



Review

Introduction to early *in vitro* identification of metabolites of new chemical entities in drug discovery and development

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Abstract:

The introduction of combinatorial chemistry and robotics for high throughput screening has changed the way drugs are discovered today compared with 10–15 years ago when fewer compounds were tested in animal or organ models. The introduction of new analytical techniques, especially liquid chromatography/mass spectrometry (LC/MS) has made it possible to characterize the chemical properties, permeability, metabolic stability and metabolic fate of a large number of screening hits for further development in a funnel-like manner. The purpose of this contribution is to discuss principles and recent strategies for metabolite identification and to give an introduction to biotransformation studies. Metabolites are experimentally generated with the use of animal and human recombinant expressed enzymes, and different liver and other tissue fractions like microsomes and slices. For separation and identification of structurally diverse metabolites, LC/MS and tandem mass spectrometry (LC/MS/MS) techniques are commonly used. The LC/MS and LC/MS/MS techniques are rapid, sensitive, easy to automate and robust, and therefore, they are the methods of choice for these studies. The outcome of the metabolite identification studies is detection of metabolites that could be pharmacologically active and contribute to the efficacy of a new chemical entity (NCE), and elimination of compounds that form reactive intermediates and/or toxic metabolites that could cause adverse effects of NCE. If such information is available at an early stage during the drug discovery process, the chemical structure of the compound may be modified to reduce the risk of idiosyncratic and/or adverse drug reactions during clinical development.

Key words:

metabolite identification, reactive metabolite, liquid chromatography, mass spectrometry, Q-Tof

Abbreviations: APCI – atmospheric pressure chemical ionization, API – atmospheric pressure ionization, APPI – atmospheric pressure photo ionization, CYP – cytochrome P450, ESI – electrospray ionization, GSH – L-glutathione, HLM – human liver microsomes, LC – liquid chromatography, MS – mass spectrometry, NCE – new chemical entity, NMR – nuclear magnetic resonance spectroscopy, Q-Tof – quadrupole time-of-flight mass analyser, Q-trap – quadrupole ion trap mass analyser

Introduction

Drug discovery and development is a time and costs consuming process. On average, it takes approximately 12 years and costs about 1 billion USD to register a new drug, and attrition of the process is high

[20]. Several factors contribute to this high attrition, e.g. lack of efficacy and adverse effects in clinical studies of new chemical entities (NCEs) are responsible for approximately 60% of all failures. Insufficient knowledge of metabolic and pharmacokinetic properties of NCEs was responsible for approximately 10% of failures during drug development in 2000 [20].

During the drug discovery and development process, there is a need for metabolite identification at different stages. This is one of the most time consuming and costly parts of metabolic studies and until recently, the metabolite identification studies were generally only performed at a late stage, when an NCE was chosen for clinical development. However, recent progress in *in vitro* metabolic methods [32], and especially in analytical techniques makes it possible to test NCEs at earlier stages of drug discovery and development [21]. In early preclinical phase, during the lead generation phase there is a need to identify "metabolic soft spots" of compounds that show low metabolic stability, which makes it possible to alter the structure of the compound in order to reduce the metabolic rate. There is also a need for identification of metabolites of promising NCEs prior to the performance of toxicological studies in animals. Metabolite identification is usually performed with a combination of *in vitro* and *in vivo* experiments using material from different species in order to compare metabolite exposure and to identify the most suitable species to be used for animal toxicity studies. The species chosen for toxicity studies should preferably form the same metabolites as humans since unique human metabolites otherwise have to be tested in animals in additional studies. Metabolite identification studies are also important for identification of compounds that can contribute to the pharmacological effect or give rise to toxicological effects. Studies with radiolabeled drugs are needed for a complete metabolic profile and mass balance, such studies are required before the NCE can enter large clinical trials (phase III).

