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Liquid Chromatography-Mass Spectrometry in Studies of Drug Metabolism and Permeability

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

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I Hakala, K., Laitinen, L., Kaukonen, A.M., Hirvonen, J., Kostiainen, R., Kotiaho, T., Development of LC/MS/MS methods for cocktail dosed Caco-2 samples using atmospheric pressure photoionization and electrospray ionization. *Analytical Chemistry* 2003, 75, 5969-5977.

II Hakala, K.S., Suchanova B., Luukkanen L., Ketola R.A., Finel M., Kostiainen R., Rapid simultaneous determination of metabolic clearance of multiple compounds catalyzed *in vitro* by recombinant human UDP-glucuronosyltransferases. *Analytical Biochemistry* 2005, 341, 105-12.

III Hakala, K.S., Kostiainen, R., Ketola, R.A., Feasibility of different mass spectrometric techniques and programs for automated metabolite profiling of tramadol in human urine. *Rapid Communications in Mass Spectrometry* 2006, 20, 1-10.

IV Koljonen, M., Hakala, K.S., Ahola-Sätilä, T., Laitinen, L., Kostiainen, R., Kotiaho, T., Kaukonen, A.M., Hirvonen, J., Evaluation of cocktail approach to standardize Caco-2 permeability experiments. *European Journal of Pharmaceutics and Biopharmaceutics* 2006, 64, 379-387.

V Hakala, K.S., Link M., Szotakova, B., Skalova, L., Kostiainen, R., Ketola, R.A., Characterisation of metabolites of sibutramine in primary cultures of rat and human hepatocytes with liquid chromatography-ion trap mass spectrometry. Submitted to *Journal of Mass Spectrometry.*

ABBREVIATIONS

ADME	adsorption, distribution, metabolism, excretion
AP-BL	apical-to-basolateral
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
BL-AP	basolateral-to-apical
C18	n-octadecylsilane reverse-phase packing
Cl_{int}	intrinsic clearance
CYP	cytochrome P450
ESI	electrospray ionization
FT-ICR	Fourier-transform ion cyclotron resonance
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[-2-ethanesufonic acid]
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
IAM	immobilized artificial membrane
K_m	Michaelis-Menten constant for a substrate
LC	liquid chromatography
LLE	liquid-liquid extraction
LC/MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
log P	partition coefficient
log D	distribution coefficient
LOQ	limit of quantification
LTQ	linear ion trap
MCT	monocarboxylic acid trasporter
$[M-H]^-$	deprotonated molecule
$[M+H]^+$	protonated molecule
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple stage mass spectrometry
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide
MUX	multiplexed ionization source
m/z	mass-to-charge ratio
NL	neutral loss
PAMPA	parallel artificial membrane permeability assay
P_{app}	apparent permeability coefficient
PRE	precursor ion
Q-TOF	quadrupole time-of-flight
QqQ	triple quadrupole
Q-Trap	quadrupole-linear ion trap
RAM	restricted access media
S/N	signal-to-noise ratio
SPE	solid phase extraction

SRM	selected reaction monitoring
TEER	trans-epithelial electrical resistance
UDPGA	uridine 5’-diphosphoglucuronic acid
UGT	uridine 5’-diphospho-glucuronosyltransferase
UPLC	ultra performance liquid chromatography
V_{max}	maximal reaction velocity

ABSTRACT

Poor pharmacokinetics is one of the reasons for the withdrawal of drug candidates from clinical trials. There is an urgent need for investigating *in vitro* ADME (absorption, distribution, metabolism and excretion) properties and recognising unsuitable drug candidates as early as possible in the drug development process. Current throughput of *in vitro* ADME profiling is insufficient because effective new synthesis techniques, such as drug design *in silico* and combinatorial synthesis, have vastly increased the number of drug candidates. Assay technologies for larger sets of compounds than are currently feasible are critically needed.

The first part of this work focused on the evaluation of cocktail strategy in studies of drug permeability and metabolic stability. N-in-one liquid chromatography-tandem mass spectrometry (LC/MS/MS) methods were developed and validated for the multiple component analysis of samples in cocktail experiments. Together, cocktail dosing and LC/MS/MS were found to form an effective tool for increasing throughput. First, cocktail dosing, i.e. the use of a mixture of many test compounds, was applied in permeability experiments with Caco-2 cell culture, which is a widely used *in vitro* model for small intestinal absorption. A cocktail of 7-10 reference compounds was successfully evaluated for standardization and routine testing of the performance of Caco-2 cell cultures. Secondly, cocktail strategy was used in metabolic stability studies of drugs with UGT isoenzymes, which are one of the most important phase II drug metabolizing enzymes. The study confirmed that the determination of intrinsic clearance (Cl_{int}) as a cocktail of seven substrates is possible. The LC/MS/MS methods that were developed were fast and reliable for the quantitative analysis of a heterogenous set of drugs from Caco-2 permeability experiments and the set of glucuronides from *in vitro* stability experiments. The performance of a new ionization technique, atmospheric pressure photoionization (APPI), was evaluated through comparison with electrospray ionization (ESI), where both techniques were used for the analysis of Caco-2 samples. Like ESI, also APPI proved to be a reliable technique for the analysis of Caco-2 samples and even more flexible than ESI because of the wider dynamic linear range.

The second part of the experimental study focused on metabolite profiling. Different mass spectrometric instruments and commercially available software tools were investigated for profiling metabolites in urine and hepatocyte samples. All the instruments tested (triple quadrupole, quadrupole time-of-flight, ion trap) exhibited some good and some bad features in searching for and identifying of expected and non-expected metabolites. Although, current profiling software is helpful, it is still insufficient. Thus a time-consuming largely manual approach is still required for metabolite profiling from complex biological matrices.

1 INTRODUCTION

The development of effective drugs is an expensive and long process (Figure 1). It has been estimated that putting a drug on the market takes over ten years and costs approximately one billion US dollars.[1] In past decades, the main reasons for the withdrawal of drug candidates from clinical trials have been poor efficiency, unacceptable adverse effects and poor pharmacokinetics.[2] Nevertheless, comparison of attrition rates between the early 1990s and 2000 shows that many improvements have been achieved in preventing failures due to undesirable pharmacokinetic attributes. The proportion of failures due to poor pharmacokinetics has dropped from 40% to 10% of all failures.[3] Major efforts have been made in developing more effective technologies for the screening of ADME (absorption, distribution, metabolism and excretion) and toxicity properties and recognizing poor candidates as early as possible in the drug discovery process.[4,5] Nonetheless, current throughput of *in vitro* ADME profiling is by no means sufficient because new and highly effective synthesis techniques, such as drug design *in silico* and combinatorial synthesis, have dramatically increased the number of drug candidates. Today, hundreds to thousands of compounds may be involved at the lead identification stage. Assay technologies capable of handling a larger set of compounds than is currently possible are in high demand.

Only *in vitro* experiments are realistic for large numbers of samples. Animal tests are expensive and time consuming and often unethical, as well. It is, of course, important that erroneous results do not slip through and that *in vitro* results correlate with the *in vivo*. Successful prediction of *in vivo* performance depends upon full *in vitro* characterization of the drug candidate making use of toxicological, formulation and biopharmaceutical data. Pre-clinical experiments with animals as the next step in the drug discovery process provide further confirmation of ADME properties of a drug candidate.

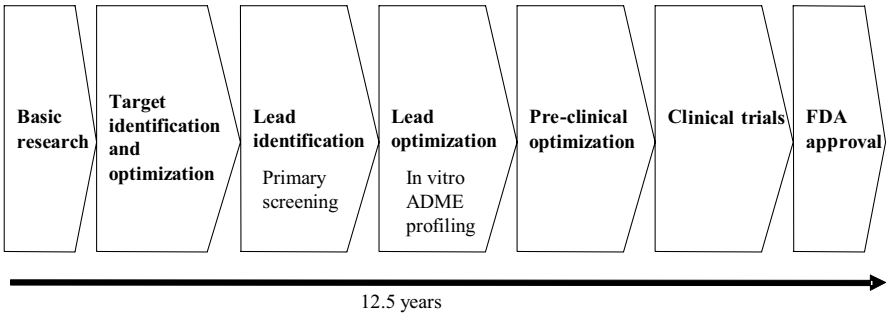

Figure 1. Drug discovery process.

An effective approach to increase throughput in ADME studies is to use cocktails of several drugs or to pool samples together and analyse them by an n-in-one method.[6] Analysis of several compounds, possibly over a large concentration range, requires a selective and sensitive analytical technique. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) qualifies for this and offers a good tool for n-in-one analysis. The main limitation of cocktail dosing is possible drug-drug interactions. Validation and examination of beneficial conditions for minimizing such undesirable effects should, therefore, be performed.

Major metabolites will be identified during the lead optimization stage of the drug discovery process. Structural information, especially on reactive or toxic and pharmacologically active metabolites, is essential. Given the requirements of the drug industry, high throughput is not as easily applied in this step, and typically much manual and specific work is required, not least in interpretation of the results. LC/MS and LC/MS/MS have been extensively used for metabolite profiling and identification.[7,8] Useful structural information on metabolites can be obtained by combining special features of different mass spectrometric instruments. Despite the high sensitivity and selectivity of LC/MS, however, identification of the metabolites in complex biological matrices remains challenging owing to the presence of interfering endogenous material. Furthermore, structural diversity of the metabolites, and often their very low concentrations in biological fluids, compound the difficulties of reliable identification.

This study focused on the development of effective methods for the determination of ADME properties of drug candidates. Liquid chromatography (LC) and mass spectrometry (MS) were the main techniques employed. Cocktail dosing together with n-in-one LC/MS/MS were utilized in *in vitro* permeability and metabolic stability studies of selected drugs. Metabolite profiling in complex biological matrices was explored with different mass spectrometric instruments and commercially available software packages.

2 ADME (absorption, distribution, metabolism and excretion)

ADME is an abbreviation for four physiological processes constituting the pharmacokinetic properties of drugs: absorption, distribution, metabolism and excretion. In addition to ADME parameters, pharmaceutical profiling data typically includes toxicity of drug candidates, and thus ADMETox and ADMET are commonly used abbreviations.

Absorption describes movement of the drug from the site of administration to the bloodstream, and *distribution* the movement of the drug from the bloodstream to the tissues. *Metabolic reactions* typically convert drugs into more hydrophilic, more easily excreted species. *C*ompounds exits from the body most commonly by renal or biliary *excretion*. All these processes are affected by passive diffusion, and therefore molecular properties such as lipophilicity, degree of ionization, polarity, solubility and molecular weight strongly affect the ADME properties of drugs.

The most important ADME properties for *in vitro* screening of drug candidates at the lead optimization stage of drug discovery are permeability and metabolic stability of the candidates and inhibition of the major drug metabolizing enzymes. Permeability and metabolic stability will be discussed in sections 2.1 and 2.2.2.

2.1 Intestinal absorption of drugs

Most drug candidates are evaluated for oral delivery and the major site for absorption is the epithelium of the intestine. Drug molecules permeate by many kinds of active and passive mechanisms across the intestinal cell. In active transport, specialized proteins (e.g amino acid carriers) carry drug molecules from the intestinal lumen to the bloodstream or pump (efflux transport, e.g. P-glycoprotein) them back to the intestinal lumen. Passive permeation can occur through the cell (transcellular) or through the intercellular space (paracellular). Passive paracellularly permeating drugs are typically small hydrophilic compounds, whereas transcellularly permeating drugs are small lipophilic compounds.[9] The transcellular pathway dominates since lumenal folding and small projections provide a larger cellular absorbing surface. Metabolism inside the cell may complicate the permeation process. For example, some of the major drug metabolizing enzymes in liver (CYPs and UGTs) are also expressed in the gut wall epithelium.[10-13]

In vivo measurements of drug disappearance from the gastrointestinal tract and appearance in the blood are the most reliable methods for the determination of drug absorption. However, in early stages of drug discovery, predictive *in situ* and *in vitro* cell-free and cell-based methods are useful for mimicking the situation in the human intestine. Passive permeability properties of a compound are often easily estimated from the compound physicochemical properties, such as molecular weight, ionization state, partition coefficient (log P) and distribution coefficient (log D). Computational (*in silico*) prediction methods are mainly based on these physicochemical properties.[14,15] Liposome partitioning is used to mimic a lipid bilayer of intestinal enterocytes and is a very simple method for the determination of drug absorption and interactions with the lipid bilayer.[16] Immobilized artificial membrane (IAM) columns[17,18] and parallel artificial membrane permeability assay (PAMPA)[19] are good methods for predicting passive permeation and very suitable for higher throughput analysis.

Biological cell-based methods offer many advantages over biophysical methods. They allow studies of absorption mechanism, drug transport rate and uptake to cells. Additionally, they are easily adapted for screening purposes since they require relatively small quantities of test compounds and are relatively clean for direct analysis.[20]Cell-based methods utilize either cultured cells or subcellular fractions, such as brush border membrane vesicles (BBMV) and freshly isolated cells.[21] Many laboratories have successfully used cultured human intestinal adenocarcinoma cell lines (Caco-2, HT29, T84) in permeability experiments.[22-24] An indisputable advantage of these cell lines is these capability to differentiate during culturing. In addition to human-derived cell lines, also animal cell lines, such as MDCK (Madin Darby canine kidney),

2/4/A1 (rat intestine) and CHO (Chinese hamster ovary), are widely used for *in vitro* determination of intestinal absorption.[25-27]

2.1.1 Caco-2 cells for predicting intestinal absorption

The Caco-2 cell line is one of the most reliable and widely used *in vitro* systems for predicting gastrointestinal absorption.[28] Caco-2 cells are derived from human colorectal carcinoma[29] and differentiate spontaneously in culture into polarised cell monolayers with microvilli and tight junctions.[30] Many enzymes (e.g. CYP, UGT, sulphotransferases) and transporters (e.g. monocarboxylic acid transporter, P-glycoprotein) present in the small intestine are expressed in Caco-2 cells, though in different quantities than in the small intestine.[30-33] P-glycoprotein is overexpressed, whereas peptide transporters and many CYP enzymes are underexpressed. Caco-2 cells nevertheless share many properties with the absorptive cells in human intestine, and several studies have demonstrated excellent correlation between Caco-2 permeability and intestine absorption in humans.[34-36] Caco-2 cells have been used in studies of the mechanisms of passive paracellular,[37,38] passive transcellular,[39]active efflux and carrier mediated transport.[40,41] Transcellularly passively permeating drugs typically yield the best *in vivo* correlation. High polarization of the cell monolayer and formation of tight junctions (high trans-epithelial electrical resistance [TEER] value) lead to too low permeability of hydrophilic, paracellularly permeating compounds.[37,42] In addition to tighter junctions and different expression levels of enzymes and transporters from those in human intestine the main disadvantage of Caco-2 cells is long culturing time (20 days).

The upper part of the intestine is characterized by an acidic microclimate, and better *in vivo* correlation can be achieved by using a pH gradient (acidic pH at apical side, neutral at basolateral side) in the Caco-2 cell experiments.[36] This is not always true in studies of active efflux mechanism because ionization differences between apical and basolateral sides may result in unwanted polarised permeation.[43]

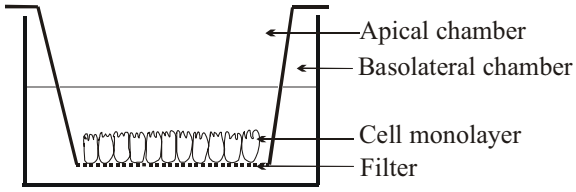

Figure 2. Caco-2 cell monolayer grown on polycarbonate filter.

Figure 2 shows a schematic picture of an experiment container in which a cell monolayer grows on a polycarbonate filter. The apical side (AP) of the cell monolayer represents the intestinal lumen and the basolateral side (BL) the bloodstream. A typical permeability experiment is simple: drug or drug mixture is loaded to the AP chamber and the permeated

sample is collected from the BL chamber at regular periods. When efflux transport is investigated, permeability experiments are carried out in both directions. Substrates for efflux transporters possess a higher permeability in BL-AP than AP-BL direction (larger than two-fold).

The rate of drug permeation is typically presented as apparent permeability coefficients (P_{app}, cm/s) :

$$P_{app} = (dQ/dt) / (A \times C_0 \times 60),$$

where dQ/dt (μmol/min, nmol/min or μg/min) is the cumulative transport rate, A (cm^2) is the surface area of the monolayers, Co (μmol/ml, nmol/ml or μg/ml) is the initial concentration of the compounds on the donor side, and 60 is the coefficient when minutes are converted to seconds.

