0.492	12.502	12.010	0.493	12.480	11.987
6 (area)	0.493	12.667	12.174	0.491	12.595	12.104	0.494	12.556	12.062	0.492	12.528	12.036	0.493	12.477	11.984	0.493	12.484	11.991
Average	0.493	12.664	12.171	0.509	12.634	12.128	0.491	12.537	12.046	0.493	12.532	12.038	0.493	12.482	11.988	0.492	12.465	11.973
St.dev.	0.001	0.013	0.013	0.010	0.028	0.018	0.002	0.029	0.028	0.001	0.019	0.018	0.001	0.011	0.012	0.001	0.024	0.023
RSD (%)	0.280	0.101	0.106	1.976	0.221	0.147	0.374	0.235	0.234	0.209	0.150	0.151	0.153	0.090	0.100	0.246	0.193	0.191

Table B.20: Raw data for the calibration curve for day 5 of the validation of the linearity curve for LC-UV.

Day 5	Phenacetin							Acetaminophen						
Conc. (ng/mL)	0	0.5	10	20	30	40	50	0	0.75	10	20	30	40	50
1 (area)	n.d.	2.260	44.645	90.201	142.939	195.894	264.860	n.d.	2.279	53.942	118.170	190.569	256.532	314.9632
2 (area)	n.d.	2.099	43.889	90.178	136.441	181.512	242.880	n.d.	n.d.	56.059	114.636	184.970	237.541	297.3262
3 (area)	n.d.	2.325	44.693	87.395	139.586	178.119	242.613	n.d.	2.391	53.253	116.916	187.335	231.485	289.2688
4 (area)	n.d.	2.103	44.679	89.882	132.892	175.946	243.557	n.d.	2.133	55.609	118.113	180.057	230.304	289.8195
5 (area)	n.d.	2.107	44.294	90.363	128.084	178.000	247.170	n.d.	n.d.	55.810	115.580	176.154	233.336	294.3264
6 (area)	n.d.	2.088	44.227	91.028	128.775	179.270	244.626	n.d.	2.082	52.209	118.157	171.511	232.683	292.8375
Average	-	2.1636	44.40457	89.84123	134.7863	181.4568	247.6177	-	2.22129	54.48006	116.9286	181.7661	236.9799	296.4236
St.dev.	-	0.1021	0.324336	1.257685	5.947805	7.303201	8.607465	-	0.14045	1.580338	1.518307	7.188284	9.890505	9.556095
RSD (%)	-	4.72	0.73	1.40	4.41	4.02	3.48	-	6.32	2.90	1.30	3.95	4.17	3.22
Day 5	Tolbutamide							4-hydroxytolbutamide						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	n.d.	17.861	37.626	61.931	81.609	104.187	n.d.	n.d.	27.243	54.781	86.470	121.148	159.4963
2 (area)	n.d.	n.d.	17.822	38.436	59.380	74.601	96.617	n.d.	n.d.	27.084	54.637	83.724	110.234	146.7516
3 (area)	n.d.	n.d.	17.999	36.977	60.074	73.772	97.681	n.d.	n.d.	27.115	53.360	85.051	108.427	146.7685
4 (area)	n.d.	n.d.	17.594	37.770	57.436	74.246	97.188	n.d.	n.d.	27.132	54.645	81.691	107.304	147.167
5 (area)	n.d.	n.d.	17.726	38.193	55.143	73.984	98.956	n.d.	n.d.	27.015	55.137	78.679	109.735	149.7663
6 (area)	n.d.	n.d.	17.787	38.581	55.480	74.956	98.163	n.d.	n.d.	26.747	56.441	78.786	109.438	148.6777
Average	-	-	17.79837	37.93027	58.24065	75.5278	98.79852	-	-	27.05571	54.83342	82.40014	111.0475	149.7712
St.dev.	-	-	0.135427	0.595928	2.688663	3.009143	2.759181	-	-	0.168681	0.992261	3.248146	5.057363	4.91301
RSD (%)	-	-	0.76	1.57	4.62	3.98	2.79	-	-	0.62	1.81	3.94	4.55	3.28
Day 5	Fluoxetine							Norfluoxetine						
Conc. (ng/mL)	0	0.75	10	20	30	40	50	0	0.5	10	20	30	40	50
1 (area)	n.d.	n.d.	25.002	54.346	89.495	118.737	153.010	n.d.	n.d.	40.396	83.875	134.669	183.748	248.8277
2 (area)	n.d.	n.d.	24.790	53.782	85.123	108.786	140.552	n.d.	n.d.	39.429	84.700	128.092	168.572	227.0651
3 (area)	n.d.	n.d.	25.219	52.316	87.337	107.297	140.291	n.d.	2.147	40.455	81.923	130.699	166.029	227.6311
4 (area)	n.d.	n.d.	25.138	53.700	81.987	105.456	140.708	n.d.	n.d.	39.996	82.616	122.213	163.516	228.885
5 (area)	n.d.	n.d.	24.937	53.974	79.497	107.414	144.313	n.d.	n.d.	39.713	83.880	119.077	168.048	232.9141
6 (area)	n.d.	n.d.	24.835	54.912	80.596	107.395	141.318	n.d.	n.d.	39.459	84.426	120.141	167.089	230.0071
Average	-	-	24.98675	53.83831	84.00554	109.181	143.3654	-	2.147	39.90796	83.56997	125.8152	169.5004	232.555
St.dev.	-	-	0.16823	0.868374	3.962552	4.800297	4.950475	-	-	0.450453	1.078915	6.292684	7.205905	8.237411
RSD (%)	-	-	0.67	1.61	4.72	4.40	3.45	-	-	1.13	1.29	5.00	4.25	3.54

Table B.21: Raw data for the retention time for phenacetin and acetaminophen for day 5 of the validation of the linearity curve for LC-UV.

Dag 1	Phenacetin																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
Rep 1	0.482	5.472	4.99	0.483	5.486	5.003	0.482	5.475	4.993	0.482	5.491	5.009	0.481	5.46	4.979	0.481	5.462	4.981
Rep 2	0.482	5.48	4.998	0.482	5.478	4.996	0.482	5.469	4.987	0.483	5.494	5.011	0.482	5.491	5.009	0.482	5.473	4.991
Rep 3	0.482	5.479	4.997	0.482	5.492	5.01	0.482	5.484	5.002	0.481	5.463	4.982	0.481	5.475	4.994	0.482	5.478	4.996
Rep 4	0.481	5.454	4.973	0.482	5.478	4.996	0.482	5.485	5.003	0.481	5.467	4.986	0.483	5.468	4.985	0.482	5.48	4.998
Rep 5	0.481	5.466	4.985	0.482	5.476	4.994	0.483	5.486	5.003	0.482	5.474	4.992	0.483	5.471	4.988	0.481	5.467	4.986
Rep 6	0.482	5.479	4.997	0.482	5.482	5	0.483	5.475	4.992	0.481	5.468	4.987	0.483	5.475	4.992	0.482	5.489	5.007
Gj.snitt(x)	0.482	5.472	4.990	0.482	5.482	5.000	0.482	5.479	4.997	0.482	5.476	4.995	0.482	5.473	4.991	0.482	5.475	4.993
St.dev(s)	0.001	0.010	0.010	0.000	0.006	0.006	0.001	0.007	0.007	0.001	0.013	0.012	0.001	0.010	0.010	0.001	0.010	0.009
RSD (%)	0.107	0.187	0.196	0.085	0.111	0.119	0.107	0.127	0.138	0.170	0.240	0.249	0.204	0.188	0.205	0.107	0.176	0.185
Dag 1	Acetaminophen																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	0.482	0.881	0.399	0.483	0.878	0.395	0.482	0.874	0.392	0.482	0.875	0.393	0.481	0.87	0.389	0.481	0.866	0.385
2 (area)	0.482	0.883	0.401	0.482	0.879	0.397	0.482	0.874	0.392	0.483	0.874	0.391	0.482	0.871	0.389	0.482	0.87	0.388
3 (area)	0.482	0.882	0.4	0.482	0.878	0.396	0.482	0.877	0.395	0.481	0.875	0.394	0.481	0.869	0.388	0.482	0.868	0.386
4 (area)	0.481	0.88	0.399	0.482	0.878	0.396	0.482	0.875	0.393	0.481	0.871	0.39	0.483	0.872	0.389	0.482	0.87	0.388
5 (area)	0.481	0.876	0.395	0.482	0.876	0.394	0.483	0.874	0.391	0.482	0.873	0.391	0.483	0.871	0.388	0.481	0.868	0.387
6 (area)	0.482	0.881	0.399	0.482	0.876	0.394	0.483	0.879	0.396	0.481	0.869	0.388	0.483	0.87	0.387	0.482	0.871	0.389
Average	0.482	0.881	0.399	0.482	0.878	0.395	0.482	0.876	0.393	0.482	0.873	0.391	0.482	0.871	0.388	0.482	0.869	0.387
St.dev.	0.001	0.002	0.002	0.000	0.001	0.001	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.002	0.001
RSD (%)	0.107	0.276	0.512	0.085	0.140	0.306	0.107	0.237	0.494	0.170	0.275	0.546	0.204	0.120	0.210	0.107	0.211	0.380

Table B.22: Raw data for the retention time for tolbutamide and 4-hydroxytolbutamide for day 5 of the validation of the linearity curve for LC-UV.

Dag 5	Tolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	n.d.	n.d.	-	0.480	11.870	11.390	0.485	11.972	11.487	0.492	12.093	11.601	0.491	12.059	11.568	0.491	12.076	11.585
2 (area)	n.d.	n.d.	-	0.480	11.830	11.350	0.485	11.987	11.502	0.492	12.089	11.597	0.492	12.059	11.567	0.491	12.070	11.579
3 (area)	n.d.	n.d.	-	0.480	11.901	11.421	0.487	11.907	11.420	0.493	12.105	11.612	0.491	12.071	11.580	0.490	12.064	11.574
4 (area)	n.d.	n.d.	-	0.484	11.945	11.461	0.486	12.010	11.524	0.492	12.088	11.596	0.491	12.059	11.568	0.491	12.058	11.567
5 (area)	n.d.	n.d.	-	0.485	11.933	11.448	0.487	12.080	11.593	0.490	12.071	11.581	0.491	12.045	11.554	0.492	12.074	11.582
6 (area)	n.d.	n.d.	-	0.483	11.933	11.450	0.492	12.105	11.613	0.491	12.070	11.579	0.491	12.074	11.583	0.492	12.053	11.561
Average	-	-	-	0.482	11.902	11.420	0.487	12.010	11.523	0.492	12.086	11.594	0.491	12.061	11.570	0.491	12.066	11.575
St.dev.	-	-	-	0.002	0.045	0.043	0.003	0.073	0.071	0.001	0.013	0.012	0.000	0.010	0.010	0.001	0.009	0.009
RSD (%)	-	-	-	0.473	0.375	0.375	0.535	0.606	0.618	0.210	0.111	0.108	0.083	0.086	0.090	0.153	0.076	0.080
Dag 5	4-hydroxytolbutamide																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	n.d.	n.d.	-	0.480	5.732	5.252	0.485	5.779	5.294	0.492	5.870	5.378	0.491	5.837	5.346	0.491	5.863	5.372
2 (area)	n.d.	n.d.	-	0.480	5.678	5.198	0.485	5.789	5.304	0.492	5.855	5.363	0.492	5.851	5.359	0.491	5.853	5.362
3 (area)	n.d.	n.d.	-	0.480	5.724	5.244	0.487	5.751	5.264	0.493	5.863	5.370	0.491	5.853	5.362	0.490	5.853	5.363
4 (area)	n.d.	n.d.	-	0.484	5.761	5.277	0.486	5.805	5.319	0.492	5.867	5.375	0.491	5.858	5.367	0.491	5.849	5.358
5 (area)	n.d.	n.d.	-	0.485	5.766	5.281	0.487	5.844	5.357	0.490	5.851	5.361	0.491	5.838	5.347	0.492	5.859	5.367
6 (area)	n.d.	n.d.	-	0.483	5.767	5.284	0.492	5.863	5.371	0.491	5.861	5.370	0.491	5.856	5.365	0.492	5.843	5.351
Average	-	-	-	0.482	5.738	5.256	0.487	5.805	5.318	0.492	5.861	5.370	0.491	5.849	5.358	0.491	5.853	5.362
St.dev.	-	-	-	0.002	0.035	0.033	0.003	0.042	0.040	0.001	0.007	0.007	0.000	0.009	0.009	0.001	0.007	0.007
RSD (%)	-	-	-	0.473	0.603	0.624	0.535	0.720	0.753	0.210	0.122	0.123	0.083	0.156	0.169	0.153	0.121	0.135

Table B.23: Raw data for the retention time for fluoxetine and norfluoxetine for day 5 of the validation of the linearity curve for LC-UV.

Dag 5	Fluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	n.d.	n.d.	-	0.480	12.872	12.392	0.485	12.903	12.418	0.492	12.959	12.467	0.491	12.909	12.418	0.491	12.892	12.401
2 (area)	n.d.	n.d.	-	0.480	12.841	12.361	0.485	12.920	12.435	0.492	12.965	12.473	0.492	12.911	12.419	0.491	12.901	12.410
3 (area)	n.d.	n.d.	-	0.480	12.894	12.414	0.487	12.860	12.373	0.493	12.979	12.486	0.491	12.923	12.432	0.490	12.898	12.408
4 (area)	n.d.	n.d.	-	0.484	12.925	12.441	0.486	12.931	12.445	0.492	12.969	12.477	0.491	12.909	12.418	0.491	12.882	12.391
5 (area)	n.d.	n.d.	-	0.485	12.912	12.427	0.487	12.987	12.500	0.490	12.957	12.467	0.491	12.903	12.412	0.492	12.894	12.402
6 (area)	n.d.	n.d.	-	0.483	12.905	12.422	0.492	13.003	12.511	0.491	12.947	12.456	0.491	12.924	12.433	0.492	12.881	12.389
Average	-	-	-	0.482	12.892	12.410	0.487	12.934	12.447	0.492	12.963	12.471	0.491	12.913	12.422	0.491	12.891	12.400
St.dev.	-	-	-	0.002	0.031	0.029	0.003	0.053	0.052	0.001	0.011	0.010	0.000	0.008	0.009	0.001	0.008	0.009
RSD (%)	-	-	-	0.473	0.237	0.232	0.535	0.412	0.415	0.210	0.085	0.082	0.083	0.065	0.068	0.153	0.064	0.069
Dag 5	Norfluoxetine																	
Conc. (µg/mL)	0.5			10			20			30			40			50		
	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R	t_M	t_R	t'_R
1 (area)	n.d.	n.d.	-	0.480	12.412	11.932	0.485	12.434	11.949	0.492	12.483	11.991	0.491	12.421	11.930	0.491	12.389	11.898
2 (area)	n.d.	n.d.	-	0.480	12.380	11.900	0.485	12.449	11.964	0.492	12.489	11.997	0.492	12.425	11.933	0.491	12.401	11.910
3 (area)	0.482	12.417	11.935	0.480	12.434	11.954	0.487	12.390	11.903	0.493	12.503	12.010	0.491	12.438	11.947	0.490	12.401	11.911
4 (area)	n.d.	n.d.	-	0.484	12.468	11.984	0.486	12.466	11.980	0.492	12.493	12.001	0.491	12.424	11.933	0.491	12.383	11.892
5 (area)	n.d.	n.d.	-	0.485	12.455	11.970	0.487	12.519	12.032	0.490	12.483	11.993	0.491	12.418	11.927	0.492	12.394	11.902
6 (area)	n.d.	n.d.	-	0.483	12.448	11.965	0.492	12.535	12.043	0.491	12.473	11.982	0.491	12.439	11.948	0.492	12.381	11.889
Average	0.482	12.417	11.935	0.482	12.433	11.951	0.487	12.466	11.979	0.492	12.487	11.996	0.491	12.428	11.936	0.491	12.392	11.900
St.dev.	-	-	-	0.002	0.032	0.030	0.003	0.054	0.053	0.001	0.010	0.010	0.000	0.009	0.009	0.001	0.009	0.009
RSD (%)	-	-	-	0.473	0.259	0.254	0.535	0.434	0.439	0.210	0.082	0.079	0.083	0.071	0.075	0.153	0.070	0.076

B.1.5. Raw data for the comparison of two NADPH regenerating systems

Table B.24 shows the raw data samples incubated with HLM, and the negative control samples for both NADPH regenerating systems.

Table B.24: Raw data for the phenacetin incubation with HLM experiment at DFS for LC-UV with two separate NADPH regenerating systems.

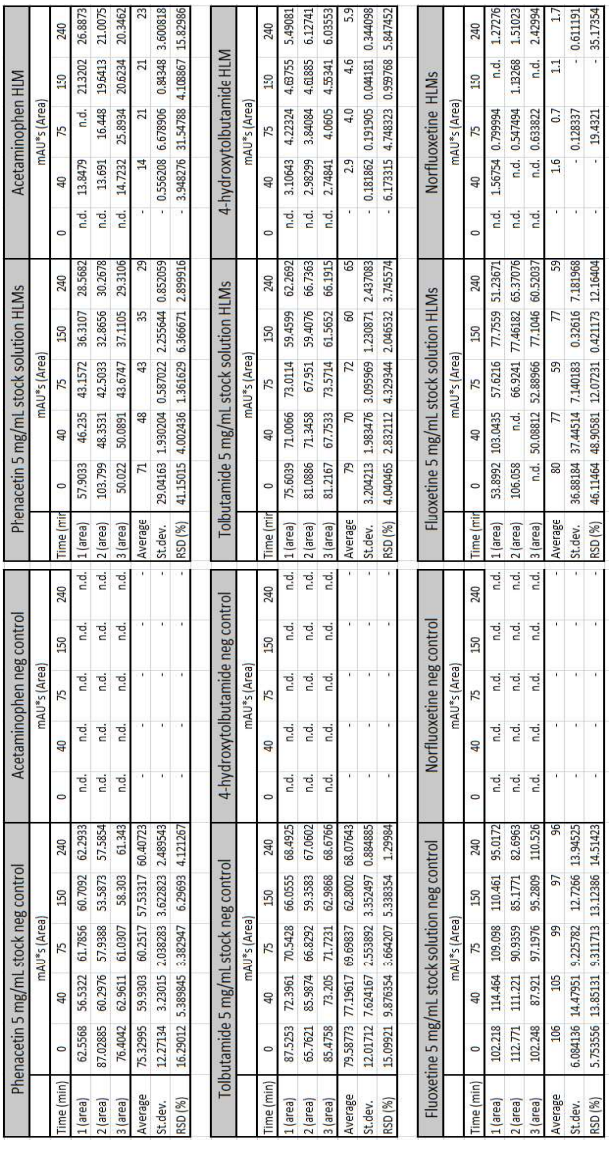
	Phenacetin NADPH UiO neg control				Acetaminophen NADPH UiO neg control			
	mAU				mAU			
Time (min)	0	40	75	120	0	40	75	120
Rep1	77.4434	63.1816	66.7953	61.2355	0	0	0	0
Rep2	68.4747	65.2951	62.3631	59.4369	0	0	0	0
Rep3	65.6798	63.2596	59.4369	60.6522	0	0	0	0
Average	71	64	63	60.4	0	0	0	0
St.dev.	6.145884	1.198348	3.704796	0.91762	0	0	0	0
RSD(%)	8.713532	1.874994	5.893248	1.518194	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	Phenacetin NADPH DFS neg control				Acetaminophen NADPH DFS neg control			
	mAU				mAU			
Time (min)	0	40	75	120	0	40	75	120
Rep1	91.2446	78.1274	63.4168	63.023	0	0	0	0
Rep2	68.4712	72.4101	60.0538	61.811	0	0	0	0
Rep3	73.039	56.7032	58.846	64.208	0	0	0	0
Average	78	69	61	63	0	0	0	0
St.dev.	12.04807	11.09347	2.368571	1.198525	0	0	0	0
RSD(%)	15.52888	16.05882	3.897458	1.901999	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

	Phenacetin NADPH UiO HLMs				Acetaminophen NADPH UiO HLMs			
	mAU				mAU			
Time (min)	0	40	75	120	0	40	75	120
Rep1	63.4958	41.6573	44.9389	42.4579	0	4.75507	8.765	10.1888
Rep2	64.7844	40.5727	45.1566	47.9209	0	5.15641	7.56235	9.33762
Rep3	65.6043	52.8421	43.4659	47.0014	0	6.28885	7.79861	9.18412
Average	65	45	44.5	46	0	5.4	8.0	9.6
St.dev.	1.062897	6.792328	0.919745	2.924986	0	0.795401	0.637194	0.54121
RSD(%)	1.644634	15.086	2.065893	6.387352	#DIV/0!	14.72935	7.923338	5.655174
	Phenacetin NADPH DFS HLMs				Acetaminophen NADPH DUS HLMs			
	mAU				mAU			
Time (min)	0	40	75	120	0	40	75	120
Rep1	68.6613	53.8156	45.9227	40.4079	0	4.10027	8.29337	9.91862
Rep2	62.7874	51.4292	44.8135	42.5975	0	4.23307	7.69953	9.93884
Rep3	63.9843	39.5228	45.7215	43.2004	0	3.39861	7.52058	9.4623
Average	65	48	45.5	42	0.0	3.9	7.8	9.8
St.dev.	3.104019	7.656603	0.590941	1.469461	0	0.448383	0.404531	0.269483
RSD(%)	4.764833	15.86668	1.299175	3.493011	#DIV/0!	11.4657	5.161269	2.757354

B.1.6. Raw data for the human liver microsome experiment with 0-240 min incubation periods

Table B.25 shows the raw data for drug incubation for 5 time periods from 0-240 min in 1 mg/mL HLM.