Development of the atmospheric pressure ionization (API) source allowed for direct coupling of liquid chromatography (LC) to mass spectrometry (MS) and the LC/MS technique was introduced into drug metabolism laboratories in the mid 1990s [5, 8]. API includes three soft ionization techniques: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and photo ionization (APPI) which gives protonated or deprotonated molecules in the positive or negative mode, respectively [19, 21]. APPI has been considered to be a more universal ionization method compared to ESI, especially for neutral

analytes [6]. The high selectivity and sensitivity of LC/MS and tandem mass spectrometry (LC/MS/MS) techniques enabled the use of those methods for both qualitative and quantitative analysis. Further introduction of rapid gradient elution and short LC columns additionally reduced the time needed for chromatographic separation of the metabolites. A turning point in drug metabolite identification was the development of the API interface for LC/MS and later development of hybrid instruments, like the Q-ToF (Waters Corp./Micromass Ltd., MA, USA) consisting of a quadrupole mass analyser coupled to a time-of-flight mass analyser [25, 34] and the Q-trap (Applied Biosystems/MDS Sciex, CA, USA) combining a quadrupole with a linear ion trap [15]. Ion traps are used frequently due to high sensitivity and low cost. Both the ion trap and the Q-ToF instruments can produce product-ion spectra but not all have the scan functions typical of a "true" MS/MS system like neutral loss or parent ion scanning. The registration of MSⁿ spectra on an ion trap instrument can simplify the interpretation of the fragmentation of the precursor ion in many cases at the expense of time and loss of sensitivity for each fragmentation cycle. The Q-ToF technology is especially useful for accurate mass measurements of precursor and product-ions with high sensitivity. Determination of the elemental composition from accurate mass measurement of product-ions facilitates the interpretation of product-ion spectra generated by collision induced dissociation (CID). Thus, metabolite structures can be proposed with higher certainty, which opens new horizons for rational and logical metabolite identification studies.

The process of *in vitro* metabolite identification can be divided into two parts:

1. Generation of the metabolites. This part involves incubations of NCEs in the presence of different *in vitro* metabolic sub- and/or cellular systems, e.g. liver microsomes, intact cells and tissue slices.
2. Separation and identification of the metabolites. This part involves analytical methods and techniques, e.g. LC/MS and LC/MS/MS, used for detection, separation and characterization of the metabolites.

Incubation systems and procedures for generation of metabolites

Liver microsomes from different animal species and strains as well as from human donors are commer-

cially available, and are a very common and widely used *in vitro* model. The major advantage of this model is its simplicity. The phase I and to some extent phase II enzyme activities e.g. cytochrome P450 (CYP), flavin-containing monooxygenases (FMOs) and UDP-glucuronosyltransferases (UGTs), respectively, can be assayed by supplementing the liver microsomes with relevant cofactors and other activators [28]. The incubations for determination of NCE metabolites in our laboratory are routinely performed at 37°C with 1 µg/µl of microsomal protein and 1 mM NADPH in a total volume of 1 ml of 100 mM-potassium phosphate buffer, pH 7.4. A final concentration of a test compound is usually 10 µM, but some laboratories perform such studies at higher concentrations, 20–50 µM, of the test compounds. In order to investigate the formation of a chemically reactive metabolite, the incubation mixture can be supplemented with *L*-glutathione (GSH) to trap electrophilic metabolites. In our laboratory, GSH is used at a final concentration of 10 mM. The controls are incubated with solvent alone, and the final concentration of organic solvent should be ≤ 1%. The reactions are started by addition of NADPH or other co-factors and terminated after 1 h with 1 volume of ice-cold acetonitrile. Precipitated proteins are removed by centrifugation at 2,800 × g for 15 min at 4°C.

Hepatocytes are commercially available and they can be isolated from different animal species and strains, as well as from human donors. The hepatocytes are a more complete and complex *in vitro* model compared to liver microsomes. The activities of enzymes from phases I and II as well as all cellular transporters are present in this system [28, 32]. Cryopreserved hepatocytes are thawed and washed in Williams's medium E with Glutamax-I (Gibco Ltd., UK). After centrifugation at 50 × g over 5 min, at 20°C, the cells are re-suspended and the viability and cell density are measured by trypan blue exclusion. The cells are diluted in Williams E medium to 1 × 10⁶ cells/ml, and the cell suspensions are incubated for up to 24 h with test compound usually at a final concentration of 10 µM. The incubations are terminated with 1 volume of ice-cold acetonitrile. Precipitated proteins are removed by centrifugation at 2,800 × g for 15 min at 4°C. The acetonitrile used to terminate the incubations is evaporated under nitrogen and the samples are filtered through 0.45 µm micro-spin cellulose filters.

Tissue slices can be prepared from different species and strains of animals as well as from human tissues.

The slices are the most complete and complex *in vitro* model. This model not only contains all drug-metabolizing enzymes, transporters etc., but also intercellular membranes and transport system, which is a major advantage of this model [32].