The integrity and maturity of monolayers are typically confirmed by measurement of the (TEER). Monolayer integrity can also be assessed by measuring the permeability of hydrophilic and hydrophobic marker molecules, such as [^{14}C]-mannitol and fluorescein. High variability in Caco-2 monolayer permeability between laboratories and also within laboratories has been reported. Characterization of cell transport properties using several reference compounds with known absorption characteristics and bioavailabilities is recommended.

2.2 Metabolism of drugs

Metabolism is a process by which xenobiotics, such as drugs, and endogenous compounds are converted enzymatically into more hydrophilic, more easily excreted species. Metabolism takes place in various tissues, of which liver and intestine are the main sites for an orally administered drug. Drug metabolism reactions can be divided into phase I and phase II reactions. Oxidation, hydrolysis and reduction are common phase I reactions, oxidation being the most important because the majority of marketed drugs go through oxidation, catalysed by cytochrome P450 (CYP) enzymes. Phase II reactions, also called conjugation reactions, are catalyzed by enzymes like glucuronosyl-, sulphate-, gluthatione-S-, acetyl- and methyltransferases. UDP-glucuronosyltransferases (UGTs) are quantitatively the most important phase II enzymes for hepatically cleared drugs.[44]A compound often goes through phase I before phase II transformation. Although metabolism is typically a detoxifying process, it can also lead to toxic and pharmacologically more active species,[45] and knowledge of the metabolism of a new drug is therefore highly important. Besides enzymatic phase I and phase II systems, the phase III detoxification system needs to be mentioned as a part of drug metabolism since it has the important task of eliminating toxic metabolites from cells. Phase III includes the action of drug transporters (uptake and efflux transporters) such as ABC efflux carrier transporters, which are considered to be major players in the phase III detoxification system.[46,47]

At the early stage of drug discovery, *in vitro* metabolism studies focus on determining induction/inhibition of at least CYP enzymes and the metabolic stability of new drug candidates.[48-50] Determination of induction is important because increased enzyme activity typically shortens exposure and could cause non-response to the drug therapy. Induction can also activate inactive compounds to an active form with undesirable (e.g. carcinogenic) properties.[51] In enzyme inhibition decreased enzyme activity slows down the elimination of drugs and can result in increased *in vivo* drug concentrations and exaggeration of adverse drug effects.[52] Metabolic stability or clearance, particularly hepatic, is an important parameter for predicting the bioavailability and toxicity of a given drug. An unstable compound might have no desired biochemical effects. On the other hand, a compound that is not metabolized at high enough rate might prove toxic because of its accumulation in the body.

Identification of the major metabolites will be central during the lead optimization of drug discovery. Structural information, especially of reactive or toxic and pharmacologically active metabolites, is essential. That information will be of use in optimizing the ADME properties of new chemical entities.[50]

2.2.1 *In vitro* models in drug metabolism studies

Subcellular fractions, and microsomes in particular, are the most widely used *in vitro* systems in drug metabolism studies of new drug candidates.[53,54] They are useful for evaluation of metabolic stability, metabolite identification and drug-drug interactions. Microsomes are fractionated from other organelles by differential ultracentrifugation. Microsomes have many advantages including easy adaptation to higher throughput assays, easy preparation and use, good stability during storage, high CYP concentration and high rate of metabolite turnover.[54,55] The majority of drug metabolizing enzymes (e.g. CYPs, UGTs, flavin-monooxygenases, epoxide hydrolase) are present in the microsomal fraction. A major disadvantage of microsomes is the absence of cofactors and certain drug metabolizing enzymes. In addition, compound-dependent protein binding in the microsomal fraction may complicate the choice of relevant drug concentration for the incubations and may have a significant impact on the *in vitro-in vivo* correlation.[56,57]

As whole cell systems, hepatocytes and liver slices, offer a more reliable *in vivo/in vitro* correlation than subcellular systems since they contain all cofactors and metabolizing enzymes.[58] In the past decades, isolated (fresh or cryopreserved) hepatocytes have overtaken liver slices for use in the prediction of pharmacokinetic properties since comparative studies have shown generally lower uptake and enzyme activities in liver slices.[59] However, liver slices are still a very interesting whole cell model for metabolite identification because of the fast preparation and use.

Primary cultures of hepatocytes have shown good in vitro-in vivo correlations in metabolic activity for a number of drugs and they allow a good view of whole cell metabolism since drug transporters and both Phase I and II enzymes are present.[60-62] Additionally, primary cultures of

hepatocytes are suitable for assessing the regulation of drug metabolizing enzymes since regulatory pathways remain functional for several days after the isolation of the hepatocytes. The disadvantages of hepatocyte cultures are decreased expression level of specific CYPs during culturing and high isolation-to-isolation differences in enzyme activity levels. Various culturing techniques have been applied to improve the properties of hepatocyte cultures: co-culturing with other cell types, adding specific additives to the culture medium and use of perfusion culture and sandwich techniques.[63,64] Hepatocytes in suspension are technically the easiest of all hepatocyte systems. The major limitation of use of suspensions is that hepatocytes in suspensions stay viable for a short period (up to 4 h) which prevents investigation of slowly metabolizing compounds. However, interspecies comparison seems to be more reliable with hepatocytes in suspensions than in culture due to potential damage of cytotoxic substances and variations in expression levels of drug metabolizing enzymes in cultures.[64] The limited availability of fresh human tissue is the biggest limitation to the use of primary human hepatocytes. To overcome this, cryopreservation of hepatocytes has been developed.[65]

Many drug metabolizing enzymes, e.g. CYPs and UGTs, exist as gene "superfamilies", and the individual isoforms differ in terms of substrate and inhibitor selectivities, regulation and drug interactions.[12,66] Determination of CYP/UGT isoform specific differences is important for the prediction of hepatic clearance and clinical response of a drug. Although microsomes and hepatocytes are traditionally used as *in vitro* systems for determining reaction phenotype, they have some significant disadvantages such as the presence of multiple CYP/UGT with overlapping substrate specificity. Cloned and expressed human drug metabolizing enzymes are now widely used for the study of drug metabolism.[67] Recombinant drug metabolizing enzymes (e.g. CYP, UGT) are nowadays well established and commercially available (e.g. Xenomes™, Supersomes™). Enzymes are expressed in yeast, human lymphoblast or baculovirus infected insect cells. Expressed recombinant enzymes are the only *in vitro* model that gives structure-activity information on individual isoenzymes involved in the metabolism of drug candidates. Furthermore, through mixing of exact proportions of different isoforms, it is possible to keep the enzyme levels constant. Recombinant enzymes are also easily applied with higher throughput, which makes them a valuable model for early metabolism studies.[68] The major disadvantage of recombinant UGTs is that their quantitation in expression systems is problematic. In addition, the relative expression of UGTs *in vivo* is currently unknown.

2.2.2 *In vitro* determination of drug interaction and metabolic stability

Microsomes, hepatocytes and recombinant enzymes are widely used as *in vitro* systems in enzyme inhibition studies, while human primary hepatocytes are considered the best model for assessment of enzyme induction in human. In inhibition studies, typically several concentrations of the substrate and the inhibitor are used to assess concentration dependent changes and determine the inhibition constant Ki. A simpler approach is to determine the concentration of inhibitor required to cause 50% inhibition (IC50). In induction studies, the catalytic activity of

enzymes or specific mRNA content is assayed using specific probe substrates in treated cells and untreated cells. Additionally, changes in immunoreactive protein levels are typically determined in induction studies using CYP/UGT specific antibodies when, these are available. The induction screens are also routinely performed using appropriate reporter gene assays for certain receptors, such as the aryl hydrocarbon and the pregnane X receptors, which are involved in regulation of metabolic enzymes.[58,69] *In vitro* drug interaction studies do not predict *in vivo* interactions if alternative metabolic or excretory routes play the major role in the clearance of the drug *in vivo*. Furthermore, different concentrations of enzymes may lead to misinterpretation. Positive *in vitro* results should thus always be confirmed *in vivo*.[70]

The goal of early *in vitro* screening of metabolic stability is to predict *in vivo* intrinsic clearance of potential lead compounds and classify these as poorly, moderately or rapidly metabolized. Ordinarily, *in vitro* metabolic stability assays involve incubation of the compound in the presence of liver microsomes or hepatocytes. The samples are typically assayed in a compound-specific analysis based on LC/MS/MS. Metabolic stability results are usually reported as measures of parent disappearance or metabolite formation.[69,71] Parent disappearance can be reported as $t_{1/2}$ or % disappearance at a single time point. *In vitro* intrinsic clearance can be obtained by estimating the maximal reaction rate (V_{max}) of metabolite formation and the Michaelis constant (K_m) and calculating CL_{int} as V_{max}/K_m. This approach is time-consuming, however, and a large number of measurements are needed to optimizing the incubation conditions and perform final experiments at several substrate concentrations. An alternative, more rapid method for determining CL_{int} *in vitro* is the depletion method in which the substrate, at very low concentration, is incubated and the concentration of the substrate is monitored at regular intervals, and CL_{int} is estimated from the half-life.[72,73]

2.3 Increasing throughput in ADME studies by cocktail dosing

Cocktail dosing, the use of a mixture of many test compounds, is an effective method for reducing the number of routine samples and increasing the throughput in drug metabolism and pharmacokinetic studies. Cocktail dosing can be applied in both *in vitro* methods and *in vivo* animal studies. The major disadvantage of cocktail dosing is the possibility of drug-drug interactions.[74] These can be minimized in three ways: (1) by using low initial dosing concentrations, (2) by putting compounds known to interact with each other in different pools and (3) by limiting the number of drugs in the pool and making compromises in the throughput.

A typical cocktail sample requires a sensitive and selective analytical method allowing simultaneous analysis of multiple components, often present in very low concentrations. Rapidity of the method is a significant factor in early drug discovery. LC/MS/MS appears to be the most widely used and best method for n-in-one analyses.

Cocktail dosing has been successfully applied in drug permeability studies. Several papers describe the use of mixtures of drugs in Caco-2 permeability experiments,[75-80] with the

conclusion that the apparent permeability coefficients (P_{app}) of multiple drugs across Caco-2 monolayers measured by cocktail assay agree well with the coefficients measured by the standard single-drug assay strategy. The number of drugs in cocktails has been three to nine and they are well-known drugs. Typically detection is by LC/MS/MS, but there are exceptions. Palmgren *et al*.,[79] for example, successfully validated combined UV and fluorescence detection for a cocktail of nine drugs in Caco-2 permeability assay.

Cocktail dosing has also been applied in studies of drug metabolism, especially inhibition and induction. Several recent articles outline the substrate cocktail strategy for CYP inhibition[81-86] studies with microsomes. In practice, experiments on enzyme inhibition are some of the easiest to make at high throughput because of the simplicity, and the same generic assays can be utilized with the same probe substrates regardless of the test materials. In applications, a mixture of five to nine selective CYP substrates and one specific inhibitor are added to a single microsomal incubation. Bu *et al.*[82] evaluated more complicated approaches in which the inhibition of several CYP isoenzymes in microsomes was investigated simultaneously. The study reported the incubation of one substrate and a cocktail of specific inhibitors for three CYP isoenzymes (CYP3A4, 2D6, 2E1). Cocktail dosing of both substrates and inhibitors has also been described, where the incubation mixture contained three to five substrates as well as several inhibitors.[81,83] In all these studies the IC_{50} values obtained in cocktail experiments were in good agreement with values for individual incubations and/or published values.

Mohutsky *et al.*[87] used cocktail strategy in CYP induction studies with human hepatocytes. They showed that cocktails can be successfully applied when drugs do not interact with one another. In addition to microsomes and hepatocytes, recombinant CYP enzymes have been used in cocktail studies. Dierks *et al*.[86] used a cocktail of seven CYP substrates in determining the activity of several CYP isoenzymes.

Post-dose sample pooling, or mixing samples from individual experiments before multicomponent analysis by LC/MS/MS, may also be an effective way to increase throughput. Drug-drug interactions are not a concern in such an approach. However, high sensitivity of the analytical method is often demanded due to dilution of the analytes during the mixing. Sample pooling has been successfully applied in studies of CYP inhibition[88] and metabolic stability.[89]

3 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC/MS)

Liquid chromatography-mass spectrometry, LC/MS, is a well-established technique for quantitative and qualitative analyses in pharmaceutical research. Typical quantitative LC/MS applications are *in vitro* ADME assays in drug permeability, metabolic stability and inhibition studies.[90] The assays need to be fast and fulfil validation requirements at the same time. Profiling of the metabolites from different biological matrices is a prime example of qualitative LC/MS analysis in drug discovery. In that case, reliable identification of the metabolites is the major aim of the study and high throughput is usually not required.

3.1 Sample preparation

Some *in vitro* matrices, such as the incubation solutions used in metabolic stability studies, need no or only simple sample pretreatment. The main risk in LC/MS analysis is contamination of the ion source by non-volatile buffers. However, early eluting buffers can usually be separated by LC and diverted to waste before the mass spectrometric analysis. If pretreatment is required, protein precipitation is generally recommended since it is simple, usually sufficient and needs no method development.[91]A more complex biological matrix such as plasma or urine requires sample preparation, typically solid-phase extraction (SPE) or liquid-liquid extraction (LLE). Direct sample injection techniques such as column switching together with restricted access media (RAM), on-line SPE and turbulent flow chromatography are alternatives to off-line methods.[92] Besides the laborious method development, a serious disadvantage of these techniques is the complexity of the analytical system.

3.2 Liquid chromatography

Liquid chromatography in quantitative analysis

Speed, efficiency and reliability are major requirements of quantitative analysis in pharmaceutical research. Mass spectrometric detection is seldom the rate limiting step in LC/MS analysis, and thus improvements have lately been focused on the efficiency of the chromatographic separation (e.g, fast columns, high flows, parallel techniques).[91,92] Full chromatographic separation of the compounds from each other may not be necessary with a selective MS/MS detection mode since the interference between co-eluting analytes is typically not a major problem. Other interfering material such as biological matrix or buffers from the *in vitro* experiments is of concern, however, as it often causes matrix effects. Accordingly, the most important task of the LC system is to provide an efficient separation of the analytes from matrix components.

A recent trend in bioanalytical applications is to use short, typically narrow bore columns for fast chromatography with relatively high flow rates. The flow rates are typically 1.5 - 2 ml/min, offering a good compromise between throughput and chromatographic parameters. The aim has been to increase the sample throughput but at the same time reduce the time required for the chromatographic method development. Method development is especially important when many analytes with diverse chemical structures are present in a sample.

Reverse phase, especially C18 bonded, column material is the most widely used material in fast separations in pharmaceutical research. Fast separation can be achieved with a short conventional column, large particle column or monolithic column. Short ($\leq$ 5 cm) and narrow-bore (i.d. 2 mm) columns with small particles and operated at higher than optimal flow rate provide fast analysis with satisfactory chromatographic separation and are widely utilized in quantitative LC-MS/MS drug analyses. Korfmacher *et al.*[91] offer extensive data to show that

micropore columns maintain analytical accuracy similarly to conventional columns. Another type of column used in high flow rate analyses is a narrow bore (~ 1 mm) column with large particles (~ 30 μm).[93] Yet a third type of column, a monolithic column, is not a conventional particle column but contains two types of pores. High permeability of the macropores (about 2 μm) allows higher flow rates without generating high backpressure, and the mesopores (13 nm) provide extended surface area, giving good separation efficiency. Both polymer and silica based monolithic columns are available. Monolithic silica columns have been successfully employed in LC/MS/MS applications to increase analytical throughput in metabolite identification[94] and quantification[95] and quantitative bioanalysis of drugs.[96] Comparative studies of the column performance of microparticulate C18 bonded and monolithic C18 column have shown similar chromatographic performance and accuracy for the two columns.[97,98]

Fast linear gradients have been used successfully, especially in separations of multiple compounds with various chemical functionalities. The gradient step in these separations is typically from one to a few minutes. In general, the gradient provides an improved peak shape and a separation of analytes from matrix interferences.[99,100] Recent reports have described ultra-fast separations using gradient elution, in which analytes are detected in less than a minute.[101,102] Cheng *et al.*[102] were able to separate five pharmaceuticals with different functionalities in 45 s. The column was a 2.1 mm x 30 mm C18 column, the flow rate was 1.5 ml/min and the gradient step lasted one min. Even faster separations can be achieved with isocratic ultra-fast separations, but the isocratic separation is not so suitable for a complex mixture of compounds. Heinig and Bucheli[103] detected pharmaceuticals in 9 s isocratically with a C18 column of dimensions 2.1 mm x 10 mm and flow rate of 2.2 ml/min.