Table B.25: Raw data for the HLM experiment for LC-UV with incubation periods from 0-4 hours.



Phenacetin 5 mg/mL stock neg control

	mAU*s (Area)				
Time (min)	0	40	75	150	240
1 (area)	62.5568	56.5322	61.7856	60.7092	62.2933
2 (area)	87.02885	60.2976	57.9388	53.5873	57.5854
3 (area)	76.4042	62.9611	61.0307	58.303	61.343
Average	75.32995	59.9303	60.2517	57.53317	60.40723
St.dev.	12.27134	3.23015	2.038283	3.622823	2.489543
RSD (%)	16.29012	5.389845	3.382947	6.29693	4.121267

Acetaminophen neg control

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-

Tolbutamide 5 mg/mL stock neg control

	mAU*s (Area)				
Time (min)	0	40	75	150	240
1 (area)	87.5253	72.3961	70.5428	66.0555	68.4925
2 (area)	65.7621	85.9874	66.8292	59.3583	67.0602
3 (area)	85.4758	73.205	71.7231	62.9868	68.6766
Average	79.58773	77.19617	69.69837	62.8002	68.07643
St.dev.	12.01712	7.624167	2.553892	3.352497	0.884885
RSD (%)	15.09921	9.876354	3.664207	5.338354	1.29984

4-hydroxytolbutamide neg control

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-

Fluoxetine 5 mg/mL stock solution neg control

	mAU*s (Area)				
Time (min)	0	40	75	150	240
1 (area)	102.218	114.464	109.098	110.461	95.0172
2 (area)	112.771	111.221	90.9359	85.1771	82.6983
3 (area)	102.248	87.921	97.1976	95.2809	110.526
Average	106	105	99	97	96
St.dev.	6.084136	14.47951	9.225782	12.7266	13.94525
RSD (%)	5.753556	13.85131	9.311713	13.12386	14.51423

Norfluoxetine neg control

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-

Phenacetin 5 mg/mL stock solution HLMs

	mAU*s (Area)				
Time (min	0	40	75	150	240
1 (area)	57.9033	46.235	43.1572	36.3107	28.5682
2 (area)	103.799	48.3531	42.5033	32.8656	30.2678
3 (area)	50.022	50.0891	43.6747	37.1105	29.3106
Average	71	48	43	35	29
St.dev.	29.04163	1.930204	0.587022	2.255644	0.852059
RSD (%)	41.15015	4.002436	1.361629	6.366671	2.899916

Acetaminophen HLM

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	13.8479	n.d.	21.3202	26.8873
	n.d.	13.691	16.448	19.6413	21.0075
	n.d.	14.7232	25.8934	20.6234	20.3462
	-	14	21	21	23
	-	0.556208	6.678906	0.84348	3.600818
	-	3.948276	31.54788	4.108867	15.82986

Tolbutamide 5 mg/mL stock solution HLMs

	mAU*s (Area)				
Time (min	0	40	75	150	240
1 (area)	75.6039	71.0066	73.0114	59.4599	62.2692
2 (area)	81.0886	71.3458	67.951	59.4076	66.7363
3 (area)	81.2167	67.7533	73.5714	61.5652	66.1915
Average	79	70	72	60	65
St.dev.	3.204213	1.983476	3.095969	1.230871	2.437083
RSD (%)	4.040465	2.832112	4.329344	2.046532	3.745574

4-hydroxytolbutamide HLM

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	3.10643	4.22324	4.63755	5.49081
	n.d.	2.98299	3.84084	4.61885	6.12741
	n.d.	2.74941	4.0605	4.55341	6.03553
	-	2.9	4.0	4.6	5.9
	-	0.181862	0.191905	0.044181	0.344098
	-	6.173315	4.748323	0.959768	5.847452

Fluoxetine 5 mg/mL stock solution HLMs

	mAU*s (Area)				
Time (min	0	40	75	150	240
1 (area)	53.8992	103.0435	57.6216	77.7559	51.23671
2 (area)	106.058	n.d.	66.9241	77.46182	65.37076
3 (area)	n.d.	50.08812	52.88966	77.1046	60.52037
Average	80	77	59	77	59
St.dev.	36.88184	37.44514	7.140183	0.32616	7.181968
RSD (%)	46.11464	48.90581	12.07231	0.421173	12.16404

Norfluoxetine HLMs

	mAU*s (Area)				
	0	40	75	150	240
	n.d.	1.56754	0.799994	n.d.	1.27276
	n.d.	n.d.	0.547494	1.13268	1.51023
	n.d.	n.d.	0.633822	n.d.	2.42994
	-	1.6	0.7	1.1	1.7
	-	-	0.128337	-	0.611191
	-	-	19.4321	-	35.17354

B.2. Raw data for the work with liquid chromatography-mass spectrometry

B.2.1. Raw data for optimizing analyte signal area

Table B.26-B.29 shows the raw data for the optimization of the signal area by varying vaporizer temperature and emitter voltage in the ESI.

Table B.26: Raw data for signal area optimization by adjusting voltage in positive mode and vaporizer temperature simultaneously for LC-MS.

Voltage varied/Vap.temp. varied							
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
2000V/500°C	1 (area)	282882	626591	97290	50426	1700	69705
2000V/500°C	2 (area)	267092	603574	95481	48427	1273	62504
2000V/500°C	3 (area)	264124	602841	93246	47183	994	59040
2000V/500°C	Average	271366	611002	95339	48678.667	1322.3333	63749.67
2000V/500°C	St.dev	10082.953	13505.44	2025.73616	1636.082	355.57606	5440.526
2000V/500°C	RSD(%)	3.7156288	2.210376	2.12477177	3.3609836	26.890047	8.534203
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
2300V/480°C	1 (area)	291536	672230	91318	47392	1751	75327
2300V/480°C	2 (area)	291665	661651	90190	47130	1606	74313
2300V/480°C	3 (area)	292116	652943	87385	45368	1480	72130
2300V/480°C	Average	291772.33	662274.7	89631	46630	1612.3333	73923.33
2300V/480°C	St.dev	304.53298	9658.613	2025.21184	1100.747	135.61096	1633.733
2300V/480°C	RSD(%)	0.1043735	1.4584	2.25949933	2.3605984	8.4108516	2.210036
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
2500V/450°C	1 (area)	308568	683130	88242	45868	2144	82110
2500V/450°C	2 (area)	307867	680143	86555	45313	2187	81783
2500V/450°C	3 (area)	309043	688391	87131	45278	1995	79001
2500V/450°C	Average	308492.67	683888	87309.3333	45486.333	2108.6667	80964.67
2500V/450°C	St.dev	591.60826	4175.919	857.522206	330.99597	100.75879	1708.427
2500V/450°C	RSD(%)	0.1917738	0.610614	0.98216556	0.7276822	4.7783175	2.110089
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3000V/400°C	1 (area)	350548	749200	84197	45952	3877	105407
3000V/400°C	2 (area)	350896	752200	83742	46943	4082	105417
3000V/400°C	3 (area)	354337	746453	84426	45922	3936	103911
3000V/400°C	Average	351927	749284.3	84121.6667	46272.333	3965	104911.7
3000V/400°C	St.dev	2094.3617	2874.428	348.167105	581.00803	105.53199	866.6172
3000V/400°C	RSD(%)	0.5951125	0.383623	0.41388517	1.2556273	2.6615885	0.826045

Table B.27: Raw data for signal area optimization by adjusting voltage in positive mode and constant vaporizer temperature for LC-MS. The data with light grey background were also used for both voltage varied/vap.temp varied (**Table B.26**), and voltage same/Vap.temp.varied (**Table B.28**).

Voltage varied/Vap.temp.same							
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3500V/350°C	1 (area)	378615	763916	79136	45675	5285	116651
3500V/350°C	2 (area)	384125	775414	80806	47487	5651	119152
3500V/350°C	3 (area)	389754	774185	81561	47038	5403	119179
3500V/350°C	Average	384164.67	771171.7	80501	46733.333	5446.3333	118327.3
3500V/350°C	St.dev	5569.6059	6313.567	1240.93715	943.63782	186.80828	1451.81
3500V/350°C	RSD(%)	1.4497965	0.818698	1.54151768	2.0191965	3.4299825	1.226944
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3000V/350°C	1 (area)	349422	731523	81089	46554	4993	108764
3000V/350°C	2 (area)	366291	762784	83287	47926	4676	111594
3000V/350°C	3 (area)	367064	760513	83218	46604	4983	111225
3000V/350°C	Average	360925.67	751606.7	82531.3333	47028	4884	110527.7
3000V/350°C	St.dev	9969.962	17429.99	1249.57366	778.09254	180.20266	1538.483
3000V/350°C	RSD(%)	2.7623311	2.319031	1.5140597	1.6545304	3.6896532	1.391944
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
2500V/350°C	1 (area)	294781	660232	83338	47591	3789	88743
2500V/350°C	2 (area)	295141	651638	81746	46775	3997	90029
2500V/350°C	3 (area)	297372	661592	82132	48290	4012	90645
2500V/350°C	Average	295764.67	657820.7	82405.3333	47552	3932.6667	89805.67
2500V/350°C	St.dev	1403.5813	5397.354	830.451283	758.2526	124.64483	970.4686
2500V/350°C	RSD(%)	0.4745602	0.82049	1.007764	1.5945756	3.1694735	1.080632
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
2000V/350°C	1 (area)	225353	538691	84403	48140	2634	65672
2000V/350°C	2 (area)	228371	530948	83435	48603	2488	66031
2000V/350°C	3 (area)	229747	538661	84043	49264	2468	66559
2000V/350°C	Average	227823.67	536100	83960.3333	48669	2530	66087.33
2000V/350°C	St.dev	2247.5519	4461.788	489.266117	564.89911	90.620086	446.1752
2000V/350°C	RSD(%)	0.9865313	0.832268	0.58273484	1.1606959	3.5818216	0.67513

Table B.28: Raw data for signal area optimization by constant voltage in positive mode and variations in vaporizer temperature for LC-MS.

Voltage same/Vap.temp varied							
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3500V/400°C	1 (area)	393972	825520	86720	48235	4764	128877
3500V/400°C	2 (area)	396547	827333	87441	48765	4486	128022
3500V/400°C	3 (area)	395969	828758	87219	47640	4569	125372
3500V/400°C	Average	395496	827203.7	87126.6667	48213.333	4606.3333	127423.7
3500V/400°C	St.dev	1351.0933	1622.87	369.261876	562.81288	142.71066	1827.501
3500V/400°C	RSD(%)	0.34162	0.196187	0.42382188	1.1673387	3.0981402	1.434192
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3500V/450°C	1 (area)	396550	833143	87567	48527	4049	120110
3500V/450°C	2 (area)	390213	834795	88188	47686	3714	119023
3500V/450°C	3 (area)	392802	837944	87432	46711	3442	116413
3500V/450°C	Average	393188.33	835294	87729	47641.333	3735	118515.3
3500V/450°C	St.dev	3186.1156	2439.088	403.195982	908.8236	304.0444	1900.065
3500V/450°C	RSD(%)	0.8103281	0.292004	0.45959259	1.9076368	8.1404124	1.603223
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
3500V/500°C	1 (area)	397000	844347	89202	45927	2872	111385
3500V/500°C	2 (area)	390154	839415	88340	43912	2216	106323
3500V/500°C	3 (area)	390345	849389	90929	44881	2353	104751
3500V/500°C	Average	392499.67	844383.7	89490.3333	44906.667	2480.3333	107486.3
3500V/500°C	St.dev	3898.5729	4987.101	1318.36351	1007.7452	346.04094	3466.626
3500V/500°C	RSD(%)	0.9932678	0.59062	1.47319097	2.2440881	13.951389	3.225179

Table B.29: Raw data for optimization of the signal area intensity in negative mode LC-MS by variations in negative voltage while keeping the vaporizer temperature constant. The red numbers are the two replicates referred to as higher than the next two replicates in Section 4.2.3.

Optimization neg mode							
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
-2500V/350°C	1 (area)	1038099	1635514	184136	86312	5513	376606
-2500V/350°C	2 (area)	1077541	1659375	150209	69790	5689	385173
-2500V/350°C	3 (area)	1077741	1653531	149433	68336	5778	384092
-2500V/350°C	Average	1064460.3	1649473.33	161259.3333	74812.667	5660	381957
-2500V/350°C	St.dev	22829.803	12437.2555	19815.57348	9985.2155	134.8592	4665.516
-2500V/350°C	RSD(%)	2.1447303	0.75401374	12.2880165	13.346958	2.382671	1.221477
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
-3000V/350°C	1 (area)	378615	763916	79136	45675	5285	116651
-3000V/350°C	2 (area)	384125	775414	80806	47487	5651	119152
-3000V/350°C	3 (area)	389754	774185	81561	47038	5403	119179
-3000V/350°C	Average	384164.67	771171.667	80501	46733.333	5446.333	118327.3
-3000V/350°C	St.dev	5569.6059	6313.56748	1240.937146	943.63782	186.8083	1451.81
-3000V/350°C	RSD(%)	1.4497965	0.81869806	1.541517678	2.0191965	3.429982	1.226944
Voltage/Vap.temp	Analyte	A Areal (Y)	P Areal(Y)	4HT Areal(Y)	T Areal(Y)	N Areal(Y)	F Areal(Y)
-3500V/350°C	1 (area)	710755	1058380	141151	73093	4357	184720
-3500V/350°C	2 (area)	716257	1060051	141019	73641	4524	183857
-3500V/350°C	3 (area)	718976	1061828	142286	74597	4645	183380
-3500V/350°C	Average	715329.33	1060086.33	141485.3333	73777	4508.667	183985.7
-3500V/350°C	St.dev	4188.2734	1724.27154	696.5316456	761.16752	144.611	679.2027
-3500V/350°C	RSD(%)	0.5855028	0.16265388	0.49229954	1.0317138	3.2074	0.369161

B.2.2. Raw data for determining limit of quantitation

Table B.30 shows the raw data for determination of LOQ for the LC-MS method for each analyte.

Table B.30: Calculated LOQ within the acceptance criteria of ±20%. The calculated RSD ranged from 1-6%, and the LOQs were determined to be 2.5 ng/mL for acetaminophen and phenacetin, 1 ng/mL for 4-hydroxytolbutamide and tolbutamide, 50 ng/mL for norfluoxetine, and 10 ng/mL for fluoxetine.

Analyte	A	P	4HT	T	N	F
LOQ concentration (ng/mL)	2.5	2.5	1	1	50	10
Rep 1 (area)	6043	10248	841	580	654	5131
Rep 2 (area)	6190	9640	880	626	578	5009
Rep 3 (area)	6132	9637	877	610	602	4937
Average (area)	6122	9842	866	605	611	5026
Standard deviation (area)	74	352	22	23	39	98
RSD (%)	1	1	4	3	6	2

B.2.3. Raw data for investigation of matrix effects

Table B.31-B.37 show all the raw data for the investigation of matrix effects in HLM, cell medium with and without FBS, until the validation of the method in cell medium without FBS.

Table B.31: Raw data for the first initial investigation of matrix effects in HLM with three concentration levels.