Usually the tissue is washed with ice-cold Krebs-Henseleit buffer, and tissue cores of 8 mm in diameter are prepared using a motor-driven coring tool from Vitron, Inc. (Tuscon, AZ, USA). The best cores are used for slice preparation. Tissue slicing is performed in ice-cold Krebs-Henseleit buffer using Brendel/Vitron tissue slicer from Vitron, Inc. The slicing buffer is gassed before and during slicing with carbogen (95% O₂/5% CO₂). The slice diameter as well as the weight depend on the tissue, e.g. for liver slices the diameter should be 8 mm and the weight 25–30 mg. Freshly cut slices are placed in 6-well plates: one slice per well with 1.5 ml of incubation medium – Williams's medium E with Glutamax-I. Test compound is added to a final concentration of 10 µM, or higher. The slices can be incubated for up to 24 h at 37°C, under continuous carbogen flow. After incubation, slices are transferred to tubes and homogenized using an Ultra-Turrax. Ice-cold acetonitrile (1.5 ml) is added to terminate the reaction and precipitated proteins are removed by centrifugation at 2,800 × g for 15 min at 4°C. The acetonitrile is then evaporated under nitrogen and samples are filtered through 0.45 µm micro-spin cellulose filters.

Analytical methods and techniques for detection and identification of metabolites

Prediction of metabolites *in silico*

Recently, several commercial computer-based *in silico* softwares for prediction of possible metabolites of chemical entities have been developed, e.g. METEOR from Lhasa Ltd., (Leeds, UK) and Pallas from CompuDrug Inc. (San Francisco, CA, USA) [38, 39]. METEOR uses a knowledge database of structure-metabolism relationship based on literature and example data [11]. Pallas software also predicts metabolism of compounds based on a comprehensive knowledge base, which contains metabolic reaction rules. The both softwares can predict metabolites

formed during phase I and II of drug metabolism. The usefulness of the prediction software consists especially in the ability to predict metabolites which are likely to be formed rather than to predict all possible metabolites.

Liquid chromatography-mass spectrometry (LC/MS & LC/MS/MS techniques)

The first step of metabolite identification work in our laboratory is investigation of suitable conditions for ionization of the parent compound by ESI or APCI. The kinetic energy the ions are given in the ion source denoted cone voltage (Waters Corp./Micromass) or declustering potential (Applied Biosystems/MDS Sciex) is either optimized or a cone-voltage ramp from 15–40 V can be used. The samples (25–90 μ l) are injected by an auto sampler onto a small molecule trap column (1 \times 10 mm) from Michrome Bioresources, Inc. (Auburn, CA, USA) into a flow of 0.02% TFA in water at 0.2 ml/min during 2.6 minutes. The trap column is connected to a six-port valve on a Waters CapLC system (Milford, MA, USA). The analytes are separated on 5 μ m Luna C18 column (1 \times 150 mm) from Phenomenex (Torrance, CA, USA) with an acetonitrile gradient from the CapLC system after switching of the six-port valve. The standard gradient at our laboratory consists of buffer A: 2% acetonitrile and 0.02% TFA in water, buffer B: 0.02% TFA in acetonitrile. The gradient schedule is 0–80% B for 25 min at 0.04 ml/min. The column is re-equilibrated with 100% A during 5 min prior to the next injection. The sample and the control sample are then analyzed in full scan mode. Reconstructed ion current chromatograms of expected metabolites are then produced for the control and incubated samples and the chromatograms are compared. Mass shifts for expected metabolites are listed in Table 1. The total ion chromatograms are also compared in order to identify unexpected metabolites. Both these metabolite screens can be performed in an automated fashion using different software e.g. MetaboLynx software (Waters Corp./Micromass Ltd.). When the metabolites have been detected, product ion spectra of the metabolites are recorded to obtain more structural information. Electrospray mass spectra are recorded in the positive ion mode on a Q-ToF II mass spectrometer. In addition, the Q-ToF can provide accurate mass of precursor and product ions, which greatly supports the interpretation, and accuracy of the results. Differ-

ent softwares e.g. Massfrontier from ThermoQuest (Waltham, MA, USA) or MS Fragmenter from ACD Labs (Toronto, Ontario, Canada) can be used to predict possible structures of the product ions from a given structure. Mass spectra are recorded with a cone voltage ramp between 15 and 40 V with an accumulation time of 2 s. Product ion spectra are recorded at 3 different collision energies (10, 20 and 40 eV) with an accumulation time of 1 s for each energy and argon as collision gas (Fig. 1A).