The use of high flow rates and fast gradients involves several risks, of which the main ones are the impacts of splitting of flow and ion suppression on sensitivity.[101,104] Another limitation of fast chromatography is the carry-over in the autosampler, derived from the fast injection cycle time. A special high-speed autosampler that minimizes the carry-over or the use of several autosamplers in series can be used to overcome this carry-over problem.[105]

One approach to increase the throughput in chromatographic separation is to use parallel LC systems in which two or more columns are used simultaneously with one mass spectrometer. The flows from parallel columns can be directed to a multiplexed ionization source (MUX), or with use of staggered injections and a valve selector, to a standard ionization source. MUX systems contain several separate ESI sprayers and allow the analysis of up to eight separate eluent streams simultaneously. Four-channel MUX technology has been used in quantitative analyses of drugs and metabolites in different biological matrices.[93,106] Fung *et al.*[107] used a parallel four-column system with a four-sprayer MUX interface in the screening of Caco-2 permeability of drug candidates. Injection-to-injection time for four samples was 6 min, which allowed analytical throughput of 800 samples in one day. MUX systems have some limitations, such as spray-to-spray interferences and sensitivity loss, relative to single sprayers. Dual- or four-column staggered parallel systems for quantitative analysis with successful validation have

been described in several papers.[108-110] The major limitation of a staggered system is that each column requires its own pumping system, making the configuration complex and expensive.

Recently, a new chromatographic tool, ultra performance liquid chromatography (UPLC), has become commercially available. The UPLC system involves a high pressure fluidic binary pump able to work up to 1000 bar. A conventional HPLC pump has maximum operating pressure of about 400 bar. The high pressure with special small-particle (~ 1.7 μm) analytical columns provides better chromatographic resolution, resulting in faster analysis and better sensitivity. Several reports describe the application of UPLC to pharmaceutical analysis, particularly in the field of metabolism studies. [111-113]

Liquid chromatography in qualitative metabolite profiling

In the case of metabolite profiling, high resolution and sharp peak bands rather than extremely high speed of the liquid chromatography are preferred to achieve sensitive and selective metabolite identification. An adequate chromatographic separation is required not only to avoid matrix interference but also to obtain the separation of isobaric metabolites. Furthermore, some metabolites (e.g. acylglucuronides) are easily dissociated during the ionization to a fragment ion identical with the molecular ion of the parent compound, and chromatographic separation of the metabolite from the parent compound becomes necessary.[114] Metabolites are normally separated with use of a slow linear gradient. Depending on the complexity of the matrix and the number of metabolites, the chromatographic run in metabolite identification applications is typically 10-30 min.

Reversed-phase chromatography is standard technique in metabolite profiling. However, a highly aqueous mobile phase is usually needed for the retention of polar metabolites, which may cause collapse of the stationary phase. The challenge of collapse and short column lifetime, has been met by the development of special reversed-phase columns for polar compounds. In these more polar columns, a polar functional group, such as an amide or carbamate, is embedded in the alkyl chain of a silica-based alkyl-bonded phase or used as an endcapping agent. The columns used in metabolite identification are typically 10-20 cm long and their internal diameter is 3-5 mm. In addition to traditional particle columns, fast monolithic reversed-phase columns have been used to increase throughput in metabolite identification.[94] Less frequently used LC techniques in metabolism studies are ion exchange and hydrophilic interaction chromatography (HILIC), which relies on polar stationary phases (e.g. diol, amino, and underivatized silica).[115]

Capillary LC is also effective, although a relatively slow technique in metabolite profiling applications. Long (100-200 mm) and narrow (<2 mm) columns offer good separation and enhanced sensitivity which is useful for the analysis of complex *in vivo* samples. Additionally, a ultra performance LC system (UPLC) is an effective technique in metabolite identification,[113,116] especially in conjunction with mass analysers with fast scanning speed.

3.3 Mass spectrometry

3.3.1 Ionization

The most common ionization techniques in LC/MS in bioanalysis are electrospray ionization (ESI),[117-119] atmospheric pressure chemical ionization (APCI)[119,120] and, less frequently, the relatively new, atmospheric pressure photoionization (APPI).[121]All of them can be considered atmospheric pressure ionization (API) sources since the ionization occurs at atmospheric pressure. In ESI a high electric field (3.5 kV) applied to the metal capillary assists nebulizing of a liquid stream to charged droplets.[117] ESI is characterized as a "softest" ionization technique since only weak fragmentation is produced. ESI ionizes polar/non volatile compounds extremely well and thus is the most frequently used ionization technique in metabolite quantification and identification. APCI requires additional heating and high gas flow for vaporization. The electron flow produced by the corona discharge needle of APCI ionizes the gas and solvent molecules, which in turn ionize analyte molecules in gas-phase ion-molecule reactions.[122] APCI is better suited than ESI for neutral compounds but it is unsuitable for thermally unstable compounds. APPI is similar to APCI but the ionization is achieved by photoionization, using a near-UV lamp producing 10 eV photons. In dopant-assisted APPI, photons first ionize the solvent or dopant (e.g. toluene, added to the eluent flow to assist ionization) producing radical cations. Thereafter, the cations formed may ionize analyte molecules through various mechanisms, including charge and proton transfer.[121,123] The major benefit of APPI over APCI and ESI is its ability to ionize more extensively non-polar compounds.

Positive ionization mode prevails in pharmaceutical analysis since most drugs are basic and are ionized well in positive mode. In metabolite profiling, however, it is important to evaluate both positive and negative ionization modes since metabolism generally transforms drugs to more acidic metabolites. Sulphate conjugation and glucuronide conjugation of alcohol are examples of transformations that can often be detected only in the negative mode.

Not all mobile phases and flow rates are suitable for API sources. A general rule is that the solvent has to be volatile and should not exceed 20 mM since non-volatile buffers and high buffer or additive concentrations cause ion suppression and inhibit ionization, especially in ESI.[124] APPI and APCI are less susceptible to ion suppression than ESI.[125] The most common mobile phase composition in reversed-phase LC/MS is water with either methanol or acetonitrile. Sensitivity is typically improved when the proportion of organic solvent is increased because of the improved desolvation.[126] Typical mobile phase additives are ammonium buffers, acetic acid, formic acid and ammonium hydroxide. Depending on the solvent composition, present ionization sources are able to vaporize flow rates up to 1-2 ml/min.

3.3.2 Mass analysers

Mass analysers in quantitative analysis

Various mass analysers are applied for quantitative bioanalysis, including quadrupole analysers,[80,82,127] time-of-flight (TOF) instruments[127,128] and ion traps.[129,130] The most widely used analyser is the triple quadrupole (QqQ) with selected reaction monitoring (SRM) mode, which provides superior signal-to-noise ratio (S/N) for compounds and high selectivity for the quantitation of target compounds in biological fluids. QqQ also offers better accuracy and precision than the ion trap,[129] and a somewhat wider dynamic range than TOF.[127] In quantitative analysis with QqQ it is recommended to use a higher resolution setting for Q1 (e.g. 0.7) than for Q3 (e.g. 1.0), since this increases selectivity against matrix background.[131] However, higher mass resolution normally also means signal decrease, and a compromise between sensitivity and selectivity is usually required. Advantages of TOF are high mass resolution, good mass accuracy and a fast full-scanning duty cycle which allows detection of multiple analyte ions without a concomitant loss in signal intensity. The ion trap is less often applied in quantitative analysis and there are more limitations, such as a relatively slow MS/MS duty cycle.

Mass analysers in qualitative metabolite profiling

Various mass analysers have been used in metabolite profiling and each of them has its own benefits.[132] The QqQ mass spectrometer has highly advantageous tandem mass spectrometric features: namely, neutral loss and precursor ion scanning modes allow group-specific detection of unexpected as well as expected metabolites.[132-134] For example, glucuronide, sulphate, and aliphatic glutathione conjugates can be detected in the positive ionization mode by neutral loss of 176, 80, and 129 Da, respectively. Perchalski *et al.*[133] detected hydroxyl metabolites of phenytoin by scanning the product ions of the precursor ion at m/z 175, which is a specific fragment for both the parent compound and the metabolites. Accurate mass measurements with high resolution are essential to determine the elemental composition of small molecules and particularly effective for separating metabolites with isobaric structures. The quadrupole time-of-flight (Q-TOF) analyser, with a mass accuracy of $\leq$ 5 ppm, is the most popular accurate mass instrument for metabolite identification because of its ease of use, fast acquisition speed, high sensitivity and high resolution in both MS and MS/MS modes.[135,136] The Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer and the recently introduced Orbitrap instrument[137] offer highest mass accuracy ($\leq$1 ppm) and highest mass resolution and are competitive approaches for accurate mass measurements. The FT-ICRMS instruments are uncommon, however, because of their large size and high investment and maintenance cost. The advantage of FT-ICRMS, and of cheaper and simpler ion trap instruments, is the possibility to perform MS^n measurements,[138] which allow a more comprehensive estimation of the potential sites of metabolic modification.

Several new hybrid instruments are very suitable for metabolite profiling. The linear ion trap time-of-flight (LTQ-TOF)[139,140] and the linear ion trap-Fourier transform mass spectrometers (LTQ-FTMS)[141] combine ion trap MSn capabilities with high resolution, and LTQ-TOF additionally combines the fast duty cycle of the TOF-instrument. A quadrupole-linear ion trap (Q-Trap) makes possible several new informative scanning modes since the third quadrupole can be operated in the ion trap mode.[142]

3.4 Liquid chromatography-mass spectrometry in metabolite profiling

The earliest metabolite identifications in drug discovery are typically made after *in vitro* incubations with microsomes. LC/MS detection of metabolites in the microsomal incubation solution is often straightforward since the matrix is relatively clean, the number of metabolites is small and, with high initial substrate concentration in the incubation medium, the concentration of the metabolites is high. In contrast to this, the identification of metabolites in complex biological matrices (e.g. urine and plasma) is challenging due to the presence of interfering material such as proteins, lipids and other endogenous compounds. Furthermore, samples typically contain structurally diverse metabolites which may be present at very low concentrations.[132]

Each mass spectrometric instrument has its own benefits in metabolite profiling, and broad information on the metabolism of a drug or a drug candidate can be obtained by combining data obtained with several instruments. The analytical strategy for metabolite profiling typically utilizes all or some of the following experiments depending on the compound: detection is achieved by unit or high resolution full-scan, precursor ion scan, and/or neutral loss scan modes, and identification and structural characterization of the potential metabolites is done by product ion (MS/MS, MS^n) and/or accurate mass MS and MS/MS measurements.[7,143]

To increase throughput in metabolite identification it is useful to collect as much MS information as possible during a single run. Various hybrid mass spectrometers with information-dependent acquisition (IDA) programs have been introduced to obtain complementary data simultaneously and to gain maximum structural information with a minimum number of analytical runs. Basically, IDA combines two or more different scanning modes in a sequential way during the same run. One disadvantage of the IDA technique is that, as a result of limited sensitivity, minor metabolites are rarely detected and identified. Hopfgartner *et al.* [144] describe the Q-Trap instrument in detail and give examples of its use in metabolite identification. In their application, up to eight MS or MS^n experiments could be performed in the same run. IDA experiments $MS^2 \rightarrow MS^n$ were successful, but some limitations were observed in $MS \rightarrow MS^2$ experiments. Selection of relevant precursor ions from full-scan data was not possible because of intense interfering matrix peaks.

Commercial software for searching and identifying metabolites (e.g. Metabolynx and Metabolite ID) has recently been developed for the automated processing of ion chromatograms and mass spectra. The software searches for expected metabolites and the differences between

two or more LC/MS runs and can set up MS/MS experiments to confirm the identity of the metabolites. An isotope cluster analysis tool is included for halogenated compounds or other groups of compounds. A particular limitation of packages is that they do not provide all theoretically possible biotransformation reactions for a given drug and require the user to add most of the main of expected metabolites manually. Computer-based systems for the prediction of routes of drug metabolism are available and are becoming more reliable as metabolism data for different compounds is added. But it is still not possible to predict fully the metabolism of a particular compound solely on the basis of its structure.[145-147]

Mass defect filtering program, recently introduced by Zhang *et al.*[148] effectively removes interfering ions from accurate mass analyses. A mass defect is defined as the difference between the exact atomic mass and the nominal mass of a compound. Phase I and phase II reactions introduce uniquely different mass defects, which typically fall within 50 mDa. For example, mass defects relative to that of the parent drug are 5 mDa for hydroxylation, -23 mDa for demethylation and 32 mDa for glucuronidation. On the basis of that information, a mass window can be defined that filters out ions whose mass defects lie outside the window specified for the drug-related compounds.

In addition to software that searches for metabolic transformations, methods have been recently developed for automated MS/MS spectrum interpretation. Those methods still have many limitations. For example, the structure of the parent compound and hypothetical structures of the metabolites should be known beforehand. Nassar *et al*.[149] report an identification strategy for metabolites in *in vitro* microsomal incubations which utilizes PALLAS software to predict possible metabolites, Metabolynx software to find potential metabolites and ACD (Advanced Chemistry Development) software to determine the chemical structure of metabolites based on their MS/MS spectra. Used in combination with an accurate mass instrument, PALLAS, Metabolynx and ACD provided to be valuable tools to increase throughput in metabolite identification studies.

Even though MS^n experiments and accurate mass measurement are powerful tools in metabolite identification, the exact structure of a metabolite may be difficult to determine. In some cases the modification of metabolites, for example by enzymatic hydrolysis or reduction methods, combined with MS detection may be useful in structural characterization. Additionally, different derivatization methods may be useful, though time-consuming, in the characterization of chirality and the site of metabolism.[150] Hydrogen-deuterium (H/D) exchange is also another useful mass spectrometric method in metabolite identification, and on-line H/D exchange LC/MS is an especially rapid tool for metabolite identification.[150-151] H/D exchange facilitates the estimation of labile hydrogen atoms (e.g. in –OH, -SH, -NH) and structural characterization and interpretation of MS/MS data. Stable isotope labelling combined with MS is a competitive method for radiolabelling.[152] In addition to the advantage in structural characterization by MS/MS, stable isotope labelling facilitates reliable detection of metabolites from full-scan data. The major benefit over radiolabelling is that safe experiments in human and animals are possible.

4 AIMS OF THE STUDY

The main aim of this work was to increase effectiveness at different stages of the drug discovery process. There were three approaches: permeability experiments with caco-2 cells, kinetic studies with recombinant enzymes, and metabolite profiling. More specifically the aims were:

- to develop and validate fast and sensitive n-in-one LC/MS/MS methods for the quantitative analysis of drugs in permeability experiments (**I,IV**) and glucuronides in *in vitro* metabolic stability experiments (**II**)
- to investigate the suitability of atmospheric pressure photoionization (APPI) as part of routine and fast quantitative LC/MS/MS in the analysis of caco-2 samples (**I,IV**)
- to investigate the applicability of cocktails in permeability experiments with caco-2 cells (**I,IV**) and in kinetic studies with recombinant human UGT isoenzymes (**II**)
- to introduce an alternative method for the determination of intrinsic clearances (Cl_{int}), a method involving the slope of the linear portion of the V vs [S] plot, and to compare the new Cl_{int}(slope) values with the values determined traditionally by deriving the enzyme kinetic parameters V_{max} and K_m from the Michaelis-Menten curve (**II**)
- to investigate the suitability of mass spectrometric instruments and commercially available software for metabolite profiling of a drug substance in urine (**III**)
- to identify all the metabolites of sibutramine formed in primary cultures of rat and human hepatocytes by LC/MS and LC/MS/MS studies (**V**).

5 EXPERIMENTAL

The most important experimental features are described in this section. More detailed descriptions can be found in the original publications **I-V**.

5.1 Chemicals and reagents

The standard compounds and chemicals used in the study are listed in Table 1. For MS work (**I-IV**), all the glassware was flushed with 5% nitric acid and rinsed with ion-exchanged water (Millipore, Milli-Q Plus, France). The structures of the studied compounds are shown in Figure 3.

Table 1. Chemicals and reagents used in the study.