	Solvent drugs			HLM Drugs			Solvent metabolites			HLM metabolites		
Concentration(ng/mL)	8.3	20.8	41.7	83.3	208.3	416.7	8.3	20.8	41.7	8.3	20.8	41.7
Analyte	Phenacetin	Tolbutamide	Fluoxetine	Phenacetin	Tolbutamide	Fluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine
1 (area)	38139	2925	9411	376713	30566	11876	5538	4157	856	13141	7345	121
2 (area)	41640	3239	4654	383152	29895	12548	5957	4207	371	12538	6706	102
3 (area)	38258	3058	2526	381070	30166	11899	7956	5456	339	12699	7001	47.51
4 (area)	34400	3015	2231	376308	29925	12662	7877	5352	133	12168	6886	75.96
5 (area)	38440	3144	2181	376946	28884	14013	8137	5720	138	12446	6779	123
6 (area)	35144	2979	1931	386933	30383	12238	8063	5328	137	12076	6891	86.79
Average	37670.1667	3060	3822.333333	380187	29969.83333	12539.3333	7254.666667	5036.666667	329	12511.33333	6934.666667	92.71
St.dev	2607.41561	114.8146332	2912.346248	4305.78281	591.8186941	790.90522	1178.300924	676.6469291	279.7691906	385.7645223	225.1973949	28.85398135
RSD(%)	6.92169916	3.752112197	76.19289041	1.13254341	1.974714666	6.30739449	16.24197193	13.43441951	85.03622814	3.083320634	3.247414847	31.12283611
Concentration(ng/mL)	41.7	104.2	208.3	41.7	104.2	208.3	41.7	104.2	208.3	41.7	104.2	208.3
Analyte	Phenacetin	Tolbutamide	Fluoxetine	Phenacetin	Tolbutamide	Fluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine
1 (area)	171280	14356	22253	190884	14271	5495	25324	18898	1783	63559	35078	426
2 (area)	143205	11923	13441	203170	15154	7038	33675	24833	1709	61707	33139	520
3 (area)	159846	13360	12948	199861	14643	5678	35337	26169	1401	64920	35586	521
4 (area)	165701	14207	12386	200083	14236	5698	34184	26340	1214	63948	34653	554
5 (area)	164591	14132	12094	202306	14856	5994	29592	22935	1047	63815	35156	532
6 (area)	153731	12781	10862	198774	14580	5998	35034	25595	1123	64808	35858	437
Average	159725.667	13459.83333	13997.33333	199179.667	14623.33333	5983.5	32191	24128.33333	1379.5	63792.83333	34911.66667	498.3333333
St.dev	10017.6008	963.9314118	4138.01951	4380.8583	349.9060826	552.406282	3951.322816	2845.483696	308.4099544	1160.25987	964.0428759	53.30916119
RSD(%)	6.27175396	7.161540473	29.56291325	2.19945056	2.392792906	9.2321598	12.27461967	11.79312163	22.35664766	1.818793444	2.761377408	10.69749054
Concentration(ng/mL)	83.3	208.3	416.7	83.3	208.3	416.7	83.3	208.3	416.7	83.3	208.3	416.7
Analyte	Phenacetin	Tolbutamide	Fluoxetine	Phenacetin	Tolbutamide	Fluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine	Acetaminophen	4-hydroxytolbutamide	Norfluoxetine
1 (area)	308233	27250	32703	411699	31600	14175	42532	31841	1949	136499	77002	1119
2 (area)	314584	26312	27930	403053	30907	13723	39031	29560	1465	127906	71926	1033
3 (area)	316053	28776	28192	413683	31078	14091	48118	36289	1684	128535	70997	972
4 (area)	297396	27031	26655	415505	30760	13841	50820	37884	1912	125200	70119	1074
5 (area)	297765	26264	24503	413280	32606	14774	45139	33417	1828	129301	71367	1089
6 (area)	301571	27809	25899	408457	31249	13753	49182	37257	1833	127286	70233	979
Average	305933.667	27240.33333	27647	410946.167	31366.66667	14059.5	45803.66667	34374.66667	1778.5	129121.1667	71940.66667	1044.333333
St.dev	8260.36119	952.9530244	2823.634325	4533.97805	673.3354785	394.907458	4448.538487	3310.75657	178.6199877	3873.715809	2571.828351	60.13207685
RSD(%)	2.70004975	3.498316312	10.21316716	1.10330219	2.146659336	2.80883003	9.712188587	9.631385236	10.04329422	3.000062584	3.574929828	5.757939054

Table B.32: Raw data for the matrix effects with the highest concentration from the first initial investigation after the optimized solvent gradient program for the LC-MS.

Highest concentration solvent						
Concentration (ng/mL)	100	100	250	250	500	500
Analyte	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	802256	237976	44484	117476	51400	2086
2 (area)	704550	203568	38076	102338	44431	1705
3 (area)	686546	202692	36207	99541	43396	1609
Average	731117.333	214745.3333	39589	106451.6667	46409	1800
St.dev	62262.0946	20123.1148	4340.97443	9649.235531	4353.20193	252.2914981
RSD(%)	8.51601949	9.370687823	10.9651025	9.0644288	9.38008129	14.01619434
Highest concentration HLM						
Concentration (ng/mL)	100	100	250	250	500	500
Analyte	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	721659	247391	33492	98049	39429	1551
2 (area)	716703	242765	33372	99132	35083	1331
3 (area)	757890	257637	34582	102780	38971	1796
Average	732084	249264.3333	33815.33333	99987	37827.6667	1559.333333
St.dev	22485.6112	7610.921714	666.6583333	2478.680899	2387.95673	232.6119802
RSD(%)	3.07145235	3.053353688	1.971467579	2.47900317	6.31272542	14.91739933

Table B.33: Raw data for initial investigation of matrix effects in cell medium with and without FBS with three concentration levels.

Solvent matrix

Concentration (ng/mL)	8.3	20.8	41.7	8.3	20.8	41.7
Analyte	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	108839	18637	2772	8699	2401	0
2 (area)	101168	16299	2301	7670	2370	0
3 (area)	110767	18736	2755	8634	2729	0
4 (area)	104905	17216	2722	8108	2284	0
5 (area)	103106	16505	2968	8002	2425	0
6 (area)	105511	16927	3046	8439	1847	0
Average	105716	17386.66667	2760.666667	8258.666667	2342.66667	0
St.dev	3562.6361	1056.864167	259.750393	400.5983858	286.115128	0
RSD(%)	3.37000653	6.078589915	9.408973425	4.850642385	12.213224	0
Concentration (ng/mL)	41.7	104.2	208.3	41.7	104.2	208.3
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	332218	71734	10513	29409	12246	421
2 (area)	336279	71114	11653	30449	12322	317
3 (area)	332488	67859	11635	29675	12145	389
4 (area)	319995	69458	10679	29931	11374	225
5 (area)	330675	72527	10941	30686	12547	503
6 (area)	323702	71744	10655	29908	12791	313
Gj.snitt	329226.167	70739.33333	11012.66667	30009.66667	12237.5	361.3333333
St.dev	6118.21959	1747.740217	508.2100616	477.4447263	482.400974	97.24539406
RSD(%)	1.85836371	2.470676687	4.614777482	1.590969775	3.94198958	26.91293194
Concentration (ng/mL)	83.3	208.3	416.7	83.3	208.3	416.7
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	713334	144540	26070	64876	27508	1015
2 (area)	650212	142452	23951	60417	26563	1107
3 (area)	647380	138062	22663	61184	26408	1052
4 (area)	657828	143581	24687	61089	26605	1137
5 (area)	639857	143838	24435	59717	25219	1050
6 (area)	631609	139190	21338	59480	25256	881
Gj.snitt	656703.333	141943.8333	23857.33333	61127.16667	26259.8333	1040.333333
St.dev	29157.7905	2680.250915	1655.305732	1962.880681	881.128689	89.49562373
RSD(%)	4.44002475	1.888247522	6.938351864	3.211142914	3.35542377	8.602591195

Cell medium without FBS

Concentration (ng/mL)	8.3	20.8	41.7	8.3	20.8	41.7
Analyte	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	69628	12926	2159	6244	2299	0
2 (area)	67436	13199	1996	6114	1811	0
3 (area)	67540	13098	2065	5917	2083	0
4 (area)	66726	13022	2158	5865	1933	0
5 (area)	66808	12782	2025	5945	1800	0
6 (area)	66462	13038	2093	6164	2071	0
Average	67433.3333	13010.83333	2082.666667	6041.5	1999.5	0
St.dev	1154.3654	143.8254729	67.4645586	153.1231531	190.54632	0
RSD(%)	1.71186168	1.105428601	3.2393354	2.534522106	9.52969842	0
Concentration (ng/mL)	41.7	104.2	208.3	41.7	104.2	208.3
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	328708	71767	10077	29425	11459	266
2 (area)	349993	79936	10807	32224	11758	427
3 (area)	335362	75171	10952	30786	10471	520
4 (area)	336130	72965	10841	30331	10312	447
5 (area)	347285	73823	11059	31681	11517	245
6 (area)	334470	75418	10228	30363	9439	364
Gj.snitt	338658	74846.66667	10560.66667	30801.66667	10826	378.1666667
St.dev	8206.13914	2842.73943	405.2869265	1010.511883	901.197426	107.4754235
RSD(%)	2.42313459	3.798084212	3.811083671	3.280705211	8.32438043	28.42012081
Concentration (ng/mL)	83.3	208.3	416.7	83.3	208.3	416.7
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	702475	163597	23064	64078	25841	768
2 (area)	673595	152104	22133	60578	24164	986
3 (area)	656091	147572	20719	58422	24947	1179
4 (area)	656470	154133	22175	69947	24663	878
5 (area)	662488	154301	21941	61836	23443	998
6 (area)	674313	153635	23247	62357	25957	964
Gj.snitt	670905.333	154223.6667	22213.16667	62869.66667	24835.8333	962.1666667
St.dev	17402.2135	5233.693979	905.3355707	3947.492504	969.633831	136.8625832
RSD(%)	2.59384039	3.393573822	4.080172738	6.278850697	3.90417273	14.22441536

Cell medium with FBS

Concentration (ng/mL)	8.3	20.8	41.7	8.3	20.8	41.7
Analyte	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	71917	15116	2175	6626	2853	0
2 (area)	69672	14919	1964	6139	2648	0
3 (area)	70410	14907	2092	6260	2666	0
4 (area)	69334	14366	1897	6145	2547	0
5 (area)	67906	13849	2119	6092	2545	0
6 (area)	67919	13915	1908	6046	3019	0
Average	69526.3333	14512	2025.833333	6218	2713	0
St.dev	1533.84258	548.4129831	117.9956214	212.2536219	187.32325	0
RSD(%)	2.20613185	3.77903103	5.824547333	3.413535251	6.9046535	0
Concentration (ng/mL)	41.7	104.2	208.3	41.7	104.2	208.3
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	317899	76678	10197	27428	14606	571
2 (area)	361437	87999	10964	31994	14801	498
3 (area)	340671	83118	10835	30488	14694	477
4 (area)	346999	83601	10984	31239	14568	512
5 (area)	317113	80018	10571	28452	13588	517
6 (area)	336026	84747	11341	29972	14105	418
Gj.snitt	336690.833	82693.5	10815.33333	29928.83333	14393.667	498.8333333
St.dev	17154.2377	3916.975606	392.2492405	1716.460593	461.308	50.44369799
RSD(%)	5.0949524	4.736739413	3.626788268	5.735140337	3.2049373	10.11233505
Concentration (ng/mL)	83.3	208.3	416.7	83.3	208.3	416.7
	Phenacetin	Acetaminophen	Tolbutamide	4-hydroxytolbutamide	Fluoxetine	Norfluoxetine
1 (area)	743064	181257	26283	66155	32269	1305
2 (area)	679935	170213	22468	60839	30806	1091
3 (area)	661335	164158	22250	58764	28770	1039
4 (area)	679798	164647	21545	60360	29133	1050
5 (area)	679295	170890	22166	61408	30433	1058
6 (area)	671762	164323	21612	59074	28945	1203
Gj.snitt	685864.833	169248	22720.66667	61100	30059.333	1124.333333
St.dev	28939.5211	6620.821641	1782.951224	2676.758562	1366.8926	106.9086838
RSD(%)	4.21942045	3.911905394	7.847266324	4.38094691	4.5473152	9.508628856

Table B.34: Raw data for the calibration curve with solvent matrix during investigation of matrix effects with all analytes and ISTD to determine the most optimal matrix to perform a validation with.

Solvent	Phenacetin							Tolbutamide							Fluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	6096	50741	152324	244457	357594	464715	n.d.	56.86	3489	7500	12480	15596	22157	n.d.	10823	82769	175883	263059	336469	410238
2 (area)	n.d.	6306	49063	151944	247862	367940	462659	n.d.	25.41	3728	7477	12536	15689	22107	n.d.	10707	81621	175689	273602	334622	399238
3 (area)	n.d.	5880	49853	154348	242229	366344	454660	n.d.	25.71	3711	7664	12404	15348	22080	n.d.	11013	79749	177499	265413	335826	393720
Gj.snitt	-	6094	49885.67	152872	244849.3	363959.3	460678	-	35.99333	3642.667	7547	12473.33	15544.33	22114.67	-	10847.67	81379.67	176357	267358	335639	401065.3
St.dev	-	213.007	839.4768	1292.297	2836.92	5570	5312.158	-	18.07169	133.3504	101.9755	66.25204	176.2735	39.06832	-	154.4841	1524.395	993.7464	5534.075	937.5921	8409.247
RSD (%)	-	3.495357	1.682802	0.845346	1.158639	1.530391	1.153117	-	50.20843	3.660791	1.351206	0.531149	1.134005	0.176662	-	1.424123	1.87319	0.563486	2.069912	0.279345	2.096728
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	772	8571	31824	54405	80149	105519	n.d.	207	7991	15733	25598	31257	46241	n.d.	174	252	598	1038	1339	1782
2 (area)	n.d.	674	9840	31471	54576	79826	95864	n.d.	210	7767	16090	26059	32445	46056	n.d.	122	276	590	1049	1256	1746
3 (area)	n.d.	773	9326	33574	52226	77531	99594	n.d.	222	8209	16264	24896	31659	46493	n.d.	117	287	438	1032	1278	1533
Gj.snitt	-	739.6667	9245.667	32289.67	53735.67	79168.67	100325.7	-	213	7989	16029	25517.67	31787	46263.33	-	137.6667	271.6667	542	1039.667	1291	1687
St.dev	-	56.8712	638.3027	1126.182	1310.202	1427.426	4868.907	-	7.937254	221.0068	270.7046	585.6469	604.2549	219.3544	-	31.56475	17.89786	90.15542	8.621678	43	134.5771
RSD (%)	-	7.688761	6.903804	3.487748	2.438236	1.803019	4.853102	-	3.72641	2.766389	1.688843	2.295065	1.90095	0.474143	-	22.92839	6.588169	16.63384	0.829273	3.330751	7.977304
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Consentration (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	291184	255141	264540	247241	262454	244990	273168	17827	17190	17313	17018	17718	16773	17942	16853	14912	16979	16743	16828	16181	16596
2 (area)	275423	254452	258994	245951	261289	248773	268661	17959	16971	17999	17471	17602	17207	18160	16406	14670	16461	17141	16575	16035	17417
3 (area)	274210	249586	257268	250251	257047	249934	269944	17796	17186	17829	17538	17736	17004	18047	16166	15500	16063	15973	16153	15928	16524
Gj.snitt	280272.3	253059.7	260267.3	247814.3	260263.3	247899	270591	17860.67	17115.67	17713.67	17342.33	17685.33	16994.67	18049.67	16475	15027.33	16501	16619	16518.67	16048	16845.67
St.dev	9469.224	3027.945	3799.543	2206.589	2845.682	2585.284	2322.115	86.55827	125.301	357.2469	282.8716	72.7278	217.1505	109.0245	348.6589	426.8505	459.3082	593.7912	341.0078	127	496.0971
RSD (%)	3.378579	1.196534	1.459862	0.89042	1.093386	1.042878	0.858164	0.484631	0.732083	2.016787	1.631105	0.411232	1.277757	0.604025	2.11629	2.840494	2.783517	3.572966	2.064379	0.791376	2.944954

Table B.35: Raw data for the calibration curve with cell medium without FBS matrix during investigation of matrix effects with all analytes and ISTD to determine the most optimal matrix to perform a validation with.

CM WO FBS	Phenacetin							Tolbutamide							Fluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	8966	73231	194505	367769	522300	660457	n.d.	66.58	6091	10705	15101	18436	28217	n.d.	9019	19281	219775	310261	373467	487360
2 (area)	n.d.	9234	74304	199615	364245	524758	663800	n.d.	43.15	5925	10256	14725	18281	28745	n.d.	8624	20508	220825	313484	356302	503733
3 (area)	n.d.	8393	73420	200585	366149	526046	672899	n.d.	49.36	5866	10464	14733	18273	28885	n.d.	8520	20250	220398	315133	373844	488293
Gj.snitt	-	8864.333	73651.67	198235	366054.3	524368	665718.7	-	53.03	5960.667	10475	14853	18330	28615.67	-	8721	20013	220332.7	312959.3	367871	493128.7
St.dev	-	429.6188	572.7865	3266.481	1763.906	1903.209	6439.084	-	12.13849	116.6633	224.702	214.8115	91.88858	352.2802	-	263.2622	646.9227	528.0401	2478.014	10020.82	9195.463
RSD (%)	-	4.8466	0.777697	1.647782	0.48187	0.362953	0.967238	-	22.88985	1.95722	2.145127	1.44625	0.501286	1.231075	-	3.018716	3.232512	0.239656	0.791801	2.724004	1.864719
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1526	17943	46071	99881	137087	162869	n.d.	262	12605	24214	32886	40894	62726	n.d.	108	33.6	1155	1742	1854	2754
2 (area)	n.d.	1503	17581	48237	90793	131858	163850	n.d.	277	13310	23601	33126	43285	64222	n.d.	68.98	22	1120	1615	1882	2610
3 (area)	n.d.	1402	18817	48029	89326	131525	166252	n.d.	274	13085	23952	32334	41697	63613	n.d.	90.63	11.41	974	1597	1982	2825
Gj.snitt	-	1477	18113.67	47745.67	90333.33	133490	164323.7	-	271	13030	23922.33	32782	42625.33	63553.67	-	89.20333	22.33667	1083	1651.333	1906	2729.667
St.dev	-	65.96211	635.4285	678.8942	873.4852	3119.54	1740.529	-	7.937254	311.1672	307.5749	406.1133	827.3889	799.6526	-	19.54908	11.09883	96.00521	79.03375	67.29042	109.546
RSD (%)	-	4.465952	3.508006	1.421897	0.966958	2.336909	1.059208	-	2.928876	2.388083	1.285723	1.23883	1.941073	1.258232	-	21.91519	49.68884	8.864747	4.786057	3.530452	4.013165
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Consentration (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	471380	420930	379715	406014	411402	403739	388377	23737	21781	20605	19494	20083	18206	18746	23890	20589	8404	20535	23149	20243	21759
2 (area)	458891	422272	384337	407761	412906	400280	396958	24631	21525	21398	19264	19638	18721	19018	24470	20316	8464	21353	22119	20135	22213
3 (area)	463597	419054	385787	411967	412838	400609	388001	23467	21318	20796	19419	19259	18057	19185	23349	20268	8346	21357	21737	20387	21306
Gj.snitt	464622.7	420752	383279.7	408580.7	412382	401542.7	391112	23945	21541.33	20933	19392.33	19660	18328	18983	23903	20391	8404.667	21081.67	22335	20255	21759.33
St.dev	6307.359	1616.368	3171.082	3059.974	849.3857	1909.181	5066.274	609.2389	231.9317	413.8708	117.2959	412.4403	348.4064	221.5829	560.6131	173.1444	59.00282	473.4314	730.3616	126.4278	453.5001
RSD (%)	1.357523	0.384162	0.827355	0.748928	0.205971	0.475461	1.295351	2.544326	1.076682	1.977121	0.604857	2.097865	1.900951	1.16727	2.345367	0.849122	0.702025	2.245702	3.270032	0.624181	2.084164

Table B.36: Raw data for the calibration curve with cell medium with FBS matrix during investigation of matrix effects with all analytes and ISTD to determine the most optimal matrix to perform a validation with.