Nowadays, common phase I and II reactions of xenobiotics are well known [28], and during drug metabolite identification studies the chemical structure of the NCE is also known. Therefore, it is assumed that elemental formula of the possible metabolites can be proposed by comparison of parent compound and the nominal mass obtained by LC/MS instruments (Tab. 1) [27]. Such data can be obtained with any kind of mass spectrometer with unit resolution. More structural information about the metabolites can be gained using tandem mass spectrometers after collision induced dissociation (CID). A tandem mass spectrometer contains a mass filter, where the ion of interest is isolated based on its mass to charge (m/z) ratio, and a fragmentation part, where the isolated ion is passed into a collision cell. The collision cell is filled with a gas (argon or helium) and an electric voltage is applied to the cell. The ions are excited after collision with the gas molecules which results in unimolecular decomposition and formation of product ions. As a result of this dissociation, ion fragments are formed, which pass from the cell into a second mass analyzer for determination of their mass to charge ratios (m/z). The ion fragments correspond to parts of a molecule, and based on changes in masses of ion fragments compared to the parent compound, structures of the metabolites can be determined provided that the structure of the product ions can be proposed. The interpretation of a CID spectrum of a known compound can be difficult in many cases when rearrangement reactions occur or radical ions are formed. Key information can be obtained by accurate mass measurements on a Q-ToF instrument. If mass of the product ions can be measured with accuracy better than 2.5 mDa, it is often sufficient to determine the elemental composition of the product ions. Thus, it is possible to differentiate between different fragmentation pathways [14]. For accurate mass measurements, the mass spectrometer is tuned to give a resolution of about 7000 (50% valley) and calibrated with a solu-

Tab. 1. Mass shifts of expected metabolites compared to parent compound

Reaction		Phase of metabolism/enzyme	Mass shift Da (NL*, parent ion/ion mode)
Nitro reduction	R-NO ₂ > R-NH ₂	I/Amine oxidase	-30
<i>N</i> -, <i>O</i> - or <i>S</i> -demethylation	R-NH-CH ₃ > R-NH ₂	I/CYP	-14
<i>N</i> -, <i>O</i> - or <i>S</i> -dealkylation	R-NH-alkyl > R-NH ₂	I/CYP	- depends on alkyl chain length
Dehydrogenation	R-CH ₂ -OH > R-CHO	I/dehydrogenase	-2
Hydroxylation	R-CH ₂ > R-CH-OH Ar-H > Ar-OH	I/CYP	+16
<i>di</i> -Hydroxylation		I/CYP	+32
Oxidation	R ₁ -CH ₂ -R ₂ > R ₁ -CO-R ₂	I/CYP	+14
<i>N</i> -oxidation	R-NH > R-N-OH	I/CYP and/or FMO	+16
Sulfoxidation	R-S-R > R-SO-R	I	+16
Aldehyde oxidation	R-CHO > R-COOH	I/alcohol dehydrogenase	+16
Alcohol oxidation	R-CH ₂ -OH > R-COOH	I/alcohol dehydrogenase	+16
Oxidation of CH ₃ -group to carboxylic acid	R-CH ₃ > R-COOH	I/CYP	+30
Epoxide hydroxylation	R-CH(O)-R > R-CH(OH)-CH(OH)-R	I/Epoxide hydratase	+18
Epoxide formation & hydroxylation		I/Epoxide hydratase & CYP	+34 (+18, +16)
Sulfation aromatic	Ar-OH > Ar-O-SO ₃ H	II/sulfotransferase	+80 (Precursor m/z 97/-)
Sulfation aliphatic	R-OH > R-O-SO ₃ H		
Glucuronidation	R-OH > R-O-GlcA	II/ UDP-transferase	+176 (NL 176/+ or -)
Carbamoyl-glucuronide	primary & secondary amines	II/UDP-transferase	+220 (NL 176/+)
Glycosylation Hexose (Glc)		II/GDP-transferase	+162
Glutathione conjugation	R-CH=CH ₂ > R-CH ₂ -CH ₂ -SG R-CH ₂ -CH ₂ -CysOAc	II/glutathione transferase	+307 (305) Aliphatic (NL 129/+), Aromatic (NL 273/+)
<i>N</i> -Acetylcysteines Mercapturic acid	R-CH ₂ -CH ₂ -CysOAc		+163(NL 129/+)
Glutathione conjugation	Epoxide + GSH-H ₂ O > GS-parent		+305
Acetylated GSH			+347(305+42)
Glutathione conjugation	Epoxide+GSH > R-CHOH-HCSG-		+323 (129)
GSH	R-CH ₂ -CH ₂ -CysGly		178 (176)
GSH	R-CH ₂ -CH ₂ -CysGlu		250 (248)
Cysteine conjugation	R-CH=CH ₂ > R-CH ₂ -CH ₂ -Cys		+121 (119)
Mercapturic acid (from GSH conj.)			+161
Reduction of NO ₂	R-CH ₂ -NO ₂ > R-CH ₂ -SG	II/GSH transferase	+160
Gly-conjugation	R-COOH > R-CO-Gly	I or II	+57
Ala-conjugation	R-COOH > R-CO-Ala	I or II	+71
Methylation	R-OH > R-O-CH ₃	I/Methyl transferase	+14
Acetylation (1°, 2° amines)	R-NH ₂ > R-NH-CO-CH ₃	I/ <i>N</i> -acetyltransferase	+42
Phosphorylation	R-OH R-O-PO ₃ H		+79 Precursor m/z 63/- Precursor m/z 79/-