Chemical	Manufacturer	Paper
acetic acid	Sigma-Aldrich (Steinheim, Germany); Merck (Darmstadt, Germany)	III,V
acetonitrile, HPLC grade	Rathburn (Walkerburn, Scotland); Merck (Darmstadt, Germany)	I,III,IV,V
ammonium acetate	Merck (Darmstadt, Germany)	I,II,III,IV,V
antibiotic mixture (1000 IU/mL penicillin, 1000 µg/mL streptomycin)	Life Technologies Ltd., Paisley, Scotland	I, IV
antipyrine	Aldrich Chemical Co. Inc. (Milwaukee, WI)	I, IV
cephalexin hydrate	Sigma-Aldrich (Steinheim, Germany)	I
collagen	Sigma-Aldrich (Prague, Czech Republic)	V
collagenase	Sevapharma (Prague, Czech Republic)	V
demethylsibutramine hydrochloride	Faculty of Pharmacy, Charles University Hradec Kralove (Czech Rebuplic)	V
didemethylsibutramine hydrochloride	Faculty of Pharmacy, Charles University Hradec Kralove (Czech Rebuplic)	V
Disodium hydrogen phosphate dihydrate	Merck (Darmstadt, Germany)	II
Dulbecco's modified Eagle medium	Life Technologies Ltd., Paisley, Scotland	I, IV
Dulbecco´s phosphate-buffered saline	Life Technologies Ltd., Paisley, Scotland	I, IV
entacapone	Orion Pharma (Espoo, Finland)	II
entacapone 3-O-glucuronide	Faculty of Pharmacy, University of Helsinki	II
estriol	Sigma-Aldrich (Steinheim, Germany)	II
estriol-17b-D-glucuronide	Sigma-Aldrich (Steinheim, Germany)	II
fluorescein sodium	Sigma-Aldrich (Steinheim, Germany)	I, IV
foetal calf serum (FBS)	Life Technologies Ltd., Paisley, Scotland Sigma-Aldrich (Prague, Czech Republic)	I, IV, V
formic acid (98-100%)	Sigma Aldrich (Steinheim, Germany); Merck (Darmstadt, Germany)	I, II,V
L-glutamine (200 mM)	Life Technologies Ltd., Paisley, Scotland	I,IV
HAM F12 medium	Sigma-Aldrich (Prague, Czech Republic)	V
Hank's balanced salt solution (HBSS)	Life Technologies Ltd., Paisley, Scotland	I, IV
HEBES solution	Life Technologies Ltd., Paisley, Scotland	I, IV
hydrochlorothiazide	Sigma-Aldrich (Steinheim, Germany)	I, IV
8-hydroxyquinoline	Sigma-Aldrich (Steinheim, Germany)	II
8-hydroxyquinoline glucuronide	Sigma-Aldrich (Steinheim, Germany)	II
insulin	Sigma-Aldrich (Prague, Czech Republic)	V
ketoprofen	ICN Biochemicals Inc. (Aurora, OH)	I, IV

Table 1. Continues

magnesium chloride hexahydrate	Merck (Darmstadt, Germany)	II
[^{14}C]-mannitol (specific activity = 51.50 μCi/mmol)	DuPont NEN (Boston, MA)	I, IV
methanol, HPLC grade	J.T Baker (Deventer, The Netherlands)	II, III
4-methylumbelliferone	Sigma-Aldrich (Steinheim, Germany)	II
4-methylumbelliferyl-β-D-glucuronide	Sigma-Aldrich (Steinheim, Germany)	II
metoprolol tartrate	ICN Biochemicals Inc. (Aurora, OH)	I, IV
midazolam	Hoffman-La Roche (Basle, Switzerland)	I
2-(N-morpholino)-ethanesulphonic acid (MES)	Sigma Chemical Co. (St. Louis, MO)	I, IV
nonessential amino acids	Life Technologies Ltd., Paisley, Scotland	I, IV
paracetamol	Orion Pharma (Espoo, Finland)	II
paracetamol glucuronide	Sigma-Aldrich (Steinheim, Germany)	II
perchloric acid, 70-72%	Merck (Darmstadt, Germany)	II
propranolol hydrochloride	ICN Biochemicals Inc. (Aurora, OH)	I
ranitidine	Sigma-Aldrich (Steinheim, Germany)	I
D-saccharic acid 1,4-lactone	Sigma-Aldrich (Steinheim, Germany)	II
(S)- and (R)- sibutramine hydrochlorine purity > 99%	Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic)	V
sibutramine hydrochlorine	Faculty of Pharmacy, Charles University Hradec Kralove (Czech Rebuplic)	V
sodium dihydrogen phosphate dihydrate	Fluka Chemie (Buchs, Germany)	II
thiazolyl blue (MTT)	Sigma Chemical Co. (St. Louis, MO)	I, IV
tolcapone	Orion Pharma (Espoo, Finland)	II
tolcapone-3-O-glucuronide	Faculty of Pharmacy, University of Helsinki (Helsinki, Finland)	II
toluene	Baker (Phillipsburg, NJ)	I, IV
tramadol hydrochloride	Department of Forensic Medicine, University of Helsinki	III
tramadol hydrochloride tablets (Tramal 50 mg)	Orion Pharma (Espoo, Finland)	III
umbelliferone	Sigma-Aldrich (Steinheim, Germany)	II
umbelliferone glucuronide	Ultrafine (Manchester, England)	II
uridine 5'-diphosphoglucuronic acid (UDPGA)	Sigma-Aldrich (Steinheim, Germany)	II
verapamil hydrochloride	ICN Biochemicals Inc. (Aurora, OH)	I, IV
water (Milli-Q)	Millipore, Molsheim, France	I-V
William's E medium	Sigma-Aldrich (Prague, Czech Republic)	V

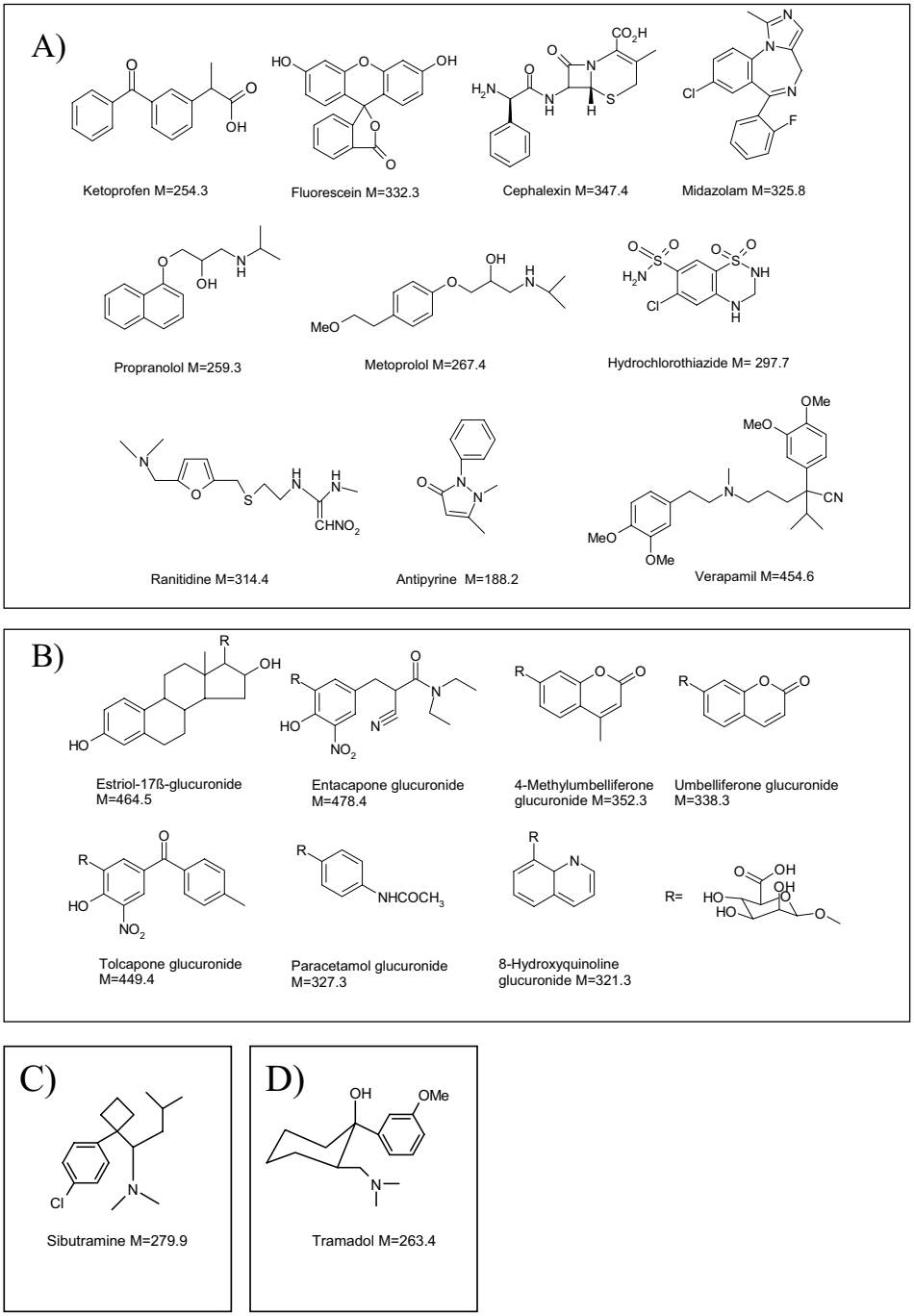

Figure 3. Structures of studied compounds. A) **I**, **IV** B) **II** C) **V** D) **III**. M= Molecular weight (g/mol).

5.2 Recombinant enzymes, hepatocytes and human urine

Human UGTs (1A1, 1A3, 1A6-1A10, 2B7, 2B15) for the determination of metabolic clearance of the test compounds (**II**) were cloned and expressed in baculovirus-infected insect cells as His-tagged proteins in our laboratory. [153,154] Protein concentrations were determined by the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

Primary cultures of hepatocytes were prepared for the incubations with sibutramine (**V**). Hepatocytes of humans and Wistar rats were isolated by two-step collagenase method.[155] Isolated hepatocytes were three times rewashed and mixed together with culture medium (1:1 mixture of Ham F12 and Williams' E). Three million viable cells in 3 ml of culture medium were placed into 60-mm plastic dishes pre-coated with collagen. Foetal calf serum was added to the culture medium (5%) to favour attachment the cells during the first four hours. After that the culture medium without serum was used. The cultures were maintained at 37 °C in a humid atmosphere of air and 5% CO_2.

For the determination of tramadol metabolites in human urine (**III**), a control urine sample (0 urine) was collected just before and a Tramadol urine sample during 12 h after the intake of a tramadol tablet (Tramal, 50 mg, Orion Pharma, Helsinki, Finland).

5.3 Caco-2 cell culture and permeability experiments

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) (**I**,**IV**). The cells were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum, 1% non-essential amino acids, 1% L-glutamine and antibiotics. The growth medium was changed every 2-3 days. Cells (6.8×10^4 cells/cm^2), at passages 32-42, were seeded on polycarbonate filter inserts (Corning Costar Corp., Cambridge, MA, mean pore size 0.4 μm, growth area 1.1 cm^2 or 0.33 cm^2). The cell monolayers were allowed to grow and differentiate for 22-27 days before the permeability experiments. The cultures were maintained at 37 °C in an atmosphere of 90% relative humidity and 5% CO_2. Prior to the experiments, integrity of the monolayers was ensured by TEER measurements. In addition, the permeability of radiolabelled mannitol (paracellular marker molecule) was measured after the experiments and MTT tests were performed to investigate possible cellular damage.

The permeability experiments were performed in apical to basolateral (AP-BL) and basolateral to apical (BL-AP) directions. The experiments were done in HEPES, which was buffered to pH 7.4 in non-gradient conditions and to pH 5.5 (apical side) and pH 7.4 (basolateral side) in pH-gradient conditions. The samples were taken at regular time intervals by transferring the cell inserts into new wells containing fresh HEPES. The samples were stored at -70 °C until analysis.

5.4 *In vitro* incubations

Incubations with UGT isoenzymes (**II**) were done in total volume of 100 µl (determination of Cl_{int} (slope)) or 250 µl (determination of V_{max} and K_m) consisting of 50 mM sodium phosphate buffer pH 7.4 (5 mM $MgCl_2$), 50 mM D-saccharic acid 1,4-lactone, 1-50 µg UGT isoform (2.5 mg/ml of protein), 10 mM stock solution of UDPGA and intermediate solution of each substrate or mixture of substrates. The incubation reaction was terminated by the addition of 4 M perchloric acid.

Cl_{int} (slope) was determined using initial substrate concentrations of 100, 150, 200, 250 and 300 µM for paracetamol and either 0.25, 0.50, 0.75, 1.00 and 1.25 µM or 2.5, 5.0, 7.5, 10.0 and 12.5 µM for the other substrates. The incubation time (60 min) and amount of protein (25µg) was the same in all experiments. The CL_{int} values were determined as the slope of the linear part of the Michaelis-Menten fitting.

Kinetic parameters V_{max} and K_m were determined using optimized incubation conditions, and reactions were linear in terms of protein concentration and time, which varied depending on the compound studied. Enzyme assays were then incubated with substrate concentrations ranging from 0.2 x K_m to 10 x K_m. Data analysis was performed by SigmaPlot Enzyme kinetics module 1.1S.

Incubations of sibutramine in primary cultures of rat hepatocytes (**V**) were done by adding water solution of sibutramine to a fresh culture medium to obtain 35 µM concentration of sibutramine. Medium of control hepatocytes was prepared by adding redistilled water in amount equivalent to the sibutramine. Aliquots of medium (1 ml) were collected after 8 and/or 24 hours incubation at 37 °C in a humid atmosphere of air and 5% CO_2. Medium samples were stored in a freezer before sample treatment and analysis.

5.5 Sample pretreatment

Caco-2 samples (**I,IV**) whose concentrations were expected to be too high (e.g. initial concentrations) for LC/MS detection were diluted with HBSS. All samples were filtered through a Millex-HV (0.45µm) PVDF membrane (Millipore Corp. Bedford, MA) prior to injection into LC/MS.

After cooling, incubation samples (**II**) were centrifuged in a 96-well plate centrifuge (Labofuge, Heraeus Sepatech, Germany) and injected to LC/MS directly from the wells.

Urine (**III**) and hepatocyte samples (**V**) were purified with various solid phase extraction (SPE) methods (Table 2).

Table 2. Solid phase extraction methods used in the purification of tramadol metabolites (**III**) in urine and the sibutramine metabolites in hepatocyte medium (**V**).

paper	cartridge	preconditioning of the cartridge	sample loading	washing step	elution
III	Oasis HBL, 30 mg (Waters, Milford, MA, USA)	1 ml MeOH, 1 ml water	1 ml urine	1 ml water	1 ml MeOH
III	Oasis MCX, 30 mg, (Waters, Milford, MA, USA)	1 ml MeOH, 1 ml water	1 ml urine acidified with 100 µl of 1 M HCl	1 ml 0.1 M HCl, 1 ml MeOH	1 ml 0.25% NH_4OH in water/MeOH (5:95)
V	Oasis HBL, 30 mg, (Waters, Milford, MA, USA)	1 ml MeOH, 1 ml 0.1% CH_3COOH	1 ml medium acified with 50 µl of CH_3COOH	1 ml 0.2% CH_3COOH	1 ml MeOH

5.6 LC/MS/MS Instruments

LC/MS instruments and ionization methods used in the study are listed in Table 3. LC separations were carried out by reverse phase chromatography with linear gradient. Columns were: Chromolith Speedrod (4.6x50 mm, C18 RPe) (**I,IV**), Purospher STAR RP-18 (55x4 mm, 3µm) (**II**), Atlantis^{TM} dC_{18} column (2.1x50 mm, 3 µm) (**III**) and Zorbax Eclipse XDB C_{18} (3.5 µm, 2.1x50 mm) (**V**). LC/MS compatible solvents such as acetonitrile, methanol, ammonium acetate and/or ion-exchanged water were used. If the eluent was adjusted to acidic this was done with acetic or formic acid. In studies **I-IV** the eluent flow from the LC (0.9-2ml/min) was postcolumn split so that flow to the mass spectrometer was 90 µl/min (**II,III**) or 200 µl/min (**I,IV**). To avoid contamination of the ion source (**I**,**II**,**V**), before mass analysis the non-volatile early eluting buffer components were diverted to waste with a switching valve and flow from an auxiliary pump.

Table 3. LC/MS instrumentation used in studies **I-V**.

Paper	HPLC	Mass Spectrometer
I-IV	Agilent 1100 well plate autosampler	API 3000 Triple Quadrupole, Sciex
III	Agilent 1100	Q-TOF Micro, Micromass
III	Agilent 1100	Esquire ion trap, Bruker Daltonics
V	Surveyor, ThermoFinnigan	LCQ Advantage ion trap, ThermoFinnigan

The main ion optics parameters, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized for SRM transitions of each compound studied (**I,II**) in order to find the best sensitivity. In studies **III** and **V** mass spectrometric behaviour of the parent drug and available standards was first examined by direct injection analysis, and similar main ion optics parameters were used further in LC/MS and LC/MS/MS studies of the metabolites.