CM W FBS	Phenacetin							Tolbutamide							Fluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	10322	69981	287416	419805	640657	729614	n.d.	56.5	4998	10608	19577	20923	29898	n.d.	19424	56687	254554	345221	442599	550146
2 (area)	n.d.	10190	70545	287980	415545	644362	730102	n.d.	60.05	5031	10741	19319	21116	29628	n.d.	18623	57088	266803	339019	441781	533834
3 (area)	n.d.	9951	71803	288670	425048	639822	727070	n.d.	74.93	5174	10618	19531	21662	30102	n.d.	19535	55431	261690	350121	447546	532175
Gj.snitt	-	10154.33	70776.33	288022	420132.7	641613.7	728928.7	-	63.82667	5067.667	10655.67	19475.67	21233.67	29876	-	19194	56402	261015.7	344787	443975.3	538718.3
St.dev	-	188.0541	932.7686	628.0541	4759.966	2416.466	1628.041	-	9.778222	93.55391	74.06979	137.613	383.2941	237.7646	-	497.6053	864.4831	6152.28	5563.71	3119.219	9931.352
RSD (%)	-	1.851959	1.31791	0.218058	1.132967	0.376623	0.223347	-	15.31996	1.846094	0.695121	0.706589	1.805124	0.795838	-	2.592504	1.532717	2.357054	1.613666	0.702566	1.843515
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1703	16636	70105	102030	161054	179644	n.d.	343	12841	25137	44489	50214	70931	n.d.	120	160	981	1313	1983	2457
2 (area)	n.d.	1544	16561	72371	101179	159163	190427	n.d.	346	12543	25380	44577	50191	71672	n.d.	134	91.16	1032	1419	2136	2393
3 (area)	n.d.	1720	17809	70385	107729	169607	178568	n.d.	359	12820	25577	45864	49255	71812	n.d.	137	96.27	1172	1681	1966	2542
Gj.snitt	-	1682.333	17002	70980.33	103646	163274.7	182879.7	-	349.3333	12734.67	25364.67	44976.67	49886.67	71471.67	-	130.3333	118.4767	1061.667	1471	2028.333	2464
St.dev	-	123.8722	699.8878	1208.497	3561.491	5564.872	6558.287	-	8.504901	166.32	220.4004	769.7119	547.1602	473.4346	-	9.073772	42.9645	98.89557	189.4307	93.6287	74.74624
RSD (%)	-	7.363121	4.116503	1.70258	3.436207	3.408289	3.586121	-	2.434609	1.306041	0.868927	1.711358	1.096807	0.662409	-	6.961973	36.2641	9.315124	12.87768	4.616041	3.033532
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Consentration (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	447288	438229	450787	458709	440143	381870	405002	19830	19672	20142	20391	19347	17356	18132	26612	26606	20522	24864	22612	20741	22834
2 (area)	455842	442830	450681	462569	438719	383767	400237	20205	19689	20823	20422	19438	17023	17602	26198	25888	20113	25142	22698	21589	22382
3 (area)	454686	440781	457626	465048	443840	381158	399893	20198	19927	20729	20894	20179	17797	17840	27046	26635	20193	24883	22911	21313	22853
Gj.snitt	452605.3	440613.3	453031.3	462108.7	440900.7	382265	401710.7	20077.67	19762.67	20564.67	20569	19654.67	17392	17858	26618.67	26376.33	20276	24963	22740.33	21214.33	22689.67
St.dev	4641.078	2305.078	3979.451	3194.473	2643.237	1348.606	2855.563	214.5142	142.5705	369.0452	281.8847	456.3599	388.2538	265.4581	424.0393	423.1576	216.7648	155.3094	153.9296	432.5244	266.6165
RSD (%)	1.025414	0.523152	0.878405	0.691282	0.599509	0.352794	0.710851	1.068422	0.721413	1.794559	1.370435	2.321891	2.23237	1.486494	1.593015	1.604308	1.069071	0.622158	0.676901	2.038831	1.175057

Table B.37: Raw data for the calibration curve with HLM matrix during investigation of matrix effects with all analytes and ISTD to determine the most optimal matrix to perform a validation with.

HLM	Phenacetin							Tolbutamide							Fluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	5631	46415	118084	217251	348760	432463	n.d.	27.74	2549	5445	8048	11513	16380	n.d.	12082	102967	206308	300037	424279	455873
2 (area)	n.d.	5682	46551	116106	224248	347806	426406	n.d.	13.52	2677	5425	8052	12186	16568	n.d.	12119	108427	218852	295929	409454	453576
3 (area)	n.d.	5893	47056	117056	223957	351612	431372	n.d.	6.16	2618	5727	8252	11922	16858	n.d.	12280	107921	202451	289371	427221	447568
Gj.snitt	-	5735.333	46674	117082	221818.7	349392.7	430080.3	-	15.80667	2614.667	5532.333	8117.333	11873.67	16602	-	12160.33	106438.3	209203.7	295112.3	420318	452339
St.dev	-	138.904	337.7381	989.2563	3958.39	1980.305	3228.485	-	10.97022	64.06507	168.8826	116.6419	339.0934	240.807	-	105.2727	3016.89	8575.364	5379.693	9522.8	4288.459
RSD (%)	-	2.4219	0.723611	0.844926	1.784516	0.566785	0.75067	-	69.40249	2.450219	3.052647	1.436949	2.855844	1.45047	-	0.865705	2.834402	4.09905	1.822931	2.265618	0.948063
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Consentration (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1252	13272	34403	69965	105913	134469	n.d.	80.1	5953	12295	16751	25156	33654	n.d.	45.56	217	383	602	1024	1315
2 (area)	n.d.	1152	13062	35032	68224	105942	135376	n.d.	92.22	5842	11924	17453	24649	34433	n.d.	34.09	138	408	495	882	1442
3 (area)	n.d.	1104	12875	34793	69301	110854	137958	n.d.	94.42	6038	12415	17229	24136	33336	n.d.	18.72	169	434	385	1055	1031
Gj.snitt	-	1169.333	13069.67	34769.33	69130	107569.7	135934.3	-	88.91333	5944.333	12211.33	17144.33	24647	33807.67	-	32.79	174.6667	408.3333	494	987	1262.667
St.dev	-	75.50717	198.611	275.2641	833.7572	2844.353	1810.271	-	7.711429	98.28699	255.9694	358.5768	510.0029	564.4133	-	13.46714	39.80368	25.50163	108.5035	92.24424	210.4384
RSD (%)	-	6.457284	1.519633	0.791686	1.206071	2.644196	1.331725	-	8.672972	1.653457	2.096163	2.091518	2.069229	1.669483	-	41.07088	22.78837	6.245298	21.96426	9.345921	16.66619
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Consentration (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	287306	249624	255897	261682	251524	264359	24768	14942	12841	12883	13649	13670	14450	14124	20822	17378	15011	14910	14173	15474	13535
2 (area)	270863	253295	254111	258510	255862	265514	246592	13893	13527	13289	13557	13801	15441	13787	19896	17304	14625	14932	14440	14911	13499
3 (area)	274089	252453	254274	258360	253819	263978	249114	14294	13587	13775	13780	14078	14504	13825	20064	17019	14804	14673	14063	15152	14368
Gj.snitt	277419.3	251790.7	254760.7	259517.3	253735	264617	173491.3	14376.33	13318.33	13315.67	13662	13849.67	14798.33	13912	20260.67	17233.67	14813.33	14838.33	14225.33	15179	13800.67
St.dev	8712.715	1923.038	987.4626	1876.156	2170.22	799.8419	128804.4	529.3244	414.4699	446.5975	112.0669	208.3083	557.2202	184.5779	493.3329	189.553	193.1692	143.6048	193.872	282.4695	491.6547
RSD (%)	3.14063	0.763745	0.387604	0.722941	0.85531	0.302264	74.24253	3.681915	3.112026	3.353925	0.820282	1.504067	3.765425	1.326753	2.434929	1.099899	1.304022	0.967796	1.362864	1.860923	3.562543

B.2.4. Raw data for validation curves from validation of the method

Table B.38-B.45 shows the raw data obtained from the validation of the LC-MS method with cell medium without FBS as matrix.

Table B.38: Raw data for the calibration curves for day 1 rep 1 and 2 without FA of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine							
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500	
1 (area)	n.d.	5600	39788	107004	213838	332419	431212	n.d.	29.94	2961	6027	8443	12481	19233	n.d.	5245	45708	98527	140560	212087	282247	
2 (area)	n.d.	5543	41987	103816	207562	318438	426561	n.d.	10.44	3111	5925	9173	12074	18842	n.d.	5508	50104	97717	146474	200342	267882	
3 (area)	n.d.	5737	39833	110018	216670	332379	429222	n.d.	4.54	2879	5589	8416	11979	19365	n.d.	5495	45272	94849	147859	206929	269552	
Average	-	5626.667	40536	106946	212690	327745.3	428998.3	-	14.97333	2983.667	5847	8677.333	12178	19146.67	-	5416	47028	97031	144964.3	206452.7	273227	
St.dev.	-	99.71125	1256.804	3101.407	4661.26	8060.412	2333.553	-	13.29298	117.6492	229.1812	429.4722	266.6702	271.9786	-	148.2329	2672.799	1932.581	3876.618	5886.971	7856.05	
RSD (%)	-	1.772119	3.100464	2.899975	2.191575	2.459352	0.543954	-	88.7777	3.943108	3.919637	4.949356	2.18977	1.420501	-	2.736945	5.683421	1.991715	2.674188	2.851487	2.875283	
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine							
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500	
1 (area)	n.d.	1418	14172	35068	65720	108706	152981	n.d.	117	7021	13977	20537	28496	44238	n.d.	8.52	48.92	179	352	694	655	
2 (area)	n.d.	1303	13187	37390	71114	115226	148367	n.d.	109	6736	13966	20939	28859	43998	n.d.	13.21	21.89	131	236	331	797	
3 (area)	n.d.	1245	14178	37132	74750	101493	150416	n.d.	120	6606	13653	20948	29509	43152	n.d.	47.25	33.94	186	282	401	754	
Average	-	1322	13845.67	36530	70528	108475	150588	-	115.3333	6787.667	13865.33	20808	28954.67	43796	-	22.99333	34.91667	165.3333	290	475.3333	735.3333	
St.dev.	-	88.05112	570.43	1272.684	4543.432	6869.414	2311.804	-	5.686241	212.2695	183.9683	234.736	513.2313	570.484	-	21.13737	13.54144	29.93883	58.41233	192.5781	72.81712	
RSD (%)	-	6.660448	4.119917	3.483941	6.442025	6.332716	1.535185	-	4.930267	3.127282	1.326822	1.128105	1.772534	1.302594	-	91.92826	38.78217	18.10816	20.14218	40.51433	9.902601	
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5							
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
1 (area)	265506	259493	275617	276192	295428	293361	291434	13900	13577	14308	15114	14996	14803	15814	21854	19106	19782	19512	18108	16921	20615	
2 (area)	260232	263235	281551	275900	291759	286602	295454	13721	14683	15283	14751	15619	15918	15694	21304	19629	20652	20466	19268	19137	21186	
3 (area)	273706	262525	279060	281716	297932	283874	291650	14058	14162	14472	14605	15737	14677	15646	22420	19551	20717	19390	19840	18562	21874	
Average	266481.3	261751	278742.7	277936	295039.7	287945.7	292846	13893	14140.67	14687.67	14823.33	15450.67	15132.67	15718	21859.33	19428.67	20383.67	19789.33	19072	18206.67	21225	
St.dev.	6789.744	1987.448	2979.7	3276.83	3104.768	4884.145	2261.175	168.609	553.3085	522.054	262.096	398.1486	683.0303	86.53323	558.0191	282.1459	522.0712	589.1768	882.4783	1149.939	630.4054	
RSD (%)	2.547925	0.759289	1.068979	1.178987	1.052322	1.696204	0.772138	1.213626	3.912889	3.55437	1.768132	2.576903	4.513615	0.550536	2.552773	1.452215	2.561223	2.977244	4.627089	6.316034	2.970108	

Rep 2	Phenacetin							Tolbutamide							Fluoxetine							
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500	
1 (area)	n.d.	5240	43291	105987	208763	318713	430986	n.d.	11.93	3072	6595	9582	13456	20190	n.d.	3099	33480	73852	108876	156563	186258	
2 (area)	n.d.	5363	41866	109238	212671	317526	418624	n.d.	18.7	2937	6300	9626	12947	20595	n.d.	3101	33729	75668	111410	149587	192479	
3 (area)	n.d.	5660	41308	106553	207870	323710	414106	n.d.	9.21	2911	6523	9890	13275	19738	n.d.	3577	33276	76249	111185	153559	190267	
Average	-	5421	42155	107259.3	209768	319983	421238.7	-	13.28	2973.333	6472.667	9699.333	13226	20174.33	-	3259	33495	75256.33	110490.3	153236.3	189668	
St.dev.	-	215.9236	1022.601	1736.787	2553.413	3281.789	8738.476	-	4.886911	86.43109	153.8062	166.5813	258.0136	428.7147	-	275.3979	226.8722	1250.402	1402.573	3499.176	3153.46	
RSD (%)	-	3.983095	2.425812	1.619241	1.217256	1.025614	2.074471	-	36.79903	2.906875	2.376241	1.717451	1.950806	2.12505	-	8.45038	0.677332	1.661524	1.269408	2.283516	1.662621	
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine							
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500	
1 (area)	n.d.	1423	12006	31332	65837	95747	119124	n.d.	100	6015	12349	18896	24869	38603	n.d.	15.08	68.89	158	251	560	841	
2 (area)	n.d.	839	12298	34422	64019	89048	136043	n.d.	82.36	5926	12281	18565	25749	38559	n.d.	37.73	63.61	139	374	616	589	
3 (area)	n.d.	1288	10887	28445	62823	98277	131838	n.d.	81.15	5622	12207	18859	24895	38282	n.d.	7.78	65.82	166	258	488	696	
Average	-	1183.333	11730.33	31399.67	64226.33	94357.33	129001.7	-	87.83667	5854.333	12279	18773.33	25171	38481.33	-	20.19667	66.10667	154.3333	294.3333	554.6667	708.6667	
St.dev.	-	305.7455	744.7982	2989.074	1517.659	4768.856	8808.901	-	10.55112	206.0688	71.02112	181.368	500.7315	174.0239	-	15.61684	2.651647	13.86843	69.08208	64.16645	126.4766	
RSD (%)	-	25.83765	6.349335	9.519447	2.362986	5.054039	6.828517	-	12.0122	3.519936	0.578395	0.966093	1.989319	0.45223	-	77.32387	4.011165	8.986023	23.47069	11.56847	17.84712	
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5							
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
1 (area)	316187	290353	330908	315593	328207	314215	322160	12865	12235	12881	12998	12939	12572	13675	17930	14674	19406	20165	21117	19153	19673	
2 (area)	304771	297385	322409	319494	334404	319121	323166	12791	12050	13177	13158	13011	12786	13918	18520	15193	20349	20878	21002	19980	21151	
3 (area)	306678	303922	330362	326082	331475	322448	317254	12837	13467	13098	12627	13097	12635	13600	18205	14792	19386	20318	21835	20327	20850	
Average	309212	297220	327893	320389.7	331362	318594.7	320860	12831	12584	13052	12927.67	13015.67	12664.33	13731	18218.33	14886.33	19713.67	20453.67	21318	19820	20558	
St.dev.	9115.319	6786.005	4757.123	5301.551	3100.045	4141.059	3103.137	37.36308	770.2746	158.2677	272.3974	79.10331	109.9742	166.2318	295.2259	272.0558	550.3057	375.3616	451.4122	603.1327	781.0691	
RSD (%)	1.977711	2.283159	1.450816	1.65472	0.935546	1.299978	0.985831	0.291194	6.121063	1.174285	2.107089	0.607755	0.868378	1.210631	1.620488	1.827554	2.791493	1.83518	2.117517	3.043051	3.799344	

Table B.39: Raw data for the retention times for day 1 rep 1 and 2 without FA of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.28	5.27	5.26	5.26	5.25	5.25	n.d.	8.11	8.11	8.11	8.11	8.09	8.09	n.d.	8.17	8.17	8.17	8.18	8.17	8.15
2 (t_R)	n.d.	5.26	5.27	5.26	5.25	5.26	5.26	n.d.	8.15	8.12	8.09	8.11	8.09	8.1	n.d.	8.16	8.18	8.18	8.17	8.14	8.16
3 (t_R)	n.d.	5.25	5.27	5.26	5.26	5.26	5.26	n.d.	8.06	8.11	8.12	8.11	8.09	8.1	n.d.	8.18	8.2	8.17	8.16	8.16	8.19
Average	-	5.263	5.270	5.260	5.257	5.257	5.257	-	8.107	8.113	8.107	8.110	8.090	8.097	-	8.170	8.183	8.173	8.170	8.157	8.167
St.dev.	-	0.015	0.000	0.000	0.006	0.006	0.006	-	0.045	0.006	0.015	0.000	0.000	0.006	-	0.010	0.015	0.006	0.010	0.015	0.021
RSD (%)	-	0.290	0.000	0.000	0.110	0.110	0.110	-	0.556	0.071	0.188	0.000	0.000	0.071	-	0.122	0.187	0.071	0.122	0.187	0.255
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.88	1.9	1.88	1.88	1.88	1.89	n.d.	5.26	5.27	5.27	5.26	5.26	5.26	n.d.	8.2	8.16	8.2	8.25	8.19	8.14
2 (t_R)	n.d.	1.89	1.89	1.88	1.88	1.87	1.88	n.d.	5.28	5.26	5.26	5.27	5.26	5.25	n.d.	8.19	8.2	8.19	8.17	8.22	8.16
3 (t_R)	n.d.	1.89	1.89	1.89	1.87	1.88	1.89	n.d.	5.25	5.26	5.27	5.27	5.26	5.25	n.d.	8.22	8.2	8.18	8.16	8.14	8.21
Average	-	1.887	1.893	1.883	1.877	1.877	1.887	-	5.263	5.263	5.267	5.267	5.260	5.253	-	8.203	8.187	8.190	8.193	8.183	8.170
St.dev.	-	0.006	0.006	0.006	0.006	0.006	0.006	-	0.015	0.006	0.006	0.006	0.000	0.006	-	0.015	0.023	0.010	0.049	0.040	0.036
RSD (%)	-	0.306	0.305	0.307	0.308	0.308	0.306	-	0.290	0.110	0.110	0.110	0.000	0.110	-	0.186	0.282	0.122	0.602	0.494	0.441
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.23	5.22	5.22	5.22	5.21	5.22	5.22	8.02	8	7.98	7.97	8	7.99	7.96	8.13	8.09	8.1	8.09	8.09	8.09	8.07
2 (t_R)	5.23	5.22	5.22	5.22	5.22	5.22	5.21	8.01	7.98	8.01	7.98	7.99	7.97	8	8.12	8.08	8.09	8.1	8.08	8.08	8.08
3 (t_R)	5.22	5.22	5.22	5.22	5.23	5.21	5.21	7.99	7.99	7.99	8	7.98	7.99	7.98	8.09	8.1	8.1	8.09	8.11	8.09	8.08
Average	5.227	5.220	5.220	5.220	5.220	5.217	5.213	8.007	7.990	7.993	7.983	7.990	7.983	7.980	8.113	8.090	8.097	8.093	8.093	8.087	8.077
St.dev.	0.006	0.000	0.000	0.000	0.010	0.006	0.006	0.015	0.010	0.015	0.015	0.010	0.012	0.020	0.021	0.010	0.006	0.006	0.015	0.006	0.006
RSD (%)	0.110	0.000	0.000	0.000	0.192	0.111	0.111	0.191	0.125	0.191	0.191	0.125	0.145	0.251	0.257	0.124	0.071	0.071	0.189	0.071	0.071