* NL = Neutral loss scanning

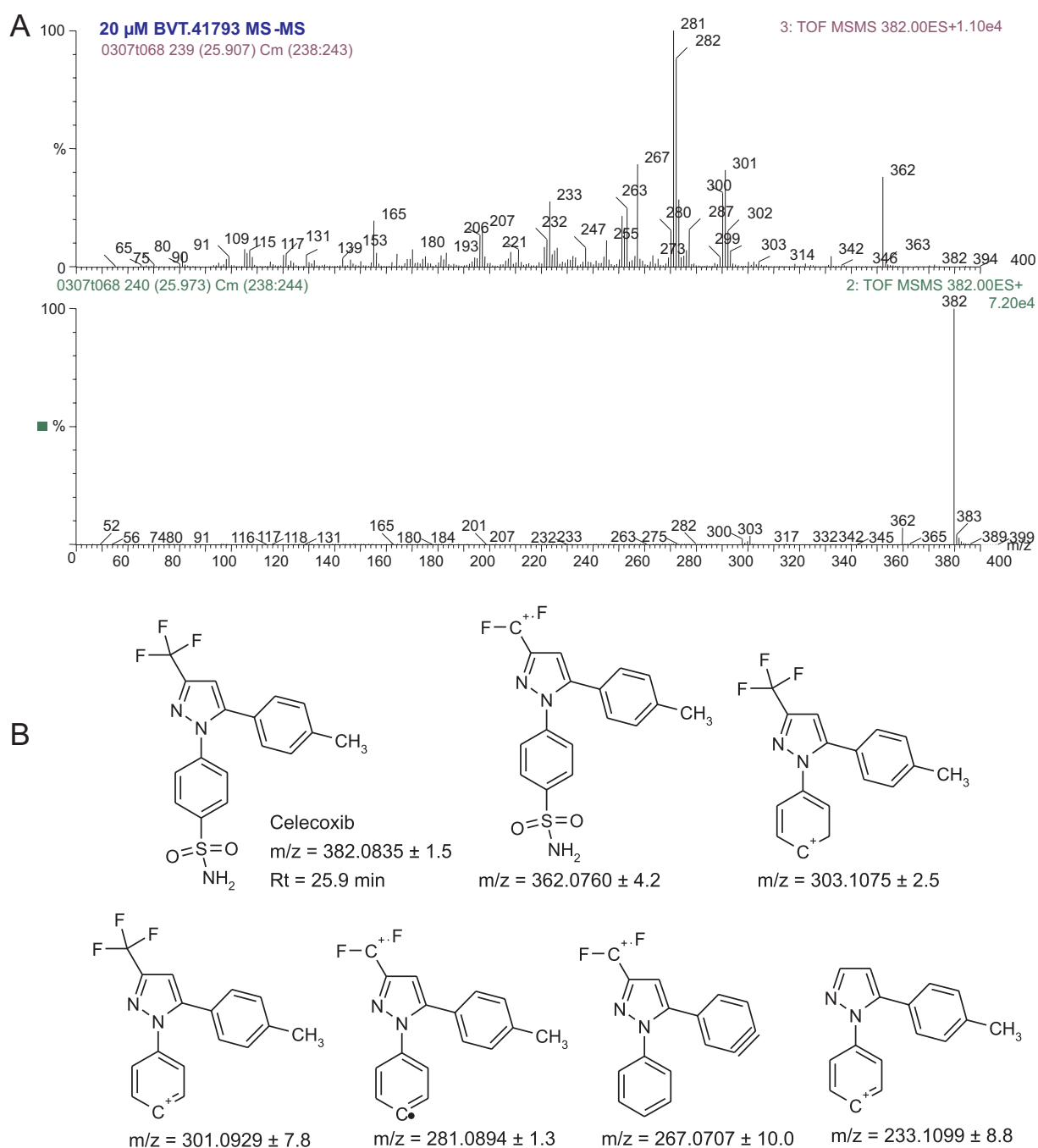


Fig. 1. A – Product ion spectra of celecoxib obtained using Q-ToF II mass spectrometer with a collision energy of 20 eV (bottom) and 40 eV (top), **B** – proposed structures of product ions of celecoxib based on accurate mass measurements with the deviation from theoretical mass given in mDa

tion of sodium formate. During accurate mass measurements of product ions from a protonated test compound, the precursor ion is first used for lock mass correction at low collision energy (10–20 eV) to determine the elemental composition of the product ions formed and then the determined product ion is used

for lock mass correction at higher collision energies (40 eV). An example of product ion spectra of celecoxib recorded on a Q-ToF II mass spectrometer is shown in Figure 1 together with proposed structures of product ions of celecoxib based on determined accurate masses. The most intense fragment at $m/z = 281$

occurred after loss of the sulfonamide group and one of the fluorine atoms at the trifluoromethyl group (Fig. 1). The fragmentation pathway was used to deduce the structures of the metabolites of celecoxib and a scheme of the proposed metabolites determined after *in vitro* incubation with hepatocytes from different species is presented in Figure 2. The structures of the metabolites were compared with those previously obtained *in vivo* [29–31]. As described previously the major metabolic pathway of celecoxib *in vivo* ($m/z = 382$) involved monohydroxylation of the methyl group

human showed good agreement between *in vitro* and *in vivo* studies (Tab. 2). Qualitatively the same pattern of the metabolites was detected *in vitro* as *in vivo* (Fig. 2). Additionally, the *in vitro* experiments showed that the major metabolite of celecoxib was the carboxylic acid (M2), which correlated well with the results from *in vivo* studies performed with [^{14}C]celecoxib and published previously (Tab. 2) [29–31]. The semi-quantitative analysis of the metabolites from *in vitro* studies with celecoxib used area percent of reconstructed ion current chromatograms.