6 RESULTS AND DISCUSSION

The main results obtained of the work are described in this section. More detailed information can be found in the original publications **I-V**.

6.1 Cocktail dosing and n-in-one analysis in permeation and metabolic stability studies

Cocktail dosing was used to increase throughput in experiments investigating drug permeation through Caco-2 cell monolayers (**I,IV**) and in drug incubations with UGT isoenzymes (**II**). To test the feasibility of cocktails, permeation and metabolic stability experiments were carried out with both cocktail and single drug dosing. N-in-one LC/APPI/MS/MS and LC/ESI/MS/MS methods were developed, validated and compared for the quantification of multiple drugs in permeability experiments (**I**), and an LC/ESI/MS/MS method was developed for the quantification of glucuronides in incubation solution (**II**).

6.1.1 Development of n-in-one LC/MS/MS

The cocktails used in studies **I, II** and **IV** contained polar, less polar, acidic and basic compounds representing structurally diverse model drugs. pK_a values of drugs in the cocktail used in permeability studies with Caco-2 cell line varied from 1.44 to 10.07 (**I**). Compromises among time efficiency, chromatographic separation and ionization efficiency of the LC/MS/MS method were therefore demanded. Furthermore, external instead of internal calibration was employed because of lack of a universal internal standard. Additionally, various permeabilities (**I**) and metabolic activities (**II**) of the drugs meant that a wide range of concentrations had to be quantified, which placed demands requirements on sensitivity, robustness and dynamic linear range of the analytical methods.

Ionization: APPI vs ESI (**I**)

APPI, introduced by Bruins and co-workers in the year 2000, is suitable for compounds of a relatively wide range of polarities and, especially for less polar compounds, which are problematic in ESI and APCI.[121] APPI was compared with ESI in paper **I,** with special attention paid the functionality of APPI.

Optimum flow rate for APPI was shown to be 200 µL/min. A specific observation made when APPI and mobile phase with high water content were used at flow 200 µL/min was that the APPI source block requires relatively high temperature (425 °C). With a temperature of 350 °C, the quartz tube inside the source was quickly contaminated, and the background noise increased.

Ionization efficiency in APPI with toluene (10%) as dopant was tested with seven compounds (verapamil, metoprolol, ketoprofen, antipyrine, propranolol, ranitidine, hydrochlorothiazide) and four solvent compositions (Table 4). In positive ion mode all

compounds except hydrochlorothiazide showed abundant $[M+H]^+$ in all solvent compositions. In negative APPI mode only ranitidine and hydrochlorothiazide were ionized. Ranitidine showed abundant $[M-H]^-$ in all solvents and hydrochlorothiazide in solvent systems 1, 2 and 4. Ionization efficiency of cephalexine, fluorescein and midazolam was tested in solvent 4. Cephalexine exhibited poor sensitivity in both positive and negative mode. Midazolam was ionized well in positive mode producing $[M+H]^+$, and fluorescein was ionized moderately intensively in both positive and negative mode producing $[M+H]^+$ and $[M-H]^-$. Acetonitrile and 10 mM ammonium acetate (solvent 4) were chosen as the final solvent system for the APPI method for the following reasons: seven analytes were ionized with high efficiency; repeatability of the chromatographic separation was good; acetonitrile gave faster separation than methanol; and neutral conditions allowed separation of the compounds at two acquisition windows with different ionization modes. Hydrochlorothiazide and fluorescein were detected in negative APPI and seven compounds in positive ion APPI (Figure 4).

Toluene is the recommended solvent to be used as dopant in APPI [121,156] and it gave successful ionization efficiency for all compounds tested. Ionization efficiency was also tested without dopant, but the intensity of the analytes was then 10-20% lower. Recently, Kauppila *et al.* [157] showed that use of anisole as dopant provides significant improvement in the signal intensity of analytes owing to the low ionization energy and proton affinity. Three of the compounds investigated (midazolam, verapamil and propranolol) were the same as in this study (**I**) and for them anisole gave more or less the same results as those obtained here with toluene in RP-LC solvents. Anisole would be worth testing for a wider range of compounds and in analyses of compounds in biological and *in vitro* matrices.

Ionization efficiency in ESI was tested with the same ten compounds as used in APPI experiments with two solvent systems (Table 4). Most of the compounds ionized well in positive ESI producing intensive $[M+H]^+$. Aqueous 0.1% HCOOH gave 10-70% better sensitivity for all compounds as compared with the ammonium acetate-based solvent system and it was used in the final ESI method. One compound of the ten (hydrochlorothiazidine) was not ionized with ESI in either of the solvents tested.

Table 4. Solvents used for testing ionization efficiency in APPI and ESI (**I**).

APPI method	ESI method
1. ACN/water	1. ACN/water 0.1% HCOOH
2. MeOH/water	2. ACN/10mM NH_4OAc pH 3.7
3. ACN/water 0.1% HCOOH	
4. ACN/10mM NH_4OAc	

APPI and ESI both gave successful ionization efficiency for nine of the ten drug molecules tested, and for both techniques detection limits were very low, mostly below 5 nM (Table 5). Similar ionization efficiencies for pharmaceuticals with APPI and ESI are reported in the literature. Cai *et al.*[158] screened 86 known drugs and 201 drug candidates by APPI and ESI. For

the most part, APPI and ESI performed similarly, both detecting over 90% of the compounds. Despite the capability of APPI to ionize different drug molecules well, LC/APPI/MS applications for small drug molecules continue to be rare. A typical pharmaceutical APPI application in the literature is the analysis of steroids.[159]

Liquid chromatography

In *in vitro* permeability and metabolite assays, the most important task for the HPLC system is to separate the analyte from the HBSS and phosphate buffers, which may cause ion suppression and contaminate the ion source of the MS instrument. To avoid these undesired effects, non-volatile early eluting buffer components were diverted to waste before the mass analysis with a switching valve and flow from an auxiliary pump. The column switching also enabled the use of relatively high injection volumes (50 µl or 80 µl) improving LODs to the degree necessary for these analyse. The use of a switching valve in a one-column system is simple, and fast and easily automated and thus very suitable for higher throughput analysis. The switching valve has become routine for removing buffer impurities in *in vitro* permeability and metabolism experiments.[75, 101, 139]

A full chromatographic separation of compounds was not the aim in developing a fast n-in-one LC/MS/MS method for the structurally diverse mixtures. At maximum, two (**I**) or three (**II**) compounds eluted concurrently but were differentiated by MS/MS. Short columns used with high eluent flow rates provided very short run times. In study **I**, a monolithic fast column (4.6x50 mm) with 2 ml flow gave separation of nine compounds in 2.8 min (Figure 4). The total analysis time per sample was 5.5 min (APPI method) or 7 min (ESI method). In study **II**, a column (2.1x50mm) with a 3 µm particle size and 1.5 ml/min flow rate gave separation of the seven glucuronides within 3.5 min, total analysis time being 10 min (Figure 5). A fast linear gradient in place of isocratic elution gave narrower peak widths and improved resolution and S/N ratios. The use of a pre-column in this kind of higher throughput application was not reasonable since the benefits of a short column would them be lost. A steeper gradient (**II**) was applied, but the robustness of the method was poor after a few dozen injections due to the impaired chromatography. It may be concluded that a certain compromise between speed and reliability of the method is important.

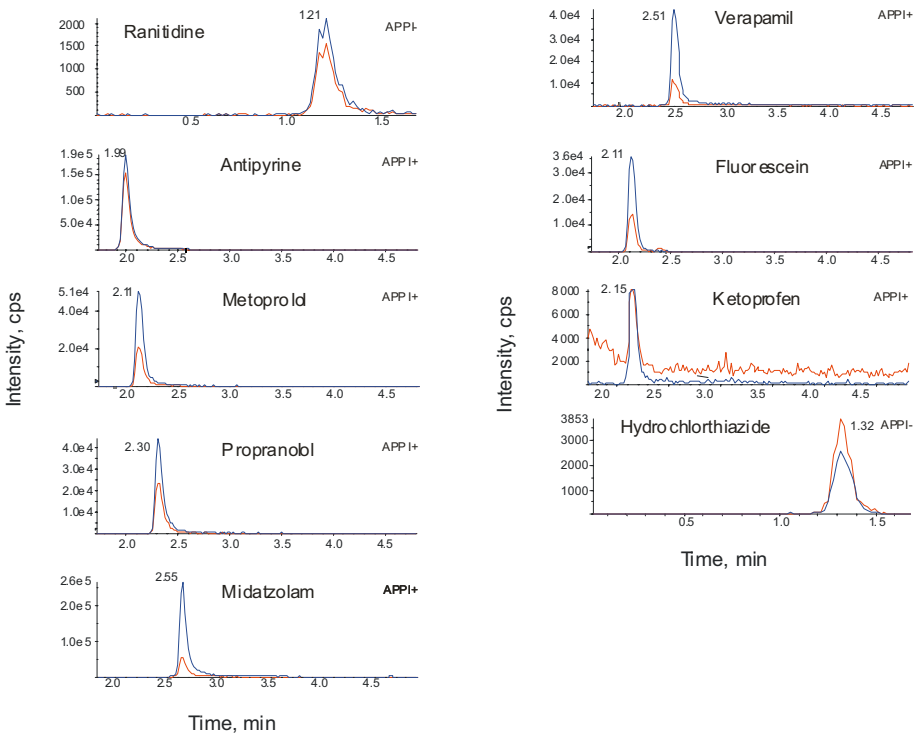

Figure 4. Extracted ion chromatograms obtained by APPI method for a typical cocktail sample with SRM.

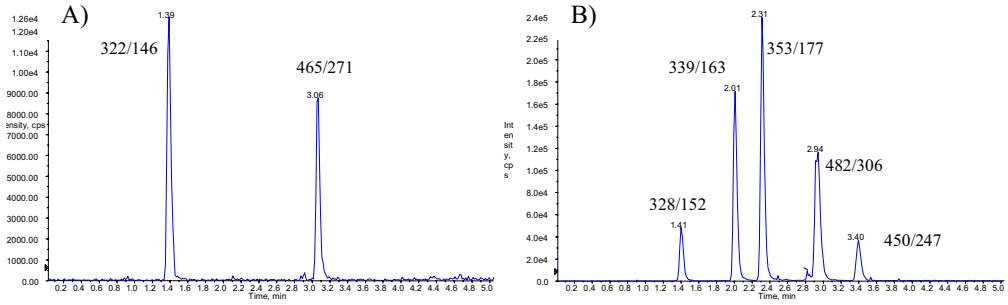

Figure 5. Extracted ion chromatograms and selected reaction monitored ion pairs for 10 nM standard containing A) glucuronides of 8-hydroxyquinoline (322/146) and estriol (465/271), and B) paracetamol (328/152), entacapone (482/306), umbelliferone (339/163), methylumbelliferone (353/177) and tolcapone (450/274).

Validation

LC/MS/MS methods for Caco-2 samples and samples from incubations with recombinant UGT enzymes were thoroughly validated. The validation step was extremely important because problems in the analytical part can result in significant quantification errors and thus major mistakes in calculating PK parameters. It was adequate to follow the guidelines for acceptable bioanalytical method validation.[160]

Table 5 shows the main validation results for the three LC/MS/MS methods developed in this work. Details of the validation results and their calculation can be found in the original publications **I-II**. Methods 1 and 2 in Table 5 are the ESI and APPI methods developed for nine drugs from permeability experiments (**I**), and method 3 is the ESI method developed for glucuronides from incubations with UGT isoenzymes (**II**).

The linearity of the response was studied from the limits of detection up to 50 000 nM (**I**) or up to 20 000 nM (**II**). The dynamic linear ranges obtained for the APPI method were typically 3-4 orders of magnitude, whereas ranges for the ESI methods were only 2-3 orders of magnitude. Dynamic linear range of 3-4 orders of magnitude for APPI has been reported also in literature.[161] The different ranges was the major difference between the APPI and ESI methods developed for Caco-2 samples (Table 5, methods 1 and 2). The wider dynamic linear range of APPI reduces sample handling because more analyses can be done with undiluted samples. That feature proved to be highly advantageous in the analysis of n-in-one cocktails containing compounds with different permeabilities. Because of the relatively wide dynamic linear range of APPI compared with ESI, APPI would also be recommended for the measurement of new drug candidates of unknown permeability. In study **I** the problem of narrow dynamic linear range of ESI was resolved through the dilution of samples, and in study **II** three calibration curves (2.5-250, 10-2000, 1000-7500 nM) were used. The correlation coefficients (r^2) of the calibration curves were higher than 0.995 for methods 2 and 3 and higher than 0.990 for method 1 (Table 5). Relative standard deviations (RSDs) for the individual data points were less than 20% in a few cases at limit of quantification but in other cases less than 15%. Theoretically, use of a calibration curve is not necessary in the determination of drug permeabilities since the permeability value is based on the ratio of the apical and basolateral samples, and MS responses can be used instead of concentrations for calculating permeability. Fung *et al.*[162] improved the throughput in Caco-2 analysis through elimination of the calibration curve and thus reduction of the number of samples. However, the method posits that apical and basolateral samples are in linear range, which is difficult to ensure, and thus the risk of false positive results exists.

Relative standard deviations (RSDs) in the within- and between-day experiments were typically less than 15 % which is acceptable for a bioanalytical method. The only exceptions were fluorescein and ketoprofen measured with the APPI method: RSD values of fluorescein varied from 15.9 to 28.6 and those of ketoprofen from 17.0 to 18.2 (Table 5). One reason for the large RSD values could be low sensitivity of these compounds in APPI mode. In addition, in the case of fluorescein, even small changes in pH may result in change in ionization state of some of

the multiple ionizing functional groups. An alternative, fast and more reliable method for the quantification of fluorescein is the fluorescence-based method which is applied in study **IV**.

Table 5. Validation results obtained for the three LC/MS/MS methods described in papers **I** and **II**.

	Linearity (nM)	R^2	LOD (nM)	Between-day repeatability RSD% (n=4)	Within-day repeatability RSD% (n=6) at three concentration levels		
1. ESI METHOD for drugs (I)							
antipyrine	50-1000	0.9964	10	7.5	6.4	3.1	5.4
fluorescein	5-1500	0.9990	2	6.5	17.5	5.3	4.7
ketoprofen	10-1500	0.9940	8.5	2.8	1.9	3.9	3.0
metoprolol	5-500	0.9958	1.2	10.2	12.3	3.1	2.7
midazolam	5-1000	0.9906	0.2	7.3	8.0	4.4	2.6
propranolol	5-500	0.9962	0.8	2.8	10.8	2.1	2.1
verapamil	5-1000	0.9984	0.4	9.7	3.1	2.0	3.0
ranitidine	50-1000	0.9900	0.2	11.3	9.3	4.4	4.1
cephalexin	50-1000	0.9932	10	6.3	5.2	5.4	6.0
2. APPI METHOD for drugs (I)							
antipyrine	10-5000	0.9996	10	7.8	10.7	6.8	2.1
fluorescein	50-50 000	0.9970	50	12.2	28.6	15.9	26.5
ketoprofen	100-7500	0.9990	100	9.0	18.2	17.6	17.0
metoprolol	50-10 000	0.9980	2.2	4.5	18.4	3.7	2.3
midazolam	5-5000	0.9940	1.4	2.9	7.1	4.6	2.6
propranolol	7.5-7500	0.9982	1.4	2.4	13.5	4.3	4.3
verapamil	5-2500	0.9984	0.8	4.2	7.4	3.4	2.9
ranitidine	5-5000	0.9978	1.2	4.9	7.4	5.4	3.9
hydrochlorothiazide	5-25000	0.9980	4	8.8	8.2	7.5	5.1
3. ESI METHOD for glucuronides (II)							
entacapone-3-O-glucuronide	10-2000	0.9980	0.5	3.4	6.5	5.3	4.6
umbelliferone glucuronide	5-2000	0.9988	0.5	3.0	14.5	6.5	7.2
4-methylumbelliferone glucuronide	10-2000	0.9968	0.5	6.0	12.3	6.7	8.1
tolcapone-3-O-glucuronide	25-10 000	0.9952	1	3.0	3.7	2.7	5.1
hydroxyquinolin glucuronide	10-2000	0.9960	0.5	6.6	6.8	3.4	5.2
estriol-17β-glucuronide	10-5000	0.9968	5	12.7	7.0	5.1	4.6
paracetamol glucuronide	10-2000	0.9962	0.5	8.2	13.1	10.4	5.7

Limits of detection (LOD) were less than 5 nM for nearly all compounds with all methods. The lowest limit of quantitation (LOQ) varied from 2.5 to 50 nM with the ESI methods and from 5 to 100 nM with the APPI method (I). The especially sensitive LC-ESI/MS/MS method was developed for quantification of glucuronides (II). LODs were less than 1 nM and LOQs less than

2.5 nM for nearly all glucuronides. Also in the literature ESI is characterized as an ionization technique which ionizes polar glucuronides extremely well. Keski-Hynnilä *et al.* found ESI to be a better ionization method than APPI and APCI for polar glucuronides of three catechols.[163]

Sensitivity of the methods developed in this work is good compared with that of corresponding methods reported in the literature. For example, LOQs of the LC/MS/MS methods developed for Caco-2 samples (**I**) are at the same level as LOQs in the most sensitive method in the literature.[164] There are several advantages of low LOQs in permeability and metabolic stability studies of drugs. Determination of permeability of low permeating compounds and intrinsic clearance of slow metabolizing compounds is feasible. The possibility to use low initial concentrations in experiments also minimizes drug-drug interactions in both permeability and metabolism studies and typically represents a more realistic physiological concentration of drug exposure. Low initial drug concentrations are also safer for the Caco-2 cell line and allow the use of smaller inserts in cocktail studies, as reported in paper **IV**. Initial concentration of individual compounds in the Caco-2 permeability experiments was 50 µM (**I**), and the total drug concentration in cocktail experiments was 500 µM. Palmgren *et al.*[79] developed a UV/fluorescence based method for a cocktail of seven drugs for Caco-2 permeability experiments. Initial concentrations of the individual compounds varied from 20-500 µM, depending on the drug transport mechanism, and the total concentration in cocktail experiments was 1100 µM. With use of LC/MS/MS it is not necessary to modify drug concentrations according to the expected transportation of the compound or its sensitivity to the analytical method, and low initial concentrations can also be used for slowly permeating samples.