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.27	5.25	5.25	5.25	5.25	5.25	n.d.	8.12	8.09	8.07	8.06	8.07	8.07	n.d.	8.13	8.13	8.13	8.14	8.17	8.11
2 (t_R)	n.d.	5.25	5.25	5.25	5.25	5.24	5.25	n.d.	8.07	8.07	8.08	8.07	8.06	8.06	n.d.	8.12	8.13	8.15	8.12	8.14	8.14
3 (t_R)	n.d.	5.25	5.24	5.24	5.25	5.25	5.25	n.d.	8.11	8.06	8.06	8.07	8.06	8.06	n.d.	8.12	8.12	8.13	8.13	8.13	8.11
Average	-	5.257	5.247	5.247	5.250	5.247	5.250	-	8.100	8.073	8.070	8.067	8.063	8.063	-	8.123	8.127	8.137	8.130	8.147	8.120
St.dev.	-	0.012	0.006	0.006	0.000	0.006	0.000	-	0.026	0.015	0.010	0.006	0.006	0.006	-	0.006	0.006	0.012	0.010	0.021	0.017
RSD (%)	-	0.220	0.110	0.110	0.000	0.110	0.000	-	0.327	0.189	0.124	0.072	0.072	0.072	-	0.071	0.071	0.142	0.123	0.256	0.213
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.91	1.87	1.88	1.89	1.88	1.88	n.d.	5.26	5.26	5.25	5.25	5.25	5.25	n.d.	8.18	8.11	8.1	8.09	8.22	8.15
2 (t_R)	n.d.	1.9	1.88	1.89	1.89	1.88	1.88	n.d.	5.26	5.25	5.25	5.24	5.25	5.25	n.d.	8.18	8.17	8.18	8.16	8.17	8.16
3 (t_R)	n.d.	1.87	1.89	1.89	1.88	1.87	1.89	n.d.	5.26	5.26	5.25	5.25	5.25	5.25	n.d.	8.11	8.18	8.15	8.12	8.16	8.16
Average	-	1.893	1.880	1.887	1.887	1.877	1.883	-	5.260	5.257	5.250	5.247	5.250	5.250	-	8.157	8.153	8.143	8.123	8.183	8.157
St.dev.	-	0.021	0.010	0.006	0.006	0.006	0.006	-	0.000	0.006	0.000	0.006	0.000	0.000	-	0.040	0.038	0.040	0.035	0.032	0.006
RSD (%)	-	1.099	0.532	0.306	0.306	0.308	0.307	-	0.000	0.110	0.000	0.110	0.000	0.000	-	0.495	0.464	0.496	0.432	0.393	0.071
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.21	5.22	5.22	5.21	5.2	5.21	5.21	7.96	7.98	7.96	7.95	7.97	7.96	7.93	8.08	8.08	8.04	8.05	8.04	8.04	8.03
2 (t_R)	5.21	5.21	5.21	5.21	5.2	5.21	5.22	8	7.96	7.95	7.94	7.94	7.96	7.95	8.09	8.04	8.05	8.04	8.05	8.03	8.06
3 (t_R)	5.22	5.21	5.21	5.21	5.21	5.2	5.21	7.99	7.96	7.96	7.95	7.95	7.95	7.96	8.09	8.07	8.04	8.03	8.05	8.06	8.05
Average	5.213	5.213	5.213	5.210	5.203	5.207	5.213	7.983	7.967	7.957	7.947	7.953	7.957	7.947	8.087	8.063	8.043	8.040	8.047	8.043	8.047
St.dev.	0.006	0.006	0.006	0.000	0.006	0.006	0.006	0.021	0.012	0.006	0.006	0.015	0.006	0.015	0.006	0.021	0.006	0.010	0.006	0.015	0.015
RSD (%)	0.111	0.111	0.111	0.000	0.111	0.111	0.111	0.261	0.145	0.073	0.073	0.192	0.073	0.192	0.071	0.258	0.072	0.124	0.072	0.190	0.190

Table B.40: Raw data for the calibration curves for day 1 rep 1 and 2 with FA of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	5625	42442	103249	205102	339133	408089	n.d.	17.4	3380	6022	9466	13711	18508	n.d.	5374	50497	109061	171154	238394	279153
2 (area)	n.d.	5529	40916	104946	207261	385314	423502	n.d.	20.46	3248	6026	9434	13334	18462	n.d.	5606	53198	105048	173843	247056	285976
3 (area)	n.d.	5642	41915	102894	209023	343424	417407	n.d.	20.35	3280	6472	9685	13636	18227	n.d.	5400	52964	108172	176362	243280	283064
Average	-	5598.667	41757.67	103696.3	207128.7	355957	416332.7	-	19.40333	3302.667	6173.333	9528.333	13560.33	18399	-	5460	52219.67	107427	173786.3	242910	282731
St.dev.	-	60.92892	775.0705	1096.702	1963.847	25514.28	7762.46	-	1.735809	68.85734	258.6607	136.6175	199.5654	150.7216	-	127.1063	1496.454	2107.679	2604.462	4342.837	3423.667
RSD (%)	-	1.088276	1.856116	1.05761	0.948129	7.167797	1.864485	-	8.945933	2.084901	4.189967	1.433802	1.471685	0.819184	-	2.327953	2.86569	1.961964	1.498658	1.787838	1.210928
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	1202	13166	33413	69287	98942	123457	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	911	11531	33372	62274	103228	119983	n.d.	68.04	6451	12210	18721	27338	38457	n.d.	79.19	99.86	356	715	987	1307
2 (area)	n.d.	1237	12499	33413	68261	95846	127709	n.d.	98.99	6525	12815	19445	23120	38279	n.d.	29.3	172	303	536	981	979
3 (area)	n.d.	1116.667	12398.67	33399.33	66607.33	99338.67	123716.3	n.d.	80.97	6075	12216	19458	27184	37400	n.d.	36.48	156	293	799	1007	1084
Average	-	178.9702	822.1048	23.67136	3787.678	3706.951	3869.523	-	82.66667	6350.333	12413.67	19208	25880.67	38045.33	-	48.32333	142.62	317.3333	683.3333	991.6667	1123.333
St.dev.	-	16.02718	6.630591	0.070874	5.686578	3.73163	3.127738	-	15.5446	241.2993	347.5778	421.8045	2392.047	565.9172	-	26.97131	37.88553	33.85754	134.3292	13.61372	167.5002
RSD (%)	-	8.955224	0.806538	0.299407	0.150134	0.100666	0.08083	-	18.80395	3.799789	2.799961	2.195983	9.242602	1.487481	-	55.81425	26.56397	10.66939	19.65793	1.372812	14.911
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	391189	366449	360300	367923	299985	374195	378278	18184	16671	16580	14158	12029	15185	15142	34006	32717	32986	32326	32688	35504	33196
2 (area)	384074	362192	368621	358340	303239	367541	375279	17903	15237	15668	14760	11847	14983	15273	33582	30960	33397	34426	32348	36695	35065
3 (area)	376218	356763	366552	359106	302885	370900	377341	16514	15117	15687	14269	12446	15121	14957	31639	31350	31702	35415	33056	35094	36775
Average	383827	361801.3	365157.7	361789.7	302036.3	370878.7	376966	17533.67	15675	15978.33	14395.67	12107.33	15096.33	15124	33075.67	31675.67	32695	34055.67	32697.33	35764.33	35012
St.dev.	7488.556	4854.803	4332.192	5325.413	1785.303	3227.051	1521.265	894.1646	864.6456	521.1452	220.3659	307.0868	103.2344	158.7671	1262.122	922.6626	884.1759	1577.447	354.0923	831.6432	1790.089
RSD (%)	1.951024	1.341842	1.186389	1.471964	0.591089	0.897073	0.407004	5.099701	5.51608	3.261574	2.225433	2.536371	0.683837	1.049769	3.815863	2.912844	2.704315	4.631967	1.082939	2.325342	5.112786

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	6678	46373	115149	225481	365197	418828	n.d.	11.86	3108	6783	10377	15253	20219	n.d.	8125	71131	145787	216082	292736	323020
2 (area)	n.d.	6255	45734	116568	227618	351842	426699	n.d.	7.09	3201	6610	10616	15175	20138	n.d.	8403	70754	137837	207790	285107	325316
3 (area)	n.d.	6046	45922	110724	216788	345540	428264	n.d.	5.52	3332	6929	10836	14458	20352	n.d.	7596	68575	141634	220580	293408	326619
Average	-	6326.333	46009.67	114147	223295.7	354193	424597	-	8.156667	3213.667	6774	10609.67	14962	20236.33	-	8041.333	70153.33	141752.7	214817.3	290417	324985
St.dev.	-	321.9819	328.3966	3048.128	5736.2	10037.17	5057.008	-	3.301853	112.5359	159.6903	229.5655	438.2157	108.0478	-	409.9541	1379.813	3976.328	6488.109	4610.854	1822.189
RSD (%)	-	5.08955	0.713756	2.670354	2.568881	2.833814	1.191014	-	40.48042	3.501792	2.357401	2.163739	2.928858	0.53393	-	5.098086	1.966853	2.805117	3.020291	1.587667	0.560699
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1164	13426	35250	72669	106643	140476	n.d.	90.77	6747	13349	21115	29069	39890	n.d.	173	175	480	1130	888	1355
2 (area)	n.d.	1195	14651	34032	72942	115775	143235	n.d.	120	6730	13875	21314	30807	41227	n.d.	61.79	172	407	910	1018	1489
3 (area)	n.d.	1138	11912	36313	74328	117924	139639	n.d.	113	6857	13793	20892	29188	39619	n.d.	82.18	243	508	1035	1458	1403
Average	-	1165.667	13329.67	35198.33	73313	113447.3	141116.7	-	107.9233	6778	13672.33	21107	29688	40245.33	-	105.6567	196.6667	465	1025	1121.333	1415.667
St.dev.	-	28.53653	1372.039	1141.377	889.551	5989.889	1881.66	-	15.26197	68.942	283.0006	211.1137	970.9073	860.8788	-	59.20542	40.15387	52.14403	110.3404	298.7195	67.89207
RSD (%)	-	2.448086	10.29312	3.242703	1.213361	5.279885	1.333407	-	14.14149	1.017144	2.069878	1.000207	3.27037	2.139077	-	56.03567	20.41722	11.21377	10.76492	26.63967	4.795767
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	380126	361603	379739	366726	313317	376574	359719	15058	14123	142032	14576	12911	15838	14945	34862	29007	27612	36374	27145	35535	33996
2 (area)	369326	365262	373928	360980	312711	383277	360349	13312	13901	14393	14860	13075	15952	14613	19122	34300	37316	36754	34158	34705	31690
3 (area)	367474	368259	374485	360940	307042	372594	359407	14299	13825	14100	14476	12702	15178	15024	33913	23699	36223	34197	33969	24798	34779
Average	372308.7	365041.3	376050.7	362882	311023.3	377481.7	359825	14223	13949.67	56841.67	14637.33	12896	15656	14860.67	29299	29002	33717	35775	31757.33	31679.33	33488.33
St.dev.	6833.045	3333.402	3206.309	3329.062	3461.224	5399.029	479.8625	875.4776	154.0462	73777.14	199.2110	186.9519	417.866	210.0925	8826.304	5300.502	5315.255	1379.733	3995.516	5973.842	1605.856
RSD (%)	1.835317	0.913179	0.852627	0.917395	1.11285	1.430276	0.13336	6.155365	1.110035	129.7941	1.360984	1.449689	2.669047	1.467582	30.12493	18.27633	15.76432	3.856696	12.58139	18.85722	4.795271

Table B.41: Raw data for the retention times for day 1 rep 1 and 2 with FA of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.25	5.24	5.25	5.25	5.25	5.24	n.d.	8.09	8.06	8.05	8.06	8.05	8.05	n.d.	8.12	8.12	8.11	8.12	8.1	8.11
2 (t_R)	n.d.	5.25	5.26	5.25	5.25	5.25	5.24	n.d.	8.05	8.06	8.06	8.06	8.06	8.06	n.d.	8.11	8.1	8.13	8.11	8.13	8.11
3 (t_R)	n.d.	5.25	5.25	5.25	5.25	5.25	5.25	n.d.	8.08	8.06	8.07	8.06	8.04	8.05	n.d.	8.13	8.11	8.11	8.11	8.1	8.11
Average	-	5.250	5.250	5.250	5.250	5.250	5.243	-	8.073	8.060	8.060	8.060	8.050	8.053	-	8.120	8.110	8.117	8.113	8.110	8.110
St.dev.	-	0.000	0.010	0.000	0.000	0.000	0.006	-	0.021	0.000	0.010	0.000	0.010	0.006	-	0.010	0.010	0.012	0.006	0.017	0.000
RSD (%)	-	0.000	0.190	0.000	0.000	0.000	0.110	-	0.258	0.000	0.124	0.000	0.124	0.072	-	0.123	0.123	0.142	0.071	0.214	0.000
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.88	1.88	1.89	1.88	1.88	1.89	n.d.	5.28	5.25	5.26	5.25	5.25	5.25	n.d.	8.07	8.16	8.14	8.12	8.11	8.11
2 (t_R)	n.d.	1.89	1.87	1.89	1.88	1.88	1.88	n.d.	5.26	5.26	5.26	5.25	5.26	5.25	n.d.	8.09	8.17	8.15	8.2	8.18	8.11
3 (t_R)	n.d.	1.88	1.88	1.88	1.88	1.89	1.89	n.d.	5.24	5.26	5.26	5.25	5.25	5.25	n.d.	8.14	8.12	8.12	8.12	8.16	8.11
Average	-	1.883	1.877	1.887	1.880	1.883	1.887	-	5.260	5.257	5.260	5.250	5.253	5.250	-	8.100	8.150	8.137	8.147	8.150	8.110
St.dev.	-	0.006	0.006	0.006	0.000	0.006	0.006	-	0.020	0.006	0.000	0.000	0.006	0.000	-	0.036	0.026	0.015	0.046	0.036	0.000
RSD (%)	-	0.307	0.308	0.306	0.000	0.307	0.306	-	0.380	0.110	0.000	0.000	0.110	0.000	-	0.445	0.325	0.188	0.567	0.442	0.000
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.21	5.21	5.22	5.21	5.21	5.21	5.21	7.95	7.93	7.95	7.95	7.94	7.94	7.94	8.06	8.01	8.05	8.06	8.02	8.04	8.05
2 (t_R)	5.21	5.21	5.21	5.21	5.21	5.21	5.2	7.94	7.95	7.95	7.95	7.96	7.95	7.95	8.05	8.05	8.05	8.04	8.04	8.04	8.04
3 (t_R)	5.22	5.21	5.21	5.21	5.21	5.21	5.21	7.97	7.93	7.95	7.94	7.94	7.94	7.95	8.06	8.08	8.03	8.03	8.05	8.04	8.01
Average	5.213	5.210	5.213	5.210	5.210	5.210	5.207	7.953	7.937	7.950	7.947	7.947	7.943	7.947	8.057	8.047	8.043	8.043	8.037	8.040	8.033
St.dev.	0.006	0.000	0.006	0.000	0.000	0.000	0.006	0.015	0.012	0.000	0.006	0.012	0.006	0.006	0.006	0.035	0.012	0.015	0.015	0.000	0.021
RSD (%)	0.111	0.000	0.111	0.000	0.000	0.000	0.111	0.192	0.145	0.000	0.073	0.145	0.073	0.073	0.072	0.436	0.144	0.190	0.190	0.000	0.259

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.25	5.24	5.24	5.26	5.25	5.26	n.d.	8.07	8.05	8.06	8.08	8.05	8.05	n.d.	8.09	8.12	8.11	8.14	8.12	8.11
2 (t_R)	n.d.	5.25	5.24	5.24	5.26	5.25	5.24	n.d.	8.04	8.06	8.07	8.07	8.07	8.06	n.d.	8.12	8.14	8.12	8.14	8.12	8.12
3 (t_R)	n.d.	5.24	5.25	5.25	5.25	5.25	5.25	n.d.	8.11	8.09	8.05	8.07	8.06	8.08	n.d.	8.14	8.13	8.12	8.14	8.12	8.16
Average	-	5.247	5.243	5.243	5.257	5.250	5.250	-	8.073	8.067	8.060	8.073	8.060	8.063	-	8.117	8.130	8.117	8.140	8.120	8.130
St.dev.	-	0.006	0.006	0.006	0.006	0.000	0.010	-	0.035	0.021	0.010	0.006	0.010	0.015	-	0.025	0.010	0.006	0.000	0.000	0.026
RSD (%)	-	0.110	0.110	0.110	0.110	0.000	0.190	-	0.435	0.258	0.124	0.072	0.124	0.189	-	0.310	0.123	0.071	0.000	0.000	0.325
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.88	1.9	1.88	1.87	1.87	1.88	n.d.	5.24	5.24	5.25	5.25	5.25	5.25	n.d.	8.07	8.14	8.13	8.13	8.14	8.1
2 (t_R)	n.d.	1.89	1.88	1.88	1.89	1.88	1.89	n.d.	5.26	5.25	5.25	5.25	5.25	5.24	n.d.	8.14	8.16	8.16	8.16	8.13	8.14
3 (t_R)	n.d.	1.88	1.88	1.88	1.89	1.88	1.89	n.d.	5.24	5.24	5.25	5.26	5.25	5.25	n.d.	8.11	8.12	8.14	8.2	8.12	8.14
Average	-	1.883	1.887	1.880	1.883	1.877	1.887	-	5.247	5.243	5.250	5.253	5.250	5.247	-	8.107	8.140	8.143	8.163	8.130	8.127
St.dev.	-	0.006	0.012	0.000	0.012	0.006	0.006	-	0.012	0.006	0.000	0.006	0.000	0.006	-	0.035	0.020	0.015	0.035	0.010	0.023
RSD (%)	-	0.307	0.612	0.000	0.613	0.308	0.306	-	0.220	0.110	0.000	0.110	0.000	0.110	-	0.433	0.246	0.188	0.430	0.123	0.284
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.21	5.21	5.2	5.21	5.2	5.21	5.22	7.94	7.94	7.95	7.94	7.95	7.95	7.94	8.03	8.01	8.03	8.04	8.05	8.05	8.01
2 (t_R)	5.21	5.21	5.21	5.21	5.21	5.21	5.21	7.95	7.95	7.96	7.95	7.96	7.94	7.95	8.06	8.03	8.07	8.04	8.06	8.05	8.02
3 (t_R)	5.21	5.2	5.21	5.21	5.21	5.21	5.21	7.92	7.94	7.95	7.93	7.96	7.95	7.96	8.03	8.04	8.04	8.02	8.05	8.05	8.05
Average	5.210	5.207	5.207	5.210	5.207	5.210	5.213	7.937	7.943	7.953	7.940	7.957	7.947	7.950	8.040	8.027	8.047	8.033	8.053	8.050	8.027
St.dev.	0.000	0.006	0.006	0.000	0.006	0.000	0.006	0.015	0.006	0.006	0.010	0.006	0.006	0.010	0.017	0.015	0.021	0.012	0.006	0.000	0.021
RSD (%)	0.000	0.111	0.111	0.000	0.111	0.000	0.111	0.192	0.073	0.073	0.126	0.073	0.073	0.126	0.215	0.190	0.259	0.144	0.072	0.000	0.259