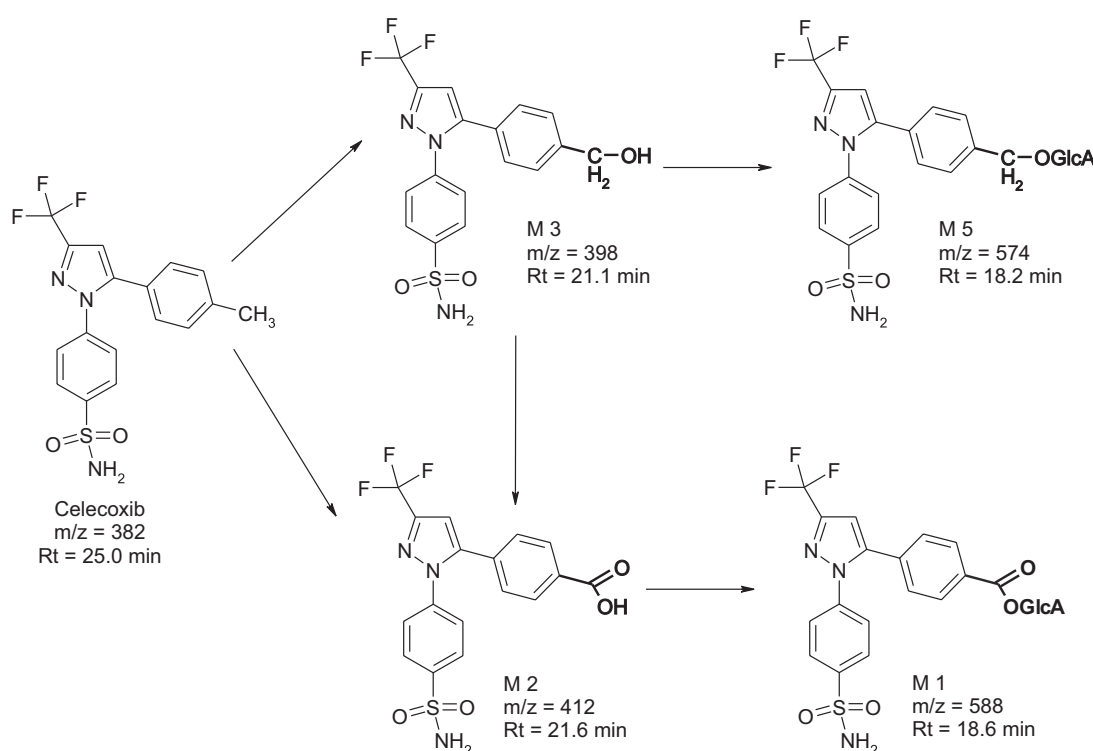


Fig. 2. Tentative identification of celecoxib metabolites after incubation with primary rat (SD male) hepatocytes and dog (Beagle male), monkey (*Cynomolgus* male) and human (pool of 5 donors) cryopreserved hepatocytes. The used incubation and analytical conditions were as described in the text. The structures of the metabolites were compared with those published previously [29–31]

(+ 16 Da) and further oxidation to the carboxylic acid (+ 30 Da), metabolites M3 and M2, respectively (Fig. 2) [29–31]. The carboxylic acid was further conjugated with glucuronic acid to form metabolite M1, an acyl glucuronide (Fig. 2). The acyl glucuronide was detected at m/z parent +30 Da + 176 Da (glucuronic acid) with a characteristic neutral loss of 176 Da. The performed *in vitro* metabolite identification experiments after incubation of celecoxib with primary and cryopreserved hepatocytes from rat, dog, monkey, and

Structural analysis is usually performed with the time-of-flight instruments but triple quadrupole instruments are used when negative mode, precursor ion and neutral loss scanning are required. The triple quadrupole instruments are used routinely for quantitative analysis of the parent compound and synthesized metabolites in biological samples by LC/MS/MS with Multiple Reaction Monitoring (MRM).

Precursor ion scanning is used to search for metabolites that give a characteristic and common frag-

Tab. 2. Percentage of celecoxib and its metabolites after *in vitro* incubation with rat, dog, monkey and human hepatocytes as well as after *in vivo* treatment of rat, dog, monkey and human with [¹⁴C]celecoxib

	Celecoxib m/z = 382	M 3 Methylhydroxy metabolite m/z = 398	M 2 Carboxylic acid metabolite m/z = 412	M 1 Acyl glucuronide metabolite m/z = 588	M 5 Glucuronide metabolite m/z = 574
Rat hepatocytes ¹⁾ Incubation 6 h	3.2	4.0	90.1	1.7	0.9
Rat urine ²⁾ 0–24 h	0.04	0.04	8.6	NR	NR
Rat feces ²⁾ 0–48 h	0.6	3.2	83.9	NR	NR
Dog hepatocytes ³⁾ Incubation 6 h	4.5	6.8	83.6	1.6	3.5
Dog EM ⁴⁾ after 4 h	50.3	27.5	5.3	2.4	NR
Dog PM ⁵⁾ after 4 h	93.8	5.8	ND	ND	NR
Monkey hepatocytes ⁶⁾ Incubation 6 h	5.4	0.3	94.3	ND	ND
Monkey <i>iv</i> ⁷⁾ after 6 h	9.2	0.7	90.1	ND	ND
Human hepatocytes ⁸⁾ Incubation 6 h	0.5	0.7	96.0	1.5	1.3
Human oral ⁹⁾ after 12 h	49.9	ND	27.0	23.3	NR