In conclusion, all three LC/MS/MS methods provided successful validation data for the bioanalytical method. Furthermore, in the case of Caco-2 studies, the permeability values (P_{app}) for the studied compounds obtained by APPI and ESI methods were very similar (Table 6), demonstrating that either method suitable for rapid permeability testing.

6.1.2 Transport of compounds across Caco-2 cell monolayer (I,IV)

To achieve good reliability of Caco-2 experiments and to enable a comparison of the results obtained in different laboratories, it is important to characterize the permeability properties of the Caco-2 cell monolayers by using reference compounds with known transport properties. The applicability of different mixtures of reference compounds was investigated in studies **I** and **IV**. The cocktail in the Caco-2 experiments of paper **I** included ten compounds, eight of which are suggested by the FDA[165] for the evaluating suitability of an *in vitro* method for the determination of drug permeabilities. Representatives of various paracellular and transcellular absorption mechanisms were used: high passive permeability (antipyrine, metoprolol and midazolam), low to moderate passive permeability (hydrochlorothiazide, ranitidine), active monocarboxylic acid transport (ketoprofen and fluorescein), active dipeptide transport (cephalexin), and active efflux (verapamil and propranolol). The cocktail in the second

study with caco-2 cell line (**IV**) included seven model drugs (antipyrine, metoprolol, ketoprofen, verapamil, ranitidine, hydrochlorothiazide, fluorescein).

Single compound versus cocktail experiments

The permeabilities measured for compounds in the cocktail of ten compounds correlated closely with those obtained in the single compound experiments (Table 6) (**I**). Note that most of the single results are from earlier work and obtained using a different cultivation batch of Caco-2 cells.[80] The good correlation between cocktail and single results also applies to the data of study **IV** where a cocktail of seven drugs was used. Even though sink conditions (i.e. overall amount transported is >10% of the amount in the donor solution) were lost in the cocktail experiments with highly permeable compounds (**IV**), the rank ordering of compound permeability and the classification into low and high permeability compounds remained the same for the single and cocktail studies, and values of 12- and 24- well formats were directly comparable. The P_{app} of ranitidine, which is able to cause a concentration-dependent decrease in its own permeability,[166] indicated a slightly lower permeability in the cocktails in both studies (**I, IV**).

Table 6. Apparent permeability coefficients ($P_{app} \cdot 10^6$ cm/s) for the studied compounds as single compounds and in a mixture of ten compounds.* Ref 80 n.d. not determined.(**I**)

Compound	$P_{app} \cdot 10^6$ cm/s Single sets		$P_{app} \cdot 10^6$ cm/s In cocktail	
	APPI	ESI	APPI	ESI
antipyrine	n.d.	52.2 ± 2.5*	37.1 ± 0.7	39.3 ± 2.5
fluorescein	24.5 ± 0.73	30.2 ± 1.2	27.5 ± 1.7	26.3 ± 0.7
hydrochlorothiazide	0.394 ± 0.025	n.d.	0.460 ± 0.030	n.d.
cephalexin	n.d.	1.53 ± 0.13*	n.d.	1.22 ± 0.07
ketoprofen	n.d.	81.6 ± 4.3*	63.2 ± 4.1	74.8 ± 4.1
metoprolol	4.14 ± 0.17	3.77 ± 0.16	6.72 ± 0.12	6.89 ± 0.19
midazolam	n.d.	14.8 ± 1.5*	16.8 ± 0.5	17.7 ± 0.4
propranolol	n.d.	2.02 ± 0.08*	4.19 ± 0.17	3.80 ± 0.09
ranitidine	0.368 ± 0.014	0.402 ± 0.047	0.243 ± 0.017	0.301 ± 0.032
verapamil	n.d.	1.82 ± 0.18*	1.92 ± 0.16	2.63 ± 0.15

The suitability of seven reference compounds for the standardization of the cell line was thoroughly investigated in study **IV**. Single and cocktail experiments were performed with pH gradient (pH 5.5 vs. 7.4) and without pH gradient (iso-pH 7.4) and in 12- and 24-well formats. Rank order correlation (order of compounds according to P_{app} values) and the permeation mechanism of the compounds were case-specifically discussed.

The rank order of the compounds was different in iso-pH 7.4 and pH-gradient conditions due to changes in ionization and the permeability of the compounds. Under pH-gradient conditions the margin between high and low permeability compounds was narrower, and the limiting P_{app} value classifying the compounds into low and high permeability classes was lower with the pH gradient ($1x10^{-6}$ cm/s) than under iso-pH 7.4 conditions ($20x10^{-6}$ cm/s).

With ketoprofen eliminated from the calculations, high correlation between the 12- and 24-well formats was found at iso-pH 7.4 and also in pH-gradient conditions. The correlation coefficients (r^2) between 0.33 cm^2 and 1.1 cm^2 monolayer inserts were 0.904 - 0.999 and the corresponding slopes of the plots were close to one (without ketoprofen). A possible reason for the poor correlation for ketoprofen could be variable expression of anion transporters (MCT) between the passages in the Caco-2 cells and/or the poor analytical precision for ketoprofen (>15%).

Both ketoprofen and fluorescein are substrates for MCT.[167] In the pH-gradient conditions, the transport of fluorescein was decreased from $17x10^{-6}$ cm/s to $6x10^{-6}$ cm/s in the presence of ketoprofen, suggesting a possible interaction. Similar inhibitory effects between ketoprofen and fluorescein were not observed (paper **I)**. However, variable expression of monocarboxylic acid transporter (MCT) transporters between the Caco-2 cell batches that were used might explain the inconsistency in the results. In any event, the use of ketoprofen and/or fluorescein as internal standards in the presence of other anionic compounds is risky in pH-gradient conditions.

Verapamil was indicated as a good marker for P-glycoprotein (P-gp) as also suggested by the FDA. However, the results varied widely between replicates and Caco-2 cell batches. More specific and sensitive markers for P-gp should perhaps be found therefore.

Of the seven compounds studied (**IV**), antipyrine, metoprolol and hydrochlorothiazide, which are high or moderate passive transporters, had very repeatable permeability characteristics and can be highly recommended for the evaluation of Caco-2 cell monolayers.

6.1.3 Determination of metabolic clearance (Cl_{int}) (II)

Determination of Cl_{int} values

Intrinsic clearance ($CL_{int} = V_{max}/K_m$) is typically determined by deriving the enzyme kinetic parameters V_{max} and K_m from the Michaelis-Menten curve (CL_{int} (M-M)). The CL_{int} value can also be determined as the slope of the linear part of the Michaelis-Menten fitting (CL_{int} (slope)). The latter approach was applied in study **II** to increase throughput in metabolism studies of seven substrates (entacapone, 17β-estriol, umbelliferone, 4-methylumbelliferone, tolcapone, hydroxyquinoline and paracetamol) with UGT isoenzymes. The CL_{int} (slope) values were compared with CL_{int} (M-M) values.

Concentration of the substrate must be well below its K_m value when Cl_{int} is determined as a slope of the linear part of the Michaelis-Menten fitting. A highly sensitive LC/MS/MS analysis enables the use of low initial substrate concentrations. The incubation conditions in this study

were not optimized separately for each experiment because the interest was increased throughput in drug discovery. On the other hand, it is impossible to select universal incubation parameters, such as substrate concentration, incubation time and protein concentration, for all the experiments, since enzyme activities can vary by several orders of magnitude in different substrates. In this work the substrate concentrations were either 0.5-2.5 μM or 5-25 μM since earlier work[168] suggested that the K_m values for the UGTs were expected to be higher. The consumption of the substrate, which is controlled by incubation time and protein concentration, should not be more than 10% because the Michaelis-Menten equation assumes that the substrate concentration is constant during the reaction. The protein concentration has to be well below the concentration of the substrate, but still high enough to allow a detectable product formation. The incubation time is also critical because the reaction rate as a function of the reaction time is linear only up to a certain point of time, and too long incubation may result in underestimation of the Cl_{int}. However, the incubation time had to be long enough for a detectable amount of metabolite is formed.

The linearity of the plots of substrate concentration [S] versus glucuronidation rate was sufficient for the compounds studied, correlation coefficients (r^2) being > 0.94. The correlation between CL_{int} (slope) and CL_{int} (M-M) values was very good (variation being less than three-fold), when the substrate consumption in the reactions determining CL_{int} (slope) values was below 10%. In cases where the substrate consumption was higher than 10%, the CL_{int} (slope) values were typically 5-20 times lower than the CL_{int} (M-M) values, indicating that too high substrate consumption caused underestimation of the CL_{int} values (Table 7). In the incubations determining CL_{int} (slope) values for 8-hydroxyquinoline with UGTs 1A1, 1A6, and 1A9, the substrate concentrations (5-25 μM) were well above the K_m values (Table 7). This resulted in significantly reduced CL_{int} (slope) values in comparison with the CL_{int} (M-M) values. The too high substrate consumptions and too high initial substrate concentrations in the incubations were recognized as clearly increased intercept values in the plots of substrate concentration [S] versus glucuronidation rate V (Table 7). Accordingly, the intercept value can be used as an indicator of the validity of the experimental conditions, and in cases where the intercept is clearly increased the experiment should be repeated under acceptable conditions. It should be noted that intercept values give only an estimation and values from should different experiments should not be compared because the magnitude of the CL_{int} values varies strongly with the substrate and enzyme.

Table 7. Comparison of Cl_{int} (slope) values determined as slope of the linear portion of the Michaelis-Menten (M-M) curve and V_{max}/K_m ratios calculated from kinetic parameters (V_{max} and K_m), determined in M-M fittings. (**II**)

		Michaelis-Menten parameters			Determination of CL_{int} as slope of the linear part of the M-M fitting			
Substrate	enzyme	K_m, µM	V_{max}, pmol/min/mg	V_{max}/K_m, µl/min/mg	Cl_{int} (slope), µl/min/mg	intercept	R^2	Substrate consumption %
4-Methylumbelliferone	1A1	115	459	4.0	2.63	+0.96	0.995	<10
Umbelliferone	1A1	294	462	1.6	2.87	-0.75	0.994	<10
Entacapone	1A3	259	622	2.40	2.58	+0.27	0.927	<10
8-Hydroxyquinoline	1A3	494	22	0.045	0.05	+0.03	0.984	<10
4-Methylumbelliferone	1A3	335	989	3.0	1.21	+0.02	0.996	<10
8-Hydroxyquinoline	1A6	6.5	705	108	9.03	+74.3	0.972	44
4-Methylumbelliferone	1A6	157	7870	50	9.23	+51.3	0.968	37
Entacapone	1A9	21	10171	351	29.10	+2.37	0.999	100
8-Hydroxyquinoline	1A9	0.48	49	102	12.68	+0.41	0.987	35
4-Methylumbelliferone	1A9	18	1388	77	10.76	+3.15	0.974	42
umbelliferone	1A9	111	2282	21	9.41	+2.26	0.956	30
8-Hydroxyquinoline	2B7	301	371	1,2	0.33	+0.28	0.987	<10
8-Hydroxyquinoline	2B15	16	33	2,1	0.31	+0.31	0.988	<10
4-Methylumbelliferone	2B15	149	40	0,27	0.071	+0.24	0.967	<10
Umbelliferone	2B15	339	25	0,074	0.032	+0.03	0.985	<10

Single-substrate versus n-in-one incubations

Intrinsic clearances of seven substrates (entacapone, 17β-estriol, umbelliferone, 4-methylumbelliferone, tolcapone, hydroxyquinoline and paracetamol) were determined as a mixture of the substrates and in single incubations. Since cross effects in n-in-one incubations can be minimized by using as low substrate concentrations as possible, the concentrations employed were well below the K_m values. The Cl_{int} (slope) values obtained from the mixture incubations correlated well (r^2 = 0.9155) with the values obtained in single-compound incubations (Figure 6). The results give no indication of cross effects between substrates and clearly show that n-in-one experiments are viable if the substrate concentrations are kept sufficiently low (<expected K_m).

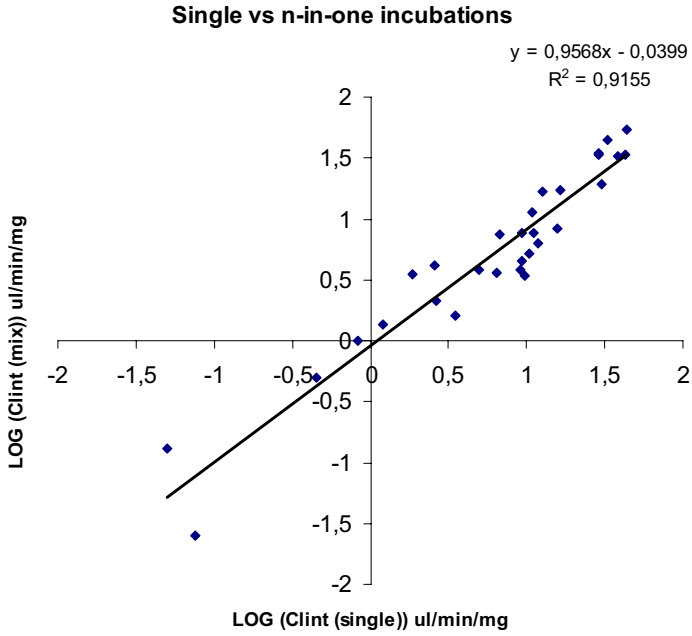

Figure 6. Correlation between single (Cl_{int} (single)) and n-in-one (Cl_{int} (mix)) incubations of seven substrates with six isoenzymes (**II**).

6.2 Metabolite profiling of biological matrices (III, V)

The aim in study **III** was to test the suitability of different mass spectrometric instruments (QqQ, Q-TOF, Ion Trap) and two commercially software packages for metabolite profiling of a relatively complex biological matrix. Search was made in urine for metabolites of tramadol, whose metabolism is previously well known (Figure 7).[169,170] In study **V** the aim was to identify metabolites of sibutramine formed in rat and human hepatocytes. Furthermore, enantioselective formation of sibutramine was investigated.

Metabolite ID (Sciex, ThermoFinnigan) and Metabolynx (Micromass) sofwares were used to search for expected metabolites (a table-dependent search) in an LC/MS run of the urine or hepatocyte sample. The default transformation table of each package was supplemented to include several other theoretically possible metabolites of drugs, based on a general understanding of drug metabolism pathways, the structure and the literature. Search was made for the expected tramadol and sibutramine metabolites as protonated molecules $[M+H]^+$. Sibutramine data was also processed for ammonium adducts of metabolites $[M+NH_4]^+$ since our previous studies of sibutramine metabolism showed a few sibutramine metabolites that appeared intensively as ammonium adducts.