Table B.42: Raw data for the calibration curves for day 2 rep 1 and 2 of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	6849	50768	128250	221887	376387	504455	n.d.	13.6	3101	6677	9516	13292	20562	n.d.	7435	75625	156584	220375	304482	354848
2 (area)	n.d.	6285	49547	131127	226676	377605	488263	n.d.	13.05	3286	6774	9892	13942	20228	n.d.	7166	74993	156480	230975	320050	353900
3 (area)	n.d.	6702	49128	131467	224431	378154	512210	n.d.	11.07	3465	6735	10071	13306	20562	n.d.	7286	75223	165540	220997	302116	356617
Average	-	6612	49814.33	130281.3	224331.3	377382	501642.7	-	12.57333	3284	6728.667	9826.333	13513.33	20450.67	-	7295.667	75280.33	159534.7	224115.7	308882.7	355121.7
St.dev.	-	292.5731	852.0565	1767.381	2396.055	904.3611	12218.7	-	1.330651	182.0082	48.80915	283.2672	371.3022	192.835	-	134.7603	319.8771	5201.031	5948.492	9743.279	1379.019
RSD (%)	-	4.42488	1.710465	1.356588	1.068088	0.239641	2.435738	-	10.58312	5.542273	0.725391	2.882736	2.747673	0.942928	-	1.847128	0.424915	3.260126	2.654206	3.154363	0.388323
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	1202	13166	33413	69287	98942	123457	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1292	13262	36026	61181	95934	144630	n.d.	122	7086	13277	19768	28168	40664	n.d.	86.74	199	483	777	1320	1448
2 (area)	n.d.	1374	14237	36339	57470	106655	144306	n.d.	94.13	6656	13516	19733	27751	40632	n.d.	53.49	165	517	739	1085	1453
3 (area)	n.d.	1215	11846	37102	61725	112582	137346	n.d.	127	7081	13690	19672	28035	40873	n.d.	57.71	187	541	843	1046	1449
Average	-	1293.667	13115	36489	60125.33	105057	142094	-	114.3767	6941	13494.33	19724.33	27984.67	40723	-	65.98	183.6667	513.6667	786.3333	1150.333	1450
St.dev.	-	79.5131	1202.259	553.4609	2315.617	8438.257	4115.079	-	17.71145	246.8299	207.3507	48.58326	213.0078	130.8854	-	18.10208	17.24336	29.14332	52.62446	148.2239	2.645751
RSD (%)	-	6.146336	9.167054	1.516788	3.851316	8.032075	2.896026	-	15.4852	3.556114	1.536576	0.246311	0.761159	0.321404	-	27.43571	9.388397	5.673587	6.692385	12.8853	0.182466
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	384838	350617	362893	354313	374915	357527	364530	16555	13353	13674	13052	13782	13542	13907	38278	34536	35605	35369	37052	36665	35690
2 (area)	387405	350747	367227	358008	375590	361212	360594	14986	13102	13458	13013	14364	13781	14032	34262	33167	32635	36019	36840	36240	34036
3 (area)	387391	352327	365588	360706	375532	356596	353731	15913	13380	13932	13271	14196	14396	14190	37382	34130	36190	34882	34274	36577	35929
Average	386544.7	351230.3	365236	357675.7	375345.7	358445	359618.3	15818	13278.33	13688	13112	14114	13906.33	14043	36640.67	33944.33	34810	35423.33	36055.33	36494	35218.33
St.dev.	1478.033	951.9629	2188.337	3209.431	374.094	2441.087	5465.212	788.8023	153.3047	237.3099	139.0719	299.5396	440.5795	141.8203	2108.138	703.1318	1906.181	570.444	1546.317	224.3279	1030.88
RSD (%)	0.382371	0.271037	0.599157	0.897302	0.099667	0.681021	1.519726	4.986738	1.154548	1.733708	1.060646	2.122287	3.168193	1.0099	5.753547	2.071426	5.475957	1.610362	4.288734	0.614698	2.927113

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	5991	44680	118763	205228	322852	428022	n.d.	23.62	3145	6559	8663	13623	19477	n.d.	6112	57291	116198	156053	212155	253557
2 (area)	n.d.	6705	45781	119982	199108	325913	431675	n.d.	29.04	3042	6259	9469	12860	19809	n.d.	6320	58898	113414	156998	210087	255097
3 (area)	n.d.	6300	44125	116723	197719	326986	437947	n.d.	17.56	3001	6550	8910	12853	19118	n.d.	5861	56567	115588	156820	212255	257865
Average	-	6332	44862	118489.3	200685	325250.3	432548	-	23.40667	3062.667	6456	9014	13112	19468	-	6097.667	57585.33	115066.7	156623.7	211499	255506.3
St.dev.	-	358.074	842.8683	1646.645	3995.18	2145.189	5019.761	-	5.742973	74.19119	170.6663	412.9419	442.5528	345.5879	-	229.8354	1193.048	1463.388	502.1617	1223.85	2182.975
RSD (%)	-	5.654991	1.878802	1.389699	1.990772	0.65955	1.16051	-	24.53563	2.422438	2.643531	4.581117	3.375174	1.775159	-	3.769236	2.071792	1.271774	0.320617	0.578655	0.854372
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	1202	13166	33413	69287	98942	123457	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1077	12636	37898	63154	105297	138244	n.d.	114	6426	13143	18732	25687	37413	n.d.	55.96	301	707	1169	1326	1893
2 (area)	n.d.	1410	13555	36184	61089	105481	140070	n.d.	122	6863	13399	18915	25533	38748	n.d.	80.97	346	745	930	1498	1780
3 (area)	n.d.	1154	13653	36355	61465	98307	141513	n.d.	113	6373	13178	18102	26171	39509	n.d.	112	245	571	998	1252	1711
Average	-	1213.667	13281.33	36812.33	61902.67	103028.3	139942.3	-	116.3333	6554	13240	18583	25797	38556.67	-	82.97667	297.3333	674.3333	1032.333	1358.667	1794.667
St.dev.	-	174.334	561.019	944.0945	1099.873	4089.829	1638.235	-	4.932883	268.9108	138.8056	426.4892	332.9204	1061.019	-	28.07384	50.59974	91.48406	123.1435	126.2115	91.88217
RSD (%)	-	14.36424	4.224117	2.564615	1.776778	3.969616	1.17065	-	4.2403	4.103002	1.048381	2.29505	1.290539	2.751842	-	33.83341	17.01785	13.56659	11.92866	9.289362	5.119735
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	344572	310841	304860	295998	306664	287444	291810	14634	12929	13183	13173	13057	13246	12942	21715	19532	15557	18640	16194	21480	21022
2 (area)	336060	310354	312868	295392	300921	287728	293721	13801	12769	12746	12471	13812	13187	13682	14871	16510	15720	17260	22054	20645	21201
3 (area)	336299	305078	305116	297993	299379	286182	300161	14228	12472	13021	13047	13630	13039	13858	22057	15246	18244	21511	21767	20969	21850
Average	338977	308757.7	307614.7	296461	302321.3	287118	295230.7	14221	12723.33	12983.33	12897	13499.67	13157.33	13494	19547.67	17096	16507	19137	20005	21031.33	21357.67
St.dev.	4846.885	3195.974	4551.32	1360.91	3839.076	822.9435	4375.399	416.3441	231.8972	220.9216	374.2673	394.0131	106.6411	486.0782	4053.72	2202.271	1506.492	2168.642	3303.541	420.9755	435.6654
RSD (%)	1.429857	1.035108	1.479552	0.459052	1.269866	0.286622	1.482027	2.929078	1.822614	1.701578	2.901972	2.918688	0.810507	3.60218	20.73762	12.88179	9.126384	11.33219	16.51358	2.001658	2.039855

Table B.43: Raw data for the retention times for day 2 rep 1 and 2 of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.24	5.25	5.24	5.24	5.24	5.24	n.d.	8.07	8.06	8.07	8.05	8.07	8.06	n.d.	8.11	8.11	8.11	8.11	8.13	8.11
2 (t_R)	n.d.	5.26	5.24	5.25	5.24	5.24	5.25	n.d.	8.09	8.04	8.07	8.07	8.06	8.05	n.d.	8.15	8.11	8.12	8.12	8.12	8.11
3 (t_R)	n.d.	5.25	5.24	5.24	5.24	5.25	5.25	n.d.	8.06	8.06	8.07	8.06	8.07	8.07	n.d.	8.14	8.14	8.12	8.13	8.13	8.12
Average	-	5.250	5.243	5.243	5.240	5.243	5.247	-	8.073	8.053	8.070	8.060	8.067	8.060	-	8.133	8.120	8.117	8.120	8.127	8.113
St.dev.	-	0.010	0.006	0.006	0.000	0.006	0.006	-	0.015	0.012	0.000	0.010	0.006	0.010	-	0.021	0.017	0.006	0.010	0.006	0.006
RSD (%)	-	0.190	0.110	0.110	0.000	0.110	0.110	-	0.189	0.143	0.000	0.124	0.072	0.124	-	0.256	0.213	0.071	0.123	0.071	0.071
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.89	1.89	1.88	1.89	1.89	1.9	n.d.	5.24	5.25	5.24	5.24	5.25	5.25	n.d.	8.13	8.12	8.16	8.14	8.19	8.17
2 (t_R)	n.d.	1.88	1.9	1.89	1.88	1.88	1.9	n.d.	5.27	5.25	5.25	5.24	5.24	5.25	n.d.	8.15	8.14	8.15	8.13	8.18	8.11
3 (t_R)	n.d.	1.89	1.89	1.88	1.88	1.88	1.89	n.d.	5.26	5.25	5.24	5.25	5.24	5.24	n.d.	8.14	8.15	8.09	8.13	8.15	8.14
Average	-	1.887	1.893	1.883	1.883	1.883	1.897	-	5.257	5.250	5.243	5.243	5.243	5.247	-	8.140	8.137	8.133	8.133	8.173	8.140
St.dev.	-	0.006	0.006	0.006	0.006	0.006	0.006	-	0.015	0.000	0.006	0.006	0.006	0.006	-	0.010	0.015	0.038	0.006	0.021	0.030
RSD (%)	-	0.306	0.305	0.307	0.307	0.307	0.304	-	0.291	0.000	0.110	0.110	0.110	0.110	-	0.123	0.188	0.465	0.071	0.255	0.369
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.2	5.21	5.21	5.2	5.2	5.21	5.21	7.95	7.94	7.95	7.94	7.92	7.96	7.93	8.03	8.06	8.02	8.03	8.04	8.05	8.05
2 (t_R)	5.21	5.22	5.21	5.21	5.2	5.2	5.21	7.94	7.93	7.94	7.95	7.95	7.93	7.94	8.05	8.04	8.06	8.04	8.05	8.04	8.03
3 (t_R)	5.2	5.2	5.21	5.2	5.21	5.2	5.21	7.93	7.95	7.95	7.93	7.94	7.93	7.96	8.04	8.06	8.05	8.05	8.06	8.05	8.05
Average	5.203	5.210	5.210	5.203	5.203	5.203	5.210	7.940	7.940	7.947	7.940	7.937	7.940	7.943	8.040	8.053	8.043	8.040	8.050	8.047	8.043
St.dev.	0.006	0.010	0.000	0.006	0.006	0.006	0.000	0.010	0.010	0.006	0.010	0.015	0.017	0.015	0.010	0.012	0.021	0.010	0.010	0.006	0.012
RSD (%)	0.111	0.192	0.000	0.111	0.111	0.111	0.000	0.126	0.126	0.073	0.126	0.192	0.218	0.192	0.124	0.143	0.259	0.124	0.124	0.072	0.144

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.25	5.26	5.26	5.26	5.27	5.26	n.d.	8.12	8.1	8.11	8.11	8.12	8.09	n.d.	8.17	8.17	8.17	8.16	8.16	8.13
2 (t_R)	n.d.	5.25	5.25	5.27	5.27	5.26	5.26	n.d.	8.08	8.12	8.1	8.1	8.11	8.08	n.d.	8.16	8.19	8.15	8.14	8.15	8.14
3 (t_R)	n.d.	5.26	5.26	5.26	5.25	5.25	5.26	n.d.	8.06	8.09	8.12	8.1	8.09	8.1	n.d.	8.17	8.17	8.15	8.15	8.15	8.15
Average	-	5.253	5.257	5.263	5.260	5.260	5.260	-	8.087	8.103	8.110	8.103	8.107	8.090	-	8.167	8.177	8.157	8.150	8.153	8.140
St.dev.	-	0.006	0.006	0.006	0.010	0.010	0.000	-	0.031	0.015	0.010	0.006	0.015	0.010	-	0.006	0.012	0.012	0.010	0.006	0.010
RSD (%)	-	0.110	0.110	0.110	0.190	0.190	0.000	-	0.378	0.189	0.123	0.071	0.188	0.124	-	0.071	0.141	0.142	0.123	0.071	0.123
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.88	1.88	1.88	1.88	1.89	1.88	n.d.	5.25	5.26	5.26	5.26	5.28	5.26	n.d.	8.19	8.19	8.16	8.15	8.16	8.17
2 (t_R)	n.d.	1.88	1.87	1.88	1.88	1.88	1.88	n.d.	5.25	5.26	5.27	5.27	5.26	5.26	n.d.	8.13	8.19	8.17	8.14	8.12	8.18
3 (t_R)	n.d.	1.88	1.88	1.88	1.89	1.86	1.89	n.d.	5.27	5.26	5.25	5.26	5.26	5.25	n.d.	8.16	8.14	8.16	8.17	8.14	8.18
Average	-	1.880	1.877	1.880	1.883	1.877	1.883	-	5.257	5.260	5.260	5.263	5.267	5.257	-	8.160	8.173	8.163	8.153	8.140	8.177
St.dev.	-	0.000	0.006	0.000	0.006	0.015	0.006	-	0.012	0.000	0.010	0.006	0.012	0.006	-	0.030	0.029	0.006	0.015	0.020	0.006
RSD (%)	-	0.000	0.308	0.000	0.307	0.814	0.307	-	0.220	0.000	0.190	0.110	0.219	0.110	-	0.368	0.353	0.071	0.187	0.246	0.071
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.21	5.22	5.22	5.22	5.22	5.23	5.22	8	7.96	7.99	7.98	7.97	7.99	7.99	8.1	8.09	8.09	8.09	8.08	8.07	8.06
2 (t_R)	5.21	5.22	5.22	5.23	5.23	5.22	5.22	7.98	7.97	7.96	7.98	7.98	8	7.99	8.07	8.09	8.07	8.09	8.07	8.08	8.05
3 (t_R)	5.21	5.22	5.22	5.21	5.21	5.21	5.21	7.97	7.96	7.96	7.99	7.99	7.98	7.98	8.09	8.08	8.09	8.07	8.1	8.07	8.09
Average	5.210	5.220	5.220	5.220	5.220	5.220	5.217	7.983	7.963	7.970	7.983	7.980	7.990	7.987	8.087	8.087	8.083	8.083	8.083	8.073	8.067
St.dev.	0.000	0.000	0.000	0.010	0.010	0.010	0.006	0.015	0.006	0.017	0.006	0.010	0.010	0.006	0.015	0.006	0.012	0.012	0.015	0.006	0.021
RSD (%)	0.000	0.000	0.000	0.192	0.192	0.192	0.111	0.191	0.073	0.217	0.072	0.125	0.125	0.072	0.189	0.071	0.143	0.143	0.189	0.072	0.258

Table B.44: Raw data for the calibration curves for day 3 rep 1 and 2 of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	6823	47278	119228	248133	412612	472291	n.d.	8.08	3305	6532	9753	13662	19596	n.d.	6915	69628	140740	215337	290451	338847
2 (area)	n.d.	6006	48452	119855	245125	375095	492224	n.d.	14.7	3224	6442	10074	13573	19479	n.d.	6934	68568	139364	219796	291834	343243
3 (area)	n.d.	6163	48660	122614	231722	387704	539030	n.d.	5.65	3049	6718	9375	13474	19094	n.d.	6716	70399	139924	210836	293709	337764
Average	-	6330.667	48130	120565.7	241660	391803.7	501181.7	-	9.476667	3192.667	6564	9734	13569.67	19389.67	-	6855	69531.67	140009.3	215323	291998	339951.3
St.dev.	-	433.5393	745.147	1801.398	8736.985	19091.54	34259.35	-	4.683869	130.8447	140.7551	349.8871	94.04432	262.6525	-	120.7518	919.2934	691.9576	4480.016	1635.18	2901.642
RSD (%)	-	6.848241	1.548196	1.494122	3.615404	4.87273	6.835716	-	49.42528	4.098289	2.14435	3.594485	0.693048	1.3546	-	1.761514	1.322122	0.494222	2.080603	0.559997	0.853546
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	1202	13166	33413	69287	98942	123457	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1487	14319	37553	79581	118588	155212	n.d.	125	6497	13272	19480	24601	39622	n.d.	126	326	708	1218	1706	2001
2 (area)	n.d.	1615	15602	40331	81658	117493	155084	n.d.	129	6817	13207	20080	26812	39334	n.d.	96.98	323	599	1231	1472	2095
3 (area)	n.d.	1700	15979	40900	78702	121348	158899	n.d.	115	7195	13746	20348	27987	36468	n.d.	59.04	278	774	1003	1815	2019
Average	-	1600.667	15300	39594.67	79980.33	119143	156398.3	-	123	6836.333	13408.33	19969.33	26466.67	38474.67	-	94.00667	309	693.6667	1150.667	1664.333	2038.333
St.dev.	-	107.221	870.2316	1790.878	1517.921	1986.523	2166.586	-	7.211103	349.4014	294.2284	444.4562	1719.212	1743.78	-	33.57888	26.88866	88.37609	128.0482	175.2551	49.89322
RSD (%)	-	6.698519	5.687788	4.523027	1.897868	1.667344	1.3853	-	5.862685	5.110947	2.194369	2.225694	6.495764	4.532281	-	35.71968	8.701831	12.74043	11.12817	10.53005	2.447746
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	342643	350615	344045	334964	353497	349145	338261	13545	14495	13977	13687	13950	14319	13505	20774	25624	22757	23855	24080	23468	22277
2 (area)	347951	352736	345256	340780	348708	340045	336617	14253	14249	13692	13436	14041	13887	13877	24274	25222	23883	23242	24198	23119	22631
3 (area)	350600	347413	347335	336210	348086	339304	339591	14225	14515	13566	13852	13553	13932	13553	26062	24496	23647	23801	23545	22989	22271
Average	347064.7	350254.7	345545.3	337318	350097	342831.3	338156.3	14007.67	14419.67	13745	13658.33	13848	14046	13645	23703.33	25114	23429	23632.67	23941	23192	22393
St.dev.	4051.87	2679.732	1663.974	3062.223	2960.865	5480.334	1489.76	400.9256	148.1396	210.5635	209.4763	259.4976	237.4932	202.3462	2689.792	571.7027	593.8114	339.4029	347.9842	247.7035	206.1359
RSD (%)	1.167468	0.765081	0.48155	0.907815	0.845727	1.598551	0.440554	2.862187	1.027344	1.531928	1.533689	1.873899	1.690824	1.482933	11.34774	2.27643	2.534515	1.43616	1.453507	1.068056	0.920537