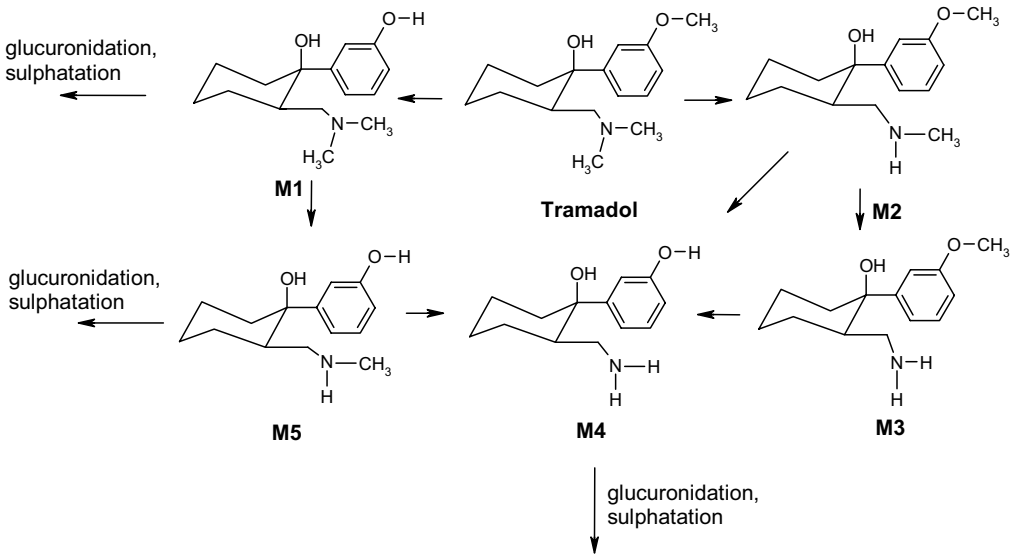

Figure 7. Main metabolism of tramadol in human.

Evaluation of software for metabolite profiling

In study **III**, the applicability of the software packages (Metabolite ID) for QqQ and (Metabolynx) for Q-TOF was thoroughly tested in search for metabolites in urine. The full scan spectra of control urine and tramadol urine samples were processed to identify expected metabolites (a table-dependent search) and non-expected metabolites (searching without a list of potential metabolites). First, full scan mass spectra were processed with different processing parameters in order to find optimal parameters for a complex urine sample where metabolites were present in different concentrations. In the case of the QqQ instrument, the threshold and sample/control parameters had a marked effect on the number of transformations found, as shown in Table 8. High values of the parameters resulted in a risk of losing many real tramadol metabolites, whereas too low values resulted in many false positives. Optimized processing parameters with the QqQ (threshold 10 000, sample/control ratio 3) and searching only for expected metabolites gave a reasonable amount of data with low risk of losing any expected metabolite. In the case of accurate mass data from Q-TOF, optimal processing parameters were easier to find and the effect of parameters on the amount of data was less significant than with QqQ. However, signal intensities of the detected ions and their ratios to the signal intensity of the lock mass peak were critical in determining the accurate mass. Optimal intensity range for both was 80-300 cps.

A search of the accurate mass measurement data for the expected metabolites by Metabolynx (Q-TOF) resulted in 24 hits, of which none proved to be false-positive. The result was the same whether or not comparison with the control sample was carried out, as useful result in *in vivo* metabolite profiling since authentic biological control samples are not always available.

A search for the non-expected metabolites resulted in a large number of candidates not only with QqQ data (Table 8) but also with accurate mass data. Searching for non-expected

metabolites may be reasonable only for metabolites present in very high concentration or in a cleaner matrix, an *in vitro* incubation matrix, for example.

Table 8. Effect of processing parameters on the number of tentative metabolites found with Metabolite ID (QqQ). The optimized parameters are in bold. (**III**)

Processing parameters		**Number of hits obtained with different searching options**			
Threshold	Sample/ control	Non-expected, comparison with control sample	Expected, comparison with control sample	Expected, no comparison with control sample	Number of true metabolites detected*
500 000	10	38	23		19
500 000	3	61	31		20
100 000	3	133	44		23
50 000	3	180	44		23
10 000	10	39	36		19
10 000	5	103	31		21
10 000	**3**	**162**	**51**		**25**
10 000	1	430	93		25
100 000				95	23
10 000				98	25

* false positive hits were eliminated after manual processing of full scan data or acquisition and interpretation of product ion spectra.

Identification of tramadol metabolites

Neutral loss, precursor ion and product ion scan modes of the triple quadrupole instrument and accurate mass measurement by Q-TOF were applied for identification of the metabolites of tramadol. MS^n of the ion trap was tested, but it proved useless because the low mass cut off of the ion trap eliminated the most informative product ions of tramadol ($[CH_2NHCH_3]^+$ at m/z 44 and $[CH_2N(CH_3)_2]^+$ at 58).

Precursor and neutral loss scan modes proved to be highly useful: 19 tramadol metabolites were identified in urine. On the basis of the product ion spectrum of tramadol and the assumption that metabolites fragment in the same way as the parent drug, ions at m/z 58 $[CH_2N(CH_3)_2]^+$ and m/z 44 $[CH_2NHCH_3]^+$ were chosen as product ions in the precursor ion scan mode for the tramadol-like and the demethylated metabolites, respectively. Neutral loss scans of 176 and 80 Da were used for the selective detection of glucuronide and sulphate conjugates, respectively.

Accurate mass measurement with the Q-TOF gave mass accuracy typically better than 20 ppm (Table 9). The concentrations of metabolites with low mass accuracy (>5 ppm) were close to the detection limit, and the signal intensity of those peaks in the mass spectra was not sufficient for an accurate mass analysis. Accordingly, they were considered uncertain metabolites.

Table 9. Analysis of a urine sample for tramadol and its metabolites with Q-TOF. Lock mass was m/z 268.1913 ($[M+H]^+$ of propranolol). (**III**)

Proposed metabolite	Transformatiom	Mass of $[M+H]^+$	Calc.mass of $[M+H]^+$	Error (ppm)	Formula of metabolite
Tramadol	unchanged	264.1968	264.1964	1.8	C16H25NO2
M1	desmethylation	250.1845	250.1807	15.3	C15H23NO2
M2	desmethylation	250.1818	250.1807	4.5	C15H23NO2
M5	didesmethylation	236.1637	236.1651	-5.7	C14H21NO2
M6	hydroxylation	280.1932	280.1913	6.6	C16H25NO3
M31	oxidation	280.1990	280.1913	27.8	C16H25NO3
M32	hydroxylation +desmethylation	266.1772	266.1756	6.1	C15H24NO3
M9	hydroxylation +dehydrogenation	278.1819	278.1756	22.8	C16H23NO3
M34	hydroxylation+ didesmethylation	252.1612	252.1600	5.0	C14H21NO3
M12	glucuronidation	440.2238	440.2284	-10.5	C22H33NO8
M13	desmethylation+ glucuronidation	426.2050	426.2128	-18.3	C21H31NO8
M2Glu	desmethylation+ glucuronidation	426.2004	426.2128	-29.1	C21H31NO8
M15	didesmethylation+ glucuronidation	412.1933	412.1971	-9.3	C20H30NO8
M6Glu	hydroxylation+ glucuronidation	456.2228	456.2234	-1.2	C22H33NO9
M6Glu	hydroxylation+ glucuronidation	456.2178	456.2234	-12.2	C22H33NO9
M32Glu	hydroxylation	442.2025	442.2077	-11.7	C21H31NO9
M32Glu	+desmethylation	442.1977	442.2077	-22.6	C21H31NO9
M32Glu	+glucuronidation	442.2089	442.2077	2.8	C21H31NO9
M17	hydroxylation	442.2185	442.2077	24.5	C21H31NO9
M17	+desmethylation +glucuronidation	442.2073	442.2077	-0.9	C21H31NO9
M34Glu	hydroxylation+ didesmethylation+	428.1742	428.1921	-41.7	C20H29NO9
M34Glu	glucuronidation	428.2057	428.1921	32.0	C20H29NO9
M22	didesmethylation+ sulphonation	316.1297	316.1219	24.9	C14H21NO5S

The product ion spectra of the protonated molecules obtained with QqQ allowed characterization of the structures of most of the metabolites. Demethylation, oxidation, hydroxylation, and dehydrogenation, as phase I metabolic reactions, were found for tramadol. Metabolites formed via demethylation (M1, M2) and di-demethylation (M5), as well as metabolites formed via hydroxylation and demethylation (M32) and via hydroxylation and di-demethylation (M34), were identified from the very intense diagnostic ions at m/z 44 or m/z 58. Two metabolites (M6 and M31) showed protonated molecules at m/z 280. Hydroxylation and oxidation (+16) are theoretically possible metabolic transformations. Structural identification of these metabolites exclusively on the basis of product ion spectra was not possible. However, metabolite M31 may be a N-oxide since MS/MS gave diagnostic product ions at m/z 58 and m/z

74, representing $[CH_2N(CH_3)_2]^+$ and N-oxide ion $[CH_2N(CH_3)_2O]^+$, respectively. The similarity of the product ion spectrum of this metabolite and that of the tramadol-N-oxide in the literature supports this suggestion of the structure.[169] Reaction for the first metabolite (M6) showing a protonated molecule at m/z 280 is probably hydroxylation. In addition, a clearly shorter retention time for this metabolite than for M32 indicates the presence of higher polarity (two hydroxyl groups) in the structure. The metabolite showing a protonated molecule at m/z 278 (M9 with retention time of 4.72 min) is the only metabolite that does not show product ions at m/z 58 or m/z 44. However, its product ion at m/z 219 represents a loss of $CH_3N(CH_3)_2$ from the protonated molecule, and the product ion at m/z 201 represents a further loss of H_2O. A structurally possible metabolic pathway for this metabolite is oxidation and followed by dehydrogenation.

Fourteen glucuronide conjugates and one sulphate conjugate were identified as phase II metabolites. The product ion spectra of all these conjugates showed diagnostic product ions at m/z 44 and/or m/z 58 (Figure 8). In addition, the loss of glucuronic acid moiety or sulphate was informative for confirmation of the structures of conjugates that were of low intensity and, owing to the low sensitivity of the neutral loss scan, were not found by a neutral loss of 176 Da.

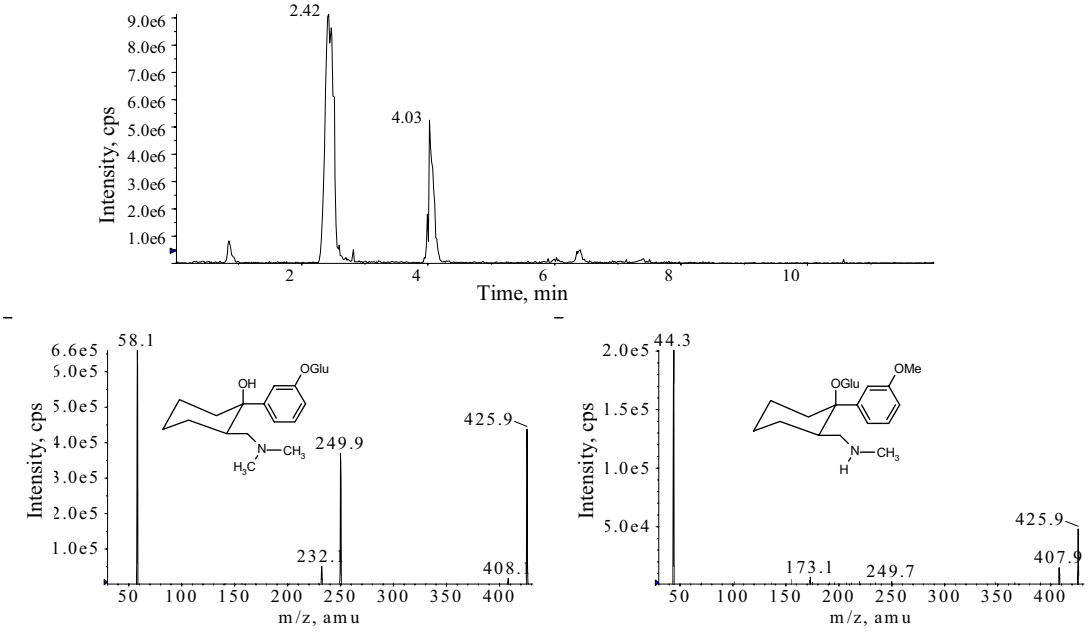

Figure 8. Extracted ion chromatogram for m/z 426 and product ion spectra of the precursor ion at m/z 426 for tramadol metabolites M1Glu and M2Glu.(**III**)

Identification of sibutramine metabolites

MS^2 and MS^3 scan modes of the ion trap instrument were used for identification of sibutramine metabolites. Twenty-three metabolites formed via demethylation, hydroxylation, dehydrogenation, acetylation, attachment of CO_2 and glucuronidation (Figure 9) were characterized even though the position of functionality, mostly hydroxylation, was not always unambiguous.

Synthetic standards of M1, M2, and M7 were used for identification of the metabolites. The metabolite M1 is formed by demethylation, M2 by further demethylation of M1, and M7 by hydroxylation of M2 at the methyl group of the hydrocarbon chain.

Metabolites M8-M10 showed $[M+H]^+$ at m/z 268 or m/z 282 (+16) indicating hydroxylated products of M1 or M2, respectively. The protonated molecule of M9 (at m/z 282) fragmented similarly to that of standard M7, suggesting that hydroxylation of metabolite M9 occurs at the methyl group of the hydrocarbon chain. In the case of M8 and M10, the position of the hydroxylation was more ambiguous. However, abundant $[M+H-H_2O-(CH_3)_2C=CH_2]^+$ ions in the product ion spectra show that hydroxylation occurs somewhere in the hydrocarbon chain. A resonance stabilized ion $[M+H-H_2O-(CH_3)_2C=CH_2]^+$ is a possible structure when hydroxylation occurs in the hydrocarbon chain and the hydroxyl group is lost, followed by the loss of $(CH_3)_2C=CH_2$ moiety (Figure 10).

Metabolites M11 and M12 showed $[M+H]^+$ at m/z 284 (+32), indicating dihydroxylated products of M2. The product ion spectra of the protonated molecules of M11 and M12 show ions at m/z 231, formed by the losses of ammonia and two water molecules and confirming that these metabolites contain two hydroxyl groups. The similarity of the fragmentations of M11 and M7 suggests that M11 is a hydroxylated product of M7. In contrast, M12 showed the abundant resonance stabilized product ion $[M+H-H_2O-(CH_3)_2C=CH_2]^+$ at m/z 210, which is formed analogously to that at m/z 194 in the case of M8 (Figure 10). Thus the same conclusions can be applied to M12, i.e. the hydroxylation occurs in the hydrocarbon chain, but not to the methyl groups or the other hydroxyl group at the cyclobutyl ring or chiral carbon.

On the basis of the product ion spectra, dihydroxylation and dehydrogenation of M1 or M2 is proposed as the metabolic route for the formation of metabolites M13-M15 (Figure 11 a-c). The product ion spectrum of $[M+H]^+$ of metabolite M13 (at m/z 296) shows $[M+H-CO]^+$ (at m/z 268), $[M+H-CH_3OH]^+$ (at m/z 264), $[M+H-CH_3OH-NH_3]^+$ (at m/z 247) and $[M+H-CH_3OH-NH_3-CO]^+$ (at m/z 219), which suggests hydroxylation of the methyl of the amino group and the carbonyl group at the end of the hydrocarbon chain (Figure 11 a). The product ion spectrum of $[M+H]^+$ of M14 shows ions $[M+H-H_2O]^+$ (at m/z 278) and $[M+H-CO]^+$ indicating the presence of hydroxyl and carbonyl groups. The ion $[M+H-(CH_3)_2C=CH_2]^+$ (at m/z 240) indicates that the hydroxyl group is not located in the alkyl chain but either at the cyclobutyl ring or at the chiral carbon. The intense product ion at m/z 233 is formed by loss of water followed by the loss of NH_2COH, indicating hydroxylation of the methyl at the amino group followed by the dehydrogenation of the hydroxyl group (Figure 11 b). The product ion spectrum of $[M+H]^+$ of

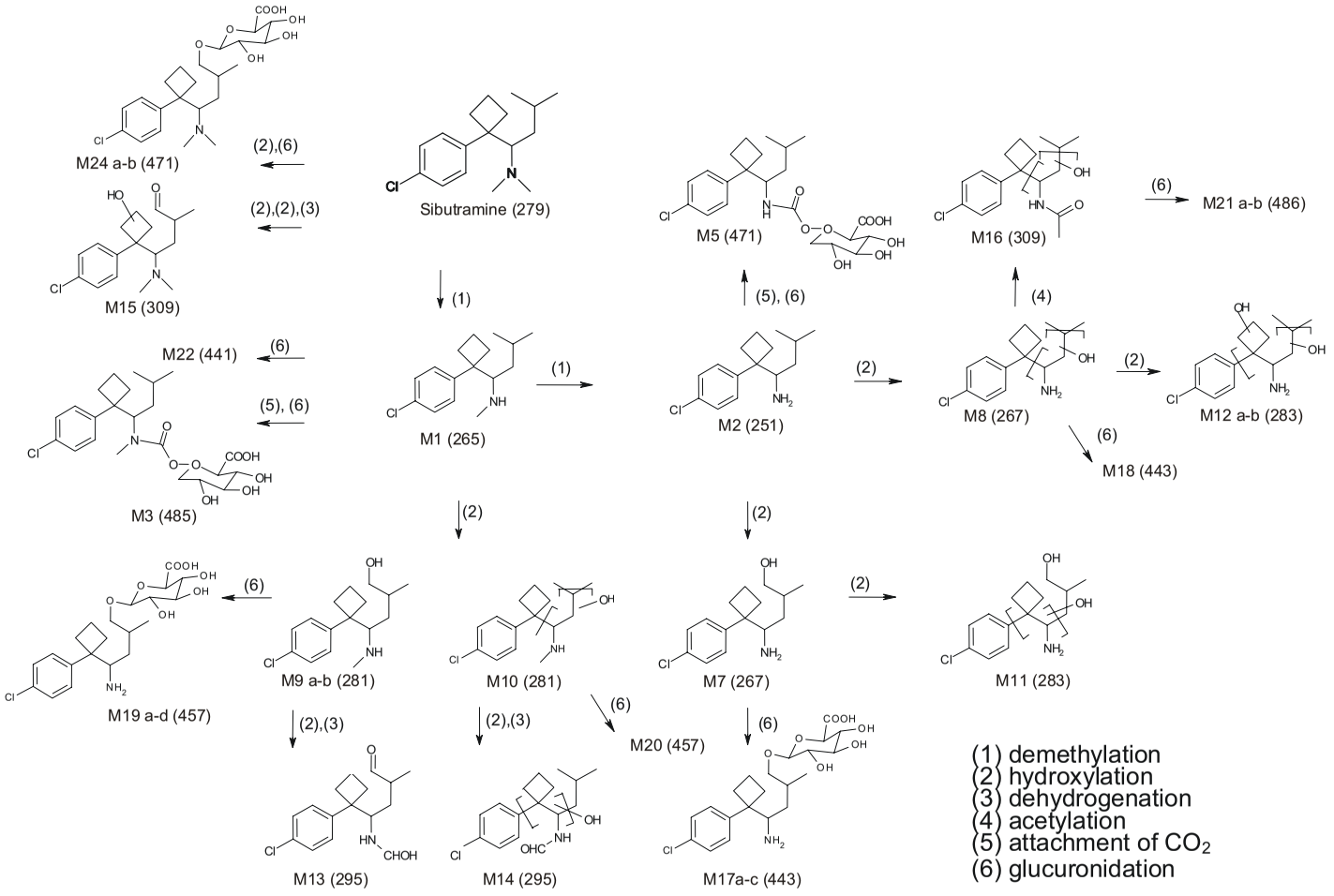

Figure 9. Proposed metabolism of sibutramine in primary cultures of rat hepatocytes. The molecular weight (g/mol) is given in parentheses after the metabolite name.

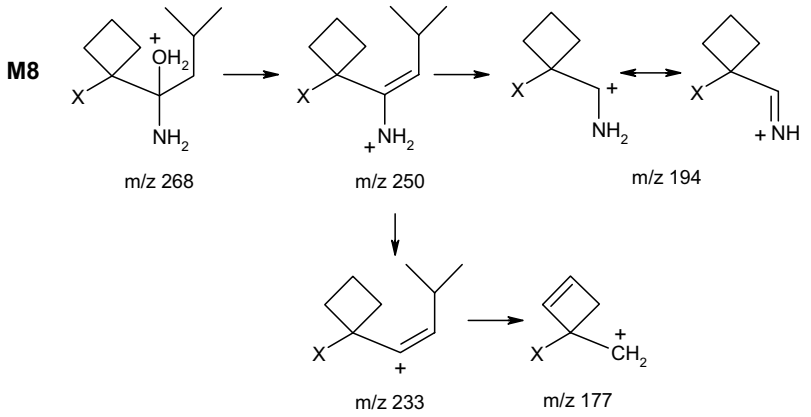

Figure 10. Proposed formation of the main ions in the product ion spectrum of $[M+H]^+$ of metabolite M8. (**IV**)

metabolite M15 (at m/z 310) showed ion $[M+H-H_2O]^+$ (at m/z 292), indicating that M15 includes one hydroxyl group (Figure 11 c). The product ion $[M+H-H_2O-(CH_3)_2NH_2]^+$ (m/z 247) shows that M15 includes a dimethylamine moiety that is not hydroxylated. The product ion at m/z 219 is formed from m/z 247 through the loss of CO and that at m/z 191 from m/z 219 through the loss of CH_2CH_2. These ions and the similarities in the fragmentations of M13 and M15 suggest that the carbonyl is located at the end of the alkyl chain.

The product ion spectrum of the protonated molecule of M16 suggests that M16 is formed from M2 by hydroxylation of the alkyl chain and acetylation of the amino group. An intense product ion $[M+H-H_2O]^+$ at m/z 292 confirms the presence of hydroxyl in the structure, and the product ion $[H_2O+(CH_3)_2C=CH_2]$ at m/z 236 indicates hydroxylation of the alkyl chain. The product ion at m/z 233 is probably formed by the loss of NH_2COCH_3 after loss of water, suggesting acetylation of the amino group (Figure 11 d).

Ten different glucuronide conjugates were identified for sibutramine. In addition, for most of the glucuronides two or more enantiomers with similar mass spectrometric behaviour were found. Mass spectrometric and chromatographic behaviour of glucuronides M3-M5 showed them to be the same carbamoyl glucuronides as found and identified in our previous studies in human urine.[171] Six other glucuronides (M17-M22) were mainly identified from MS^3 spectra, which were similar to MS^2 spectra of protonated molecules of corresponding phase I or acetylated metabolites.

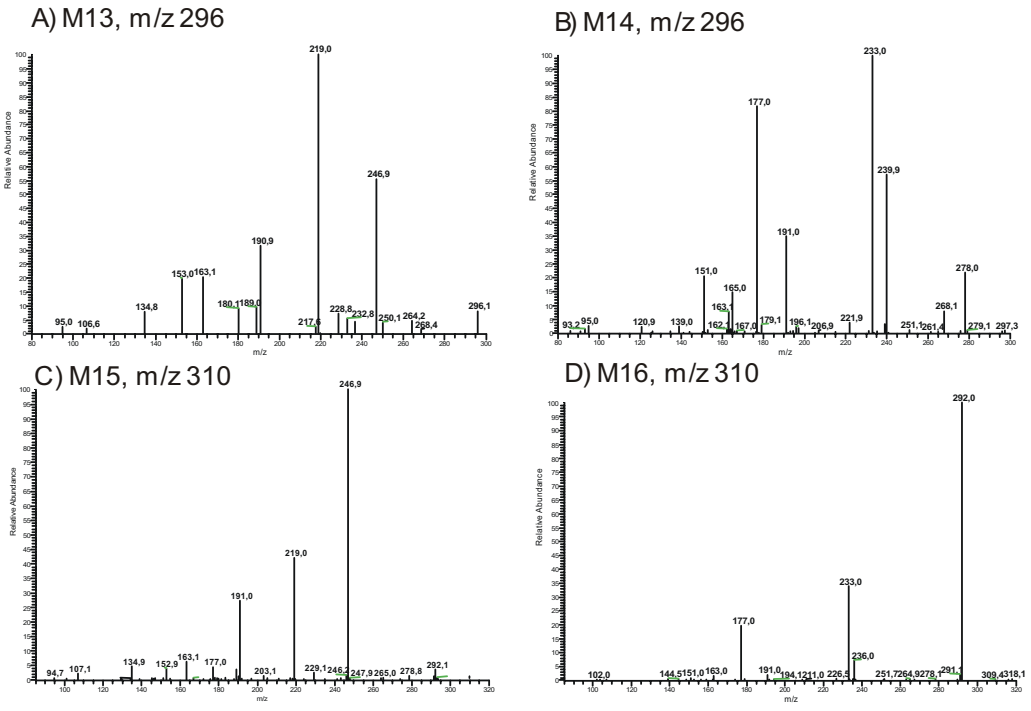

Figure 11. Product ion (MS^2) spectra of $[M+H]^+$ of metabolites M13- M16. Structures of metabolites are shown in the figure 9.

Metabolism of sibutramine

A biotransformation pathway of sibutramine in cultured rat hepatocytes (Figure 9) was proposed on the basis of the mass spectrometric identifications. All metabolites except the demethylated metabolites M1 and M2, the hydroxylated metabolite M7 and the carbamoyl glucuronides are reported for sibutramine for the first time. In addition to rat hepatocytes, incubations were done with one batch of human hepatocytes. As only a limited amount of human hepatocytes was available, the differences between human and rat hepatocytes are only tentatively proposed. The metabolisms with human and rat hepatocytes were almost identical. The main differences were with the dihydroxylated metabolites M11 and M12 and the carbamoyl glucuronide M4, as M11 and M12 were detected only with rat hepatocytes and M4 was detected only with human hepatocytes.

Semiquantitative information on the metabolites was obtained from extracted ion chromatograms (XICs) of the protonated molecules and/or ammonium adducts of the metabolites. The main metabolites formed in primary cultures of rat and human hepatocytes are

the hydroxylated metabolite M8b and the carbamoyl glucuronides M5 and M3 and, as expected from the literature, the (di)demethylated phase I metabolites M1 and M2.

Enantioselectivity was investigated with rat hepatocytes in incubations with (*R*)- and (*S*)-sibutramine. Clear enantioselectivity was found in the formation of hydroxyl derivatives M11 and M15 and glucuronide conjugates M18 and M20. The mass spectra did not confirm the exact position of hydroxyl/glucuronic acid in these structures. However, enanatioselectivity provided further indication that the position of the functional moiety is close to the chiral centre.

7 SUMMARY AND CONCLUSIONS

Cocktail dosing in *in vitro* permeability and metabolic stability experiments and n-in-one analysis were optimized to increase throughput in the early phase of drug discovery. The fast LC/MS/MS methods developed and validated were shown to be suitable for the simultaneous quantitative analysis of a set of drugs from Caco-2 permeability experiments and a set of glucuronides from *in vitro* metabolic stability experiments. In a first application, separation and detection of nine heterogeneous compounds from permeability experiments was achieved within 2.8 min with fast linear gradient and a monolithic column. The total analysis time per sample was 5.5 min (APPI method) or 7 min (ESI method). In a second application, seven glucuronides were separated in a column with 3 µm particles in 3.5 min, total time of analysis time being 10 min. Even though the methods represent highest throughput that can be achieved in sample separation and detection by existing techniques, the throughput is still insufficient for early drug discovery stages. From a future perspective, microfluidics and miniaturized techniques, especially microchip based separation with MS, are promising in terms of throughput, but significant technical enhancements will be needed to gain acceptable separation, sensitivity and reliability for a bioanalytical method.

An important feature of the LC/MS/MS methods developed in this work is the high specificity and sensitivity (LOD values typically ≤ 5nM) of the detection, which allowed the use of very low drug concentrations in *in vitro* experiments. Low concentrations are essential in cocktail dosing in both permeability and metabolic stability studies since low concentrations minimize drug-drug interactions.

The performance of a new ionization technique, atmospheric pressure photoionization (APPI), was evaluated in comparison with electrospray ionization (ESI). Quantitative LC-APPI/MS/MS and LC-ESI/MS/MS methods were validated for the analysis of Caco-2 samples. In general, the validation parameters fulfilled the requirements for a bionanalytical method and were comparable for the APPI and ESI methods, which indicates the usefulness of both ionization techniques for the reliable and sensitive testing of drug transport across the Caco-2 cell monolayer. The applicability and reliability of APPI and ESI methods were further demostrated with a real batch of Caco-2 samples. The two methods provided similar values of apparent permeability (P_{app}). The results showed that APPI is a promising ionization technique

for more extensive use in bioanalytical applications. The main difference between APPI and ESI methods is that the linear quantitative range of APPI is 3-4 orders of magnitude, whereas in ESI it is typically 2-3. This makes the APPI method a more flexible analytical method, especially where the analyte concentrations are unknown or samples with large characterized by wide differences in analyte concentrations (e.g. Caco-2 samples).

Cocktail strategy was applied in Caco-2 permeability experiments. Within the study, reference cocktails were successfully evaluated for fast testing of functionality and the viability of Caco-2 monolayers. The group of reference compounds included 7-10 representatives of various paracellular and transcellular absorption mechanisms, from which a set of 3-4 compounds, depending on the characteristic of the studied drugs, can be selected for standard testing The results confirmed that use of a cocktail of 7-10 compounds is an extremely effective way to improve throughput in Caco-2 permeability experiments if drug-drug interactions between compounds are not expected or the compounds are not substrates for the same transport proteins.

Cocktail strategy was also evaluated in metabolic stability studies with UGT isoenzymes. The determination of intrinsic clearance (Cl_{int}) for cocktails of seven substrates proved to be possible; agreement between the Cl_{int} values of single compound and mixture incubations was good, and significant cross-effects were not observed when substrate concentrations in the mixture were low enough.

In addition to cocktail dosing, throughput in metabolic stability studies was increased by applying an alternative method of determination for intrinsic clearance, which minimises the number of incubations. Cl_{int} values determined as the slope of the linear part of the Michaelis-Menten fitting correlated well with Cl_{int} (M-M) values determined as V_{max}/K_m from the Michaelis-Menten curve, providing that the substrate consumption in the reactions determining the CL_{int} (slope) values was below 10% and the substrate concentrations were well below their K_m values. Although the above-mentioned requirements for experimental conditions prevent universally accurate determination of Cl_{int}, the method would be useful at early drug discovery stages for fast, preliminary decisions about whether or not an analyte is a substrate for a particular isoenzyme. In addition, the method gives rough estimates of the reactivity levels (low, moderate or high) of substrates.

Efficiency in metabolite profiling was evaluated by testing the applicability of software tools of a triple quadrupole MS (Metabolite ID) and of quadrupole time-of-flight MS (Metabolynx) for identification of tramadol metabolites in urine. These mass spectrometers, with their identification tools, were found to be useful in a search for expected metabolites and for development of product ion methods for automated identification. Many time-consuming steps were still required, however, especially in the preparation of the transformation table for the expected metabolites and in the interpretation of the product ion spectra. In addition, because of the complexity of urine samples, many false-positive hits were obtained with the low resolution QqQ instrument, and manual evaluation of the resulting spectra was required. The high mass accuracy instrument (Q-TOF) easily gave reliable m/z values for the most expected metabolites,

and false-positive hits were not obtained even without comparison with a control urine sample. The QqQ instrument gave mass spectra of good quality in product ion experiments, and the identification was reliable, though time-consuming. Detection of non-expected metabolites in the urine sample, especially metabolites present in low concentration, was practically impossible with these commercial packages and the development of algorithms and more effective computational systems for searching differences between real and control samples remains a great challenge for drug metabolism studies. Without improvement of the software tools, routine, totally automated and reliable metabolic profiling of a specific drug or drug candidate in complex biological matrixes is not possible.

MS^n capabilites of the ion trap were utilized in the identification of tramadol and sibutramine metabolites. In the case of tramadol metabolism, the ion trap proved to be useless because the low mass cut off eliminated the most informative and often sole product ions of tramadol metabolites. In the identification of sibutramine metabolites, in contrast, the ion trap proved to be highly advantageous even though the site of functionality, mostly hydroxylation, could not be determined with MS^n in every case. This study resulted in an extended biotransformation profile for sibutramine with rat hepatocytes and, interestingly, a preliminary experiment with human hepatocytes indicated an almost identical profile. Twenty-three metabolites formed via demethylation, hydroxylation, dehydrogenation, acetylation, attachment of CO_2 and glucuronidation were characterized, and 17 of them are reported for the first time.

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Kerava, December 2007.

Kati Askola

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