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (area)	n.d.	6329	45712	122004	232668	366770	474774	n.d.	13.5	2982	6537	9653	12621	19283	n.d.	6343	65851	135514	206591	280763	323567
2 (area)	n.d.	6357	46750	120916	237394	366207	467716	n.d.	15.2	3286	6366	9642	12803	18623	n.d.	6687	70057	140634	207738	277387	320935
3 (area)	n.d.	6678	45565	119910	233048	371781	474988	n.d.	16.04	3402	6242	9826	12843	18944	n.d.	6485	66630	134386	206342	280961	329843
Average	-	6454.667	46009	120943.3	234370	368252.7	472492.7	-	14.91333	3223.333	6381.667	9707	12755.67	18950	-	6505	67512.67	136844.7	206890.3	279703.7	324781.7
St.dev.	-	193.9184	645.9203	1047.268	2625.744	3068.566	4138.098	-	1.294038	216.8994	148.1227	103.2037	118.3272	330.0409	-	172.8699	2237.618	3329.772	744.5833	2008.733	4576.536
RSD (%)	-	3.004313	1.4039	0.865916	1.120341	0.833277	0.875802	-	8.677051	6.729039	2.321066	1.063188	0.927644	1.741641	-	2.657493	3.314368	2.433249	0.359893	0.718165	1.409111
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	1202	13166	33413	69287	98942	123457	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (area)	n.d.	1537	15105	39477	79296	122446	154430	n.d.	85.29	6985	13223	19794	26289	38915	n.d.	54.15	326	761	887	1104	1689
2 (area)	n.d.	1335	15330	40957	77402	120069	157226	n.d.	99.67	6855	13635	19775	26746	37987	n.d.	71.23	379	566	1037	1470	2017
3 (area)	n.d.	1146	15584	39198	77541	122507	160697	n.d.	131	6764	13302	19723	26959	39017	n.d.	85.2	244	852	873	1148	2029
Average	-	1339.333	15339.67	39877.33	78079.67	121674	157451	-	105.32	6868	13386.67	19764	26664.67	38639.67	-	70.19333	316.3333	726.3333	932.3333	1240.667	1911.667
St.dev.	-	195.536	239.6463	945.3678	1055.666	1390.305	3139.553	-	23.37291	111.072	218.6603	36.75595	342.3249	567.5221	-	15.55094	68.01715	146.1175	90.91388	199.8233	192.9283
RSD (%)	-	14.5995	1.562265	2.37069	1.352037	1.142648	1.993987	-	22.19228	1.61724	1.633419	0.185974	1.283815	1.468755	-	22.15444	21.50173	20.11715	9.75122	16.10612	10.09215
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	326322	331371	335975	329783	340839	332725	327617	13891	13548	13665	13749	13616	13753	13053	25061	24781	24747	23332	25288	24170	23048
2 (area)	337222	337603	335999	333093	344432	342045	322781	13847	14260	14189	13786	14121	13888	13354	25568	25756	24725	22630	24962	24837	21759
3 (area)	343532	340874	340959	334893	339317	340010	331841	14350	14361	13848	13588	13849	13965	13679	25288	25495	24524	24464	23479	22580	23458
Average	335692	336616	337644.3	332589.7	341529.3	338260	327413	14029.33	14056.33	13900.67	13707.67	13862	13868.67	13362	25305.67	25344	24665.33	23475.33	24576.33	23862.33	22755
St.dev.	8706.417	4027.772	2870.611	2591.917	2626.447	4900.253	4533.444	278.5755	443.1166	265.9405	105.2727	252.7509	107.3142	313.0767	253.9613	504.7346	122.8916	925.3634	964.1962	1159.528	886.5873
RSD (%)	2.593573	1.434207	0.850188	0.779314	0.769025	1.448665	1.384625	1.985665	3.152434	1.913149	0.767984	1.823336	0.773789	2.343037	1.003575	1.991535	0.498236	3.941854	3.923271	4.859242	3.896231

Table B.45: Raw data for the retention times for day 3 rep 1 and 2 of validation for LC-MS.

Rep 1	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.27	5.26	5.25	5.25	5.25	5.25	n.d.	8.08	8.1	8.08	8.07	8.07	8.05	n.d.	8.16	8.16	8.14	8.12	8.14	8.1
2 (t_R)	n.d.	5.25	5.25	5.26	5.25	5.25	5.25	n.d.	8.07	8.09	8.06	8.08	8.07	8.07	n.d.	8.14	8.13	8.13	8.12	8.11	8.13
3 (t_R)	n.d.	5.25	5.25	5.25	5.25	5.24	5.25	n.d.	8.1	8.08	8.09	8.07	8.08	8.09	n.d.	8.14	8.14	8.13	8.12	8.13	8.13
Average	-	5.257	5.253	5.253	5.250	5.247	5.250	-	8.083	8.090	8.077	8.073	8.073	8.070	-	8.147	8.143	8.133	8.120	8.127	8.120
St.dev.	-	0.012	0.006	0.006	0.000	0.006	0.000	-	0.015	0.010	0.015	0.006	0.006	0.020	-	0.012	0.015	0.006	0.000	0.015	0.017
RSD (%)	-	0.220	0.110	0.110	0.000	0.110	0.000	-	0.189	0.124	0.189	0.072	0.072	0.248	-	0.142	0.188	0.071	0.000	0.188	0.213
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.88	1.88	1.89	1.88	1.88	1.89	n.d.	5.26	5.26	5.24	5.25	5.25	5.25	n.d.	8.14	8.19	8.18	8.16	8.16	8.13
2 (t_R)	n.d.	1.87	1.88	1.9	1.88	1.88	1.88	n.d.	5.27	5.26	5.25	5.25	5.25	5.24	n.d.	8.17	8.16	8.15	8.14	8.14	8.15
3 (t_R)	n.d.	1.89	1.88	1.89	1.88	1.88	1.88	n.d.	5.26	5.25	5.26	5.25	5.25	5.26	n.d.	8.14	8.17	8.11	8.14	8.16	8.14
Average	-	1.880	1.880	1.893	1.880	1.880	1.883	-	5.263	5.257	5.250	5.250	5.250	5.250	-	8.150	8.173	8.147	8.147	8.153	8.140
St.dev.	-	0.010	0.000	0.006	0.000	0.000	0.006	-	0.006	0.006	0.010	0.000	0.000	0.010	-	0.017	0.015	0.035	0.012	0.012	0.010
RSD (%)	-	0.532	0.000	0.305	0.000	0.000	0.307	-	0.110	0.110	0.190	0.000	0.000	0.190	-	0.213	0.187	0.431	0.142	0.142	0.123
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.22	5.21	5.21	5.2	5.21	5.21	5.21	7.98	7.97	7.97	7.97	7.95	7.96	7.94	8.06	8.07	8.08	8.06	8.04	8.05	8.05
2 (t_R)	5.22	5.22	5.21	5.21	5.21	5.22	5.2	7.98	7.96	7.96	7.96	7.95	7.96	7.96	8.07	8.05	8.08	8.04	8.03	8.03	8.05
3 (t_R)	5.21	5.22	5.21	5.21	5.21	5.21	5.21	7.99	7.98	7.97	7.97	7.95	7.97	7.97	8.08	8.06	8.06	8.04	8.05	8.03	8.04
Average	5.217	5.217	5.210	5.207	5.210	5.213	5.207	7.983	7.970	7.967	7.967	7.950	7.963	7.957	8.070	8.060	8.073	8.047	8.040	8.037	8.047
St.dev.	0.006	0.006	0.000	0.006	0.000	0.006	0.006	0.006	0.010	0.006	0.006	0.000	0.006	0.015	0.010	0.010	0.012	0.012	0.010	0.012	0.006
RSD (%)	0.111	0.111	0.000	0.111	0.000	0.111	0.111	0.072	0.125	0.072	0.072	0.000	0.073	0.192	0.124	0.124	0.143	0.144	0.124	0.144	0.072

Rep 2	Phenacetin							Tolbutamide							Fluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	10	100	200	300	400	500
1 (t_R)	n.d.	5.26	5.24	5.26	5.25	5.24	5.25	n.d.	8.04	8.07	8.1	8.08	8.08	8.06	n.d.	8.14	8.16	8.15	8.13	8.15	8.13
2 (t_R)	n.d.	5.25	5.25	5.26	5.25	5.25	5.25	n.d.	8.11	8.08	8.07	8.04	8.08	8.07	n.d.	8.16	8.14	8.14	8.12	8.13	8.13
3 (t_R)	n.d.	5.25	5.26	5.24	5.25	5.25	5.25	n.d.	8.07	8.08	8.09	8.08	8.06	8.07	n.d.	8.11	8.14	8.14	8.15	8.12	8.13
Average	-	5.253	5.25	5.25	5.25	5.247	5.25	-	8.073	8.077	8.087	8.067	8.073	8.067	-	8.137	8.147	8.14	8.133	8.133	8.13
St.dev.	-	0.006	0.01	0.01	0	0.006	0	-	0.035	0.006	0.015	0.023	0.012	0.006	-	0.025	0.012	0.01	0.015	0.015	0
RSD (%)	-	0.11	0.19	0.22	0	0.11	0	-	0.435	0.071	0.189	0.286	0.143	0.072	-	0.309	0.142	0.07	0.188	0.188	0
	Acetaminophen							4-hydroxytolbutamide							Norfluoxetine						
Conc. (ng/mL)	0	2.5	20	50	100	150	200	0	1	50	100	150	200	300	0	50	100	200	300	400	500
1 (t_R)	n.d.	1.89	1.89	1.87	1.89	1.88	1.87	n.d.	5.26	5.26	5.25	5.25	5.26	5.25	n.d.	8.12	8.14	8.16	8.18	8.15	8.16
2 (t_R)	n.d.	1.88	1.88	1.89	1.87	1.88	1.88	n.d.	5.26	5.26	5.25	5.25	5.25	5.24	n.d.	8.21	8.15	8.14	8.14	8.14	8.16
3 (t_R)	n.d.	1.87	1.88	1.87	1.88	1.88	1.88	n.d.	5.28	5.26	5.25	5.25	5.25	5.24	n.d.	8.22	8.16	8.15	8.15	8.14	8.15
Average	-	1.880	1.883	1.877	1.880	1.880	1.877	-	5.267	5.260	5.250	5.250	5.253	5.243	-	8.183	8.150	8.150	8.157	8.143	8.157
St.dev.	-	0.010	0.006	0.012	0.010	0.000	0.006	-	0.012	0.000	0.000	0.000	0.006	0.006	-	0.055	0.010	0.010	0.021	0.006	0.006
RSD (%)	-	0.532	0.307	0.615	0.532	0.000	0.308	-	0.219	0.000	0.000	0.000	0.110	0.110	-	0.673	0.123	0.123	0.255	0.071	0.071
	Phenacetin d5							Tolbutamide d9							Fluoxetine d5						
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (t_R)	5.2	5.21	5.21	5.21	5.2	5.21	5.2	7.97	7.95	7.96	7.96	7.96	7.96	7.96	8.05	8.05	8.08	8.07	8.06	8.07	8.06
2 (t_R)	5.21	5.21	5.21	5.21	5.21	5.21	5.21	7.95	7.98	7.96	7.95	7.96	7.96	7.97	8.05	8.07	8.05	8.04	8.07	8.04	8.07
3 (t_R)	5.21	5.21	5.22	5.21	5.21	5.21	5.2	7.96	7.96	7.98	7.97	7.95	7.95	7.96	8.05	8.06	8.06	8.06	8.04	8.03	8.03
Average	5.207	5.210	5.213	5.210	5.207	5.210	5.203	7.960	7.963	7.967	7.960	7.957	7.957	7.963	8.050	8.060	8.063	8.057	8.057	8.047	8.053
St.dev.	0.006	0.000	0.006	0.000	0.006	0.000	0.006	0.010	0.015	0.012	0.010	0.006	0.006	0.006	0.000	0.010	0.015	0.015	0.015	0.021	0.021
RSD (%)	0.111	0.000	0.111	0.000	0.111	0.000	0.111	0.126	0.192	0.145	0.126	0.073	0.073	0.073	0.000	0.124	0.189	0.190	0.190	0.259	0.258

B.2.5. Raw data for LoBind versus Safe Lock

Table B.46 shows the raw data from the analyses with solutions prepared in LoBind versus Safe Lock tubes for HLM matrix, and **Table B.47** shows the raw-data for the solutions prepared in cell medium without FBS in both LoBind and Safe Lock tubes.

Table B.46: Raw data for the HLM experiment comparing LoBind and safe Lock tubes

HLM	LoBind			Safe Lock		
	Phenacetin			Phenacetin		
Time (h)	1 h	2.5 h	4 h	1 h	2.5 h	4 h
1 (area)	488338	384515	330542	481873	279824	306031
2 (area)	448811	391765	295914	482648	391415	292910
3 (area)	499053	392348	303927	524958	389730	286103
Average	478734	389542.7	310127.7	496493	353656.3	295014.7
St.dev.	26462.09	4363.834	18127.63	24654.46	63946.23	10129.34
RSD (%)	5.527514	1.120245	5.845214	4.965721	18.08146	3.433504
	Acetaminophen			Acetaminophen		
Time (h)	1 h	2.5 h	4 h	1 h	2.5 h	4 h
1 (area)	23236	29493	35575	24696	22563	34027
2 (area)	20518	29403	30360	25788	30708	33048
3 (area)	22035	30381	32664	24414	29224	28907
Average	21929.67	29759	32866.33	24966	27498.33	31994
St.dev.	1362.058	540.5442	2613.381	725.7024	4338.053	2717.864
RSD (%)	6.21103	1.816406	7.951544	2.906763	15.77569	8.494919
	Phenacetin d5			Phenacetin d5		
Time (h)	1 h	2.5 h	4 h	1 h	2.5 h	4 h
1 (area)	5342126	4714261	4449483	4093127	4496747	4175233
2 (area)	4191765	4403887	4428466	4503440	4033012	4250274
3 (area)	4556645	4296809	4313915	4541906	4394339	4357840
Average	4696845	4471652	4397288	4379491	4308033	4261116
St.dev.	587856	216819.4	72963.84	248743.2	243616.8	91785
RSD (%)	12.51598	4.848753	1.659292	5.679728	5.654942	2.154013

HLM	LoBind			Safe Lock		
	Tolbutamide			Tolbutamide		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	17630	17974	18429	18023	12827	18600
2 (area)	16547	17312	16163	18465	18472	17330
3 (area)	18349	18506	18213	20180	19282	16237
Average	17508.67	17930.67	17601.67	18889.33	16860.33	17389
St.dev.	907.1066	598.1783	1250.594	1139.389	3516.37	1182.604
RSD (%)	5.1809	3.336063	7.104975	6.031915	20.85588	6.800876
	4-hydroxytolbutamide			4-hydroxytolbutamide		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	663	927	1309	712	673	1304
2 (area)	570	971	1224	740	1031	1143
3 (area)	596	1097	1258	609	1058	1139
Average	609.6667	998.3333	1263.667	687	920.6667	1195.333
St.dev.	47.98264	88.23454	42.7824	68.98551	214.9101	94.12934
RSD (%)	7.870307	8.838184	3.385576	10.04156	23.34287	7.874736
	Tolbutamide d9			Tolbutamide d9		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	161742	147400	135457	122199	139783	135497
2 (area)	127862	136494	137007	141690	127467	136100
3 (area)	133640	133516	135525	141702	142615	140885
Average	141081.3	139136.7	135996.3	135197	136621.7	137494
St.dev.	18124.39	7309.523	875.9231	11256.6	8053.633	2952.129
RSD (%)	12.84677	5.253484	0.644078	8.326072	5.894843	2.147096

HLM	LoBind			Safe Lock		
	Fluoxetine			Fluoxetine		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	124183	122293	126928	104703	86376	105676
2 (area)	139093	123162	104447	113671	113858	102134
3 (area)	128542	123931	99878	140373	114398	90025
Average	130606	123128.7	110417.7	119582.3	104877.3	99278.33
St.dev.	7666.296	819.5086	14479.72	18555.19	16024.9	8206.983
RSD (%)	5.869789	0.665571	13.11359	15.51667	15.27966	8.266641
	Norfluoxetine			Norfluoxetine		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	n.d.	-	62.45	n.d.	18.52	-
2 (area)	n.d.	-	22.21	n.d.	18.84	28.13
3 (area)	n.d.	23.02	-	n.d.	-	5.82
Average	-	23.02	42.33	-	18.68	16.975
St.dev.	-	-	28.45398	-	0.226274	15.77555
RSD (%)	-	-	67.21941	-	1.211318	92.93403
	Fluoxetine d5			Fluoxetine d5		
Time (h)	1	2.5	4	1	2.5	4
1 (area)	87526	79241	71002	56894	74090	64575
2 (area)	79141	68586	66666	65309	59408	60605
3 (area)	67022	66055	64739	71473	67558	61995
Average	77896.33	71294	67469	64558.67	67018.67	62391.67
St.dev.	10308.51	6997.685	3207.787	7318.406	7355.844	2014.506
RSD (%)	13.23363	9.815251	4.754461	11.33605	10.97581	3.228806

Table B.47: Raw data for the calibration curve from day 3 of validation comparing LoBind and safe Lock tube safter storing in 5 °C for 24 hours in Safe Lock

LB VS SL CM	Phenacetin						
Conc. (ng/mL)	0	2.5	20	50	100	150	200
1 (area)	n.d.	6686	49902	127557	236344	373784	472608
2 (area)	n.d.	6113	49619	126815	232434	365313	475929
3 (area)	n.d.	6739	48841	128183	231998	366869	488157
Average	-	6512.667	49454	127518.3	233592	368655.3	478898
St.dev.	-	347.1345	549.4079	684.8192	2393.251	4509.18	8188.655
RSD (%)	-	5.330143	1.110947	0.537036	1.024543	1.223142	1.709895
	Acetaminophen						
Conc. (ng/mL)	0	2.5	20	50	100	150	200
1 (area)	n.d.	1477	16034	43466	79217	111713	154233
2 (area)	n.d.	1254	15551	43199	74617	120403	157595
3 (area)	n.d.	1751	16369	45281	77090	118297	160555
Average	-	1494	15984.67	43982	76974.67	116804.3	157461
St.dev.	-	248.9357	411.2254	1132.861	2302.168	4533.218	3163.129
RSD (%)	-	16.66237	2.572624	2.575737	2.990812	3.881036	2.008834
	Phenacetin d5						
Conc. (ng/mL)	100	100	100	100	100	100	100
1 (area)	360710	339153	339205	341044	336805	338145	326729
2 (area)	362783	346777	337692	338104	339377	340179	328881
3 (area)	360187	342646	337350	331599	338995	337450	331315
Average	361226.7	342858.7	338082.3	336915.7	338392.3	338591.3	328975
St.dev.	1372.958	3816.447	987.181	4833.333	1387.877	1418.193	2294.445
RSD (%)	0.380082	1.113125	0.291994	1.434582	0.410138	0.418851	0.697453

	Tolbutamide						
	0	1	50	100	150	200	300
	n.d.	13.84	2997	6369	9068	12393	18023
	n.d.	17.65	3083	6171	9150	12527	17689
	n.d.	8.84	2822	6264	9396	11992	17338
	-	13.44333	2967.333	6268	9204.667	12304	17683.33
	-	4.418375	133.005	99.06059	170.6966	278.3828	342.5352
	-	32.86666	4.482308	1.580418	1.854457	2.262539	1.937051
	4-hydroxytolbutamide						
	0	1	50	100	150	200	300
	n.d.	91.56	6982	12600	19126	26115	37813
	n.d.	128	6588	13197	18737	25102	37633
	n.d.	97.88	6310	12967	19095	24964	38842
	-	105.8133	6626.667	12921.33	18986	25393.67	38096
	-	19.47233	337.6645	301.1085	216.1967	628.4921	652.2936
	-	18.40253	5.095541	2.330321	1.138716	2.474996	1.712237
	Tolbutamide d9						
	100	100	100	100	100	100	100
	13794	13537	13063	13584	13069	12632	12804
	13990	13569	13271	12962	12925	12898	12905
	13887	13445	12727	12975	12945	13208	12775
	13890.33	13517	13020.33	13173.67	12979.67	12912.67	12828
	98.04251	64.37391	274.4983	355.4185	78.00855	288.28	68.24222
	0.705833	0.476244	2.108228	2.697947	0.601006	2.232536	0.531979

	Fluoxetine						
	0	10	100	200	300	400	500
	n.d.	7007	67885	136520	204965	264984	311773
	n.d.	6648	69906	135379	203246	264563	314353
	n.d.	6849	67705	132214	203448	267122	314363
	-	6834.667	68498.67	134704.3	203886.3	265556.3	313496.3
	-	179.9287	1222.105	2230.872	939.5969	1372.15	1492.459
	-	2.632589	1.784129	1.656125	0.460843	0.516708	0.476069
	Norfluoxetine						
	0	50	100	200	300	400	500
	n.d.	114	137	685	1047	1273	1733
	n.d.	105	253	503	938	1307	1765
	n.d.	123	261	741	821	1158	1597
	-	114	217	643	935.3333	1246	1698.333
	-	9	69.39741	124.4347	113.0236	78.08329	89.20389
	-	7.894737	31.98037	19.35221	12.08378	6.266717	5.252437
	Fluoxetine d5						
	100	100	100	100	100	100	100
	22236	21258	21284	20077	23307	22242	21899
	22579	21411	21781	20568	23331	22665	22260
	22589	21558	21866	21663	21742	22291	22043
	22468	21409	21643.67	20769.33	22793.33	22399.33	22067.33
	200.9801	150.01	314.3666	811.9423	910.5605	231.3749	181.726
	0.894517	0.700687	1.452464	3.909333	3.994854	1.032954	0.823507

B.2.6. Raw data for the in-cocktail experiment with human liver microsomes

Table B.48 shows the raw data for the calibration curve used to calculate the analyte concentrations for the incubated samples, and **Table B.49** shows the raw data from the in-cocktail incubation with 1 mg/mL HLM for 8 timeperiods from 0-240 min, which also were used for investigation of the dilution integrity of the method.

Table B.48: Raw data for the calibration curves for the 8 time periods incubation experiment with HLM.

	Phenacetin						Tolbutamide						Fluoxetine					
Conc. (ng/mL)	2.5	20	50	100	150	200	1	50	100	150	200	300	10	100	200	300	400	500
1 (area)	6510	53894	144298	266830	423741	584401	4.41	2564	5481	7886	10693	16955	6603	68982	117220	157412	219427	276476
2 (area)	6819	52849	144353	265924	426597	578220	32.05	2653	5637	8308	11363	17213	6907	67825	110044	155073	212745	266281
3 (area)	6462	54043	146809	272296	424329	572839	4.67	2704	5465	7570	10430	16902	7321	67714	118290	161460	221279	273750
Average	6597	53595.33	145153.3	268350	424889	578486.7	13.71	2640.333	5527.667	7921.333	10828.67	17023.33	6943.667	68173.67	115184.7	157981.7	217817	272169
St.dev.	193.7498	650.623	1434.113	3447.23	1508.106	5785.611	15.88344	70.85431	95.0228	370.2666	481.0679	166.3801	360.4016	702.2338	4483.979	3231.382	4489.027	5278.179
RSD (%)	2.936939	1.213955	0.987999	1.284602	0.354941	1.000129	115.8529	2.683537	1.71904	4.674296	4.442541	0.977365	5.190365	1.030066	3.892861	2.045416	2.060917	1.939302
	Acetaminophen						4-hydroxytolbutamide						Norfluoxetine					
Conc. (ng/mL)	2.5	20	50	100	150	200	1	50	100	150	200	300	50	100	200	300	400	500
1 (area)	961	11793	32513	64214	104023	138499	58.65	5395	11140	15677	22142	35194	61.71	408	758	1041	1449	2124
2 (area)	894	12653	33778	64130	98412	133275	77.27	5163	10705	16709	23212	35830	58.79	355	761	1234	1445	1873
3 (area)	903	12430	33731	62813	100825	140436	88.28	5470	11191	16344	22458	35304	110	412	850	1055	1561	1864
Average	919.3333	12292	33340.67	63719	101086.7	137403.3	74.73333	5342.667	11012	16243.33	22604	35442.67	76.83333	391.6667	789.6667	1110	1485	1953.667
St.dev.	36.3639	446.2992	717.1655	785.7423	2814.637	3704.098	14.97699	160.051	267.0899	523.3128	549.7381	339.9196	28.76026	31.81719	52.27173	107.6151	65.84831	147.5816
RSD (%)	3.955464	3.630811	2.151023	1.233137	2.78438	2.695785	20.04058	2.995714	2.425444	3.221708	2.432039	0.959069	37.43201	8.123537	6.619467	9.69505	4.43423	7.554084
	Phenacetin d5						Tolbutamide d9						Fluoxetine d5					
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	355057	364280	344258	347901	349619	349062	11700	12206	12120	12133	12368	12410	20445	20222	18506	18550	17429	18066
2 (area)	364729	351066	350885	349625	351240	344154	12227	12011	11843	12793	12612	12519	19948	19746	18569	18240	17898	18509
3 (area)	357699	360140	344634	354658	352812	348948	12449	12462	12292	12502	11775	12762	19848	20975	19074	18164	17368	17878
Average	359161.7	358495.3	346592.3	350728	351223.7	347388	12125.33	12226.33	12085	12476	12251.67	12563.67	20080.33	20314.33	18716.33	18318	17565	18151
St.dev.	4999.144	6758.783	3722.309	3510.943	1596.563	2801.306	384.7107	226.1865	226.537	330.7673	430.456	180.2008	319.7442	619.6808	311.346	204.4798	289.9948	323.9738
RSD (%)	1.391892	1.88532	1.073973	1.001044	0.454571	0.806391	3.172785	1.849995	1.87453	2.651229	3.513448	1.434301	1.592325	3.050461	1.663499	1.116278	1.650981	1.784881

Table B.49: Raw data for the metabolism from the 8 time periods incubation experiment with HLM.

	Phenacetin HLM								Acetaminophen HLM								Phenacetin d5 HLM							
Time (min)	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240
1 (area)	451008	429553	349016	277140	254328	266918	165501	170578	n.d.	8343	13132	18174	15156	22180	41559	43531	340932	323034	315724	293190	273004	326221	236773	252002
2 (area)	487979	405833	336705	285043	286017	268836	150533	182696	n.d.	7479	12823	18888	17990	22444	35162	44710	318939	322381	305446	292778	328161	317264	216986	270627
3 (area)	466964	412310	367892	278557	294849	291560	136890	172126	n.d.	8317	13506	19185	19638	23427	31065	46157	311248	253692	340740	304275	312873	333197	182993	260563
Average	468650.3	415898.7	351204.3	280246.7	278398	275771.3	150974.7	175133.3	-	8046.333	13153.67	18749	17594.67	22683.67	35928.67	44799.33	323706.3	299702.3	320636.7	296747.7	304679.3	325560.7	212250.7	261064
St.dev	18543.1	12260.44	15708.24	4213.737	21307.86	13706.98	14310.61	6595.038	-	491.497	342.0151	519.6354	2267.002	657.1395	5288.841	1315.277	15405.54	39847.46	18152.61	6522.116	28476.76	7986.999	27200.91	9322.602
RSD (%)	3.956702	2.94794	4.472679	1.503582	7.653739	4.970413	9.478817	3.765724	-	6.108335	2.60015	2.771537	12.8846	2.896972	14.72039	2.93593	4.759109	13.29568	5.661427	2.197866	9.346469	2.453306	12.81547	3.571002
	Tolbutamide HLM								4-hydroxytolbutamide HLM								Tolbutamide d9 HLM							
Time (min)	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240
1 (area)	11940	14106	13473	12388	10890	13264	17079	18909	0	207	404	647	490	783	1326	1555	12420	11658	12280	11841	10359	11692	14739	14953
2 (area)	12737	13616	12513	12690	12550	12350	15300	20146	0	190	377	548	609	770	1116	1442	11538	11363	11828	11648	11953	11590	13441	16089
3 (area)	13371	13502	14747	13064	12942	13952	13518	18639	0	208	403	592	573	818	1034	1463	10798	9075	13543	11583	11449	12271	11072	15313
Average	12682.67	13741.33	13577.67	12714	12127.33	13188.67	15299	19231.33	0	201.6667	394.6667	595.6667	557.3333	790.3333	1158.667	1486.667	11585.33	10698.67	12550.33	11690.67	11253.67	11851	13084	15451.67
St.dev	717.0456	320.9133	1120.672	338.6385	1089.34	803.6525	1780.5	803.5461	0	10.11599	15.30795	49.60175	61.02732	24.82606	150.6032	60.10269	812.0353	1413.852	888.8849	134.1877	814.7548	367.2887	1859.384	580.5561
RSD (%)	5.653744	2.335387	8.253788	2.663508	8.982518	6.093508	11.63802	4.178317	#DIV/0!	5.016195	3.878704	8.327098	10.94988	3.141214	12.99798	4.042782	7.009167	13.21521	7.08256	1.147819	7.239905	3.099221	14.21113	3.757239
	Fluoxetine HLM								Norfluoxetine HLM								Fluoxetine d5 HLM							
Time (min)	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240	0	20	40	60	75	90	150	240
1 (area)	77528	98318	85064	74282	73248	93697	60314	63283	n.d.	n.d.	n.d.	0	0	8.32	n.d.	n.d.	8626	8953	8463	7991	7880	9395	5765	5452
2 (area)	93694	95914	81468	81611	82297	90572	50537	67889	n.d.	n.d.	n.d.	13.13	0	0	n.d.	n.d.	8459	8417	8052	8003	8479	9207	4662	6031
3 (area)	95825	94209	85811	79744	88526	98885	45376	64699	n.d.	n.d.	n.d.	0	4.61	0	n.d.	n.d.	8463	6813	9084	7802	8330	9346	3473	5790
Average	89015.67	96147	84114.33	78545.67	81357	94384.67	52075.67	65290.33	-	-	-	4.376667	1.536667	2.773333	-	-	8516	8061	8533	7932	8229.667	9316	4633.333	5757.667
St.dev	10005.51	2064.385	2322.028	3808.617	7682.254	4198.947	7586.935	2359.251	-	-	-	7.580609	2.661585	4.803554	-	-	95.28379	1113.531	519.5488	112.7431	311.8499	97.52436	1146.269	290.8511
RSD (%)	11.24016	2.147114	2.760561	4.84892	9.442646	4.44876	14.56906	3.613477	-	-	-	173.2051	173.2051	173.2051	-	-	1.11888	13.81381	6.088701	1.42137	3.789338	1.046848	24.73962	5.051544

B.2.7. Raw data for the experiment with primary hepatocyte spheroids

Table B.50 shows the raw data for the calibration curve used to calculate the analyte concentrations for the incubated samples, and **Table B.51** shows the raw data from the drug incubation with PHS for 6 and 24 hours.

Table B.50: Raw data for the calibration curves for the incubation experiment with PHS.

	Phenacetin						Tolbutamide						Fluoxetine					
Conc. (ng/mL)	2.5	20	50	100	150	200	1	50	100	150	200	300	10	100	200	300	400	500
1 (area)	14531	76533	223685	410361	565394	545208	115	6341	15012	22037	27956	31023	12840	96858	212994	306943	366472	355953
2 (area)	15155	74329	207922	394404	563845	529037	109	6385	14908	21334	28139	29957	11966	93598	204366	294978	367599	343781
3 (area)	13858	70820	205275	382548	521905	523957	126	5992	13022	20849	26848	29705	12171	91131	200377	291377	351117	336714
Average	14514.67	73894	212294	395771	550381.3	532734	116.6667	6239.333	14314	21406.67	27647.67	30228.33	12325.67	93862.33	205912.3	297766	361729.3	345482.7
St.dev.	648.6542	2881.234	9953.282	13956.8	24673.39	11097.39	8.621678	215.3238	1120.112	597.3243	698.5502	699.6409	457.0671	2872.636	6449.072	8148.914	9207.809	9731.728
RSD (%)	4.468957	3.899145	4.688442	3.526484	4.482962	2.083102	7.39001	3.45107	7.825293	2.790366	2.526615	2.31452	3.708255	3.060478	3.13195	2.736684	2.545497	2.81685
	Acetaminophen						4-hydroxytolbutamide						Norfluoxetine					
Conc. (ng/mL)	2.5	20	50	100	150	200	1	50	100	150	200	300	50	100	200	300	400	500
1 (area)	6256	38827	117021	213678	283427	264852	436	16815	40504	59473	76251	79998	1385	2463	5411	8033	9181	8791
2 (area)	5968	37650	113732	200843	276812	262630	454	16730	38386	56581	74365	78481	1075	2494	5021	7838	9234	8438
3 (area)	5939	36374	106355	193838	260239	246037	439	15895	37045	55417	68797	77719	1178	2480	4420	7719	8712	8302
Average	6054.333	37617	112369.3	202786.3	273492.7	257839.7	443	16480	38645	57157	73137.67	78732.67	1212.667	2479	4950.667	7863.333	9042.333	8510.333
St.dev.	175.2493	1226.833	5462.008	10061.75	11945.05	10281.61	9.643651	508.4044	1743.984	2088.448	3875.602	1160.156	157.8808	15.52417	499.2297	158.5255	287.3018	252.3972
RSD (%)	2.89461	3.261379	4.860764	4.961749	4.367596	3.987599	2.176896	3.084978	4.512833	3.65388	5.29905	1.473539	13.0193	0.626227	10.08409	2.016009	3.177297	2.965773
	Phenacetin d5						Tolbutamide d9						Fluoxetine d5					
Conc. (ng/mL)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 (area)	507789	397796	470302	447861	420283	335679	33276	27928	32744	32065	30299	25302	32188	33087	34549	34737	29870	25838
2 (area)	485871	385702	446445	424018	410621	326209	32767	27245	32309	31699	30104	24296	31653	32919	34133	34118	29483	24976
3 (area)	459131	369505	428102	416881	387549	322044	30024	26940	30073	30684	29669	23724	30470	30996	31617	32856	28700	25152
Average	484263.7	384334.3	448283	429586.7	406151	327977.3	32022.33	27371	31708.67	31482.67	30024	24440.67	31437	32334	33433	33903.67	29351	25322
St.dev.	24368.79	14195	21159.95	16223.37	16818.57	6987.386	1749.22	505.9081	1433.13	715.4651	322.5291	798.8851	879.132	1161.783	1586.397	958.6419	596.0646	455.4514
RSD (%)	5.032132	3.693399	4.720222	3.776506	4.140965	2.130448	5.462502	1.848336	4.519678	2.272568	1.074238	3.268671	2.796488	3.593068	4.745004	2.827546	2.030815	1.798639

Table B.51: Raw data for the metabolism from the incubation experiment with PHS.

	Phenacetin control		Phenacetin PHS		Acetaminophen control		Acetaminophen PHS		Phenacetin d5 control		Phenacetin d5 PHS	
Time (hour)	6	24	6	24	6	24	6	24	6	24	6	24
1 (area)	436402	447557	366136	178476	n.d.	n.d.	366136	178476	277363	296314	245004	284564
2 (area)	443075	418567	361243	183125	n.d.	n.d.	361243	183125	283364	265068	251994	251769
3 (area)	442124	430237	370696	158708	n.d.	n.d.	370696	158708	285699	273015	256449	281230
Average (area)	440533.6667	432120.3333	366025.0000	173436.3333	-	-	366025	173436.3333	282142	278132.3333	251149	272521
St.dev.	3609.5848	14586.4743	4727.4774	12965.19	-	-	4727.4774	12965.19	4300.2543	16239.4105	5769.1009	18048.9063
RSD (%)	0.8194	3.3756	1.2916	7.4755	-	-	1.2916	7.4755	1.5241	5.8387	2.2971	6.6229
Conc. (ng/mL)	1831	1822	1706	714	0	0	66	819				
	Tolbutamide control		Tolbutamide PHS		4-hydroxytolbutamide control		4-hydroxytolbutamide PHS		Tolbutamide d9 control		Tolbutamide d9 PHS	
Time (hour)	6	24	6	24	6	24	6	24	6	24	6	24
1 (area)	21173	24492	20948	20154	n.d.	n.d.	251	1588	17218	20016	18821	19376
2 (area)	19964	22165	19614	21269	n.d.	n.d.	87.45	1162	16963	19075	16977	20446
3 (area)	19902	21290	19556	19146	n.d.	n.d.	76.21	1430	16549	31755	17504	24814
Average	20346.33333	22649	20039.33333	20189.66667	-	-	138.22	1393.333333	16910	23615.33333	17767.3333	21545.3333
St.dev.	716.5851892	1654.96012	787.4625917	1061.949308	-	-	97.83190022	215.3539722	337.6344177	7064.842555	949.785414	2880.86121
RSD (%)	3.521937724	7.306989802	3.929584775	5.259865484	-	-	70.77984388	15.45602671	1.996655338	29.91633637	5.34568354	13.371161
Conc. (ng/mL)	2803	2222	2624	2169	0	0	7	59				
	Fluoxetine control		Fluoxetine PHS		Norfluoxetine control		Norfluoxetine PHS		Fluoxetine d5 control		Fluoxetine d5 PHS	
Time (hour)	6	24	6	24	6	24	6	24	6	24	6	24
1 (area)	96083	142558	114036	117783	n.d.	n.d.	n.d.	10.82	9000	12642	10613	10096
2 (area)	120954	133885	98408	114464	n.d.	n.d.	n.d.	38.42	10834	12589	8958	10265
3 (area)	108298	135127	92740	145954	n.d.	n.d.	n.d.	45.14	9458	9683	8882	12613
Average	108445	137190	101728	126067	-	-	-	31.46	9764	11638	9484.33333	10991.3333
St.dev.	12436.15162	4690.118229	11029.35646	17302.41362	-	-	-	18.1878201	954.5239651	1693.28704	978.192381	1406.94432
RSD (%)	11.46770401	3.418702696	10.84200659	13.7247762	-	-	-	57.81252416	9.775952121	14.54963946	10.3137706	12.8004882
Conc. (ng/mL)	3845	4087	3710	3974	0	0	0	41				