1) Primary hepatocytes of male Sprague-Dawley (SD) rats were incubated as described in the text. 2) Percentage of radiolabeled dose excreted as celecoxib and its metabolites in urine and feces of rats (SD) administered *iv* [¹⁴C]celecoxib at 1 mg/kg [30]. 3) Cryopreserved hepatocytes of male Beagle dogs were incubated as described in the text. 4) Metabolite profiles in plasma after *iv* treatment of dog Beagle extensive metabolizer (EM) with 5 mg/kg of [¹⁴C]celecoxib [31]. 5) Metabolite profiles in plasma after *iv* treatment of dog Beagle poor metabolizer (PM) with 5 mg/kg of [¹⁴C]celecoxib [31]. 6) Cryopreserved hepatocytes of male Cynomolgus monkeys were incubated as described in the text. 7) Metabolite profiles in plasma after *iv* treatment of monkey Cynomolgus with 1 mg/kg of [¹⁴C]celecoxib [31]. 8) Cryopreserved hepatocytes pooled from 5 human donors were incubated as described in the text. 9) Percent of radioactivity in HPLC radiochromatogram from plasma samples obtained 12 h after oral administration of 300 mg [¹⁴C]celecoxib to health male human volunteers [29]. ND – not detected, NR – not reported

ment of the parent molecule, or ion fragment altered by an expected mass shift, e.g. +16 Da for oxidation. Neutral loss scanning searches for possible metabolites based on expected neutral losses from the analyte, e.g. –176 Da for glucuronides, or –129 Da for glutathione adducts (Tab. 1). These two scanning approaches are very useful when complex matrixes like urine or bile are analyzed.

Use of stable isotopes

Mass spectrometry has a unique capability to separate stable isotopes, such as ¹H and ²H, ¹²C and ¹³C, ¹⁴N and ¹⁵N, ¹⁶O₂ and ¹⁸O₂, ³²S and ³⁴S which can be utilized in a large number of applications [4]. One example is the “isotope cluster” technique where a 1:1 mixture of labeled and unlabeled drug is administered and analyzed by LC/MS. An isotope cluster that possesses m/z value by at least two atomic mass units greater than the unlabeled parent is needed. Computer programs have been developed to identify potential metabolites by searching each scan of a chromatographic run for the characteristic isotope cluster of the parent compound mixture. Another example is to use labeled reagents which could involve the replacement of ¹⁶O

with ¹⁸O in an *in vitro* incubation where metabolites formed after oxidation of cytochrome P450 enzymes will be labeled with ¹⁸O and mechanistic information about metabolite formation can be gained [4, 18]. Stable isotope-labeled internal standards are commonly used for quantitative LC/MS work.

Deuterium exchange experiments (H/D_{exch})

If hydroxylation of a parent compound has occurred and the molecule contains nitrogen and/or sulfur, which can form *N*- or *S*-oxides, the mass shift in both cases is the same, namely + 16 Da (Tab. 1). Therefore, additional analysis with a mobile phase containing deuterium oxide instead of water is performed. The samples are reanalyzed by LC/MS in full scan mode for exchangeable hydrogen counting. The number of exchangeable hydrogens is calculated from:

$$H/D_{\text{exch}} = (M+D) - (M+H) - 1$$

Thus *N*-oxidation/*S*-oxidation can be differentiated from carbon hydroxylation based on the number of exchangeable protons. Since the hydrogen of the hydroxyl group is exchangeable, hydroxylation will give one additional proton that can be exchanged with deu-

terium. The usefulness of this approach in our laboratory has been described previously [9, 10].

Derivatization reactions

The deuterium exchange experiments discussed above can be regarded as a derivatization reaction on-line but a large number of derivatization reagents for different functional groups are available and analysis of the derivatized metabolites can provide additional structural information. This can be achieved either by the selectivity of the reactions or by altering the fragmentation properties of the product to induce charge remote fragmentation during MS/MS analysis [13, 22]. A comprehensive review on the subject is given by Liu and Hop where derivatization strategies for LC/MS/MS are discussed [22].

Differentiation between aromatic and aliphatic hydroxylations (phenolic acetylation approach)

Phenolic acetylation is frequently used in our laboratory for its simplicity and ability to differentiate between aromatic and aliphatic hydroxylation. Phenolic hydroxyl groups can be acetylated selectively in the presence of aliphatic hydroxyl groups by acetic anhydride in water at pH 9–10. Amines will also be acetylated completely or partially under such conditions. The sample (0.24 ml) is mixed with 0.06 ml of 1 M NaHCO₃ and 0.03 ml of acetic anhydride. The sample is mixed and acetylated for 15 min. Then 0.015 ml of glacial acetic acid is added to stop the reaction and the sample can be injected for analysis by LC/MS.

Quantification of metabolites

Quantitative information on metabolites which have been identified to have pharmacological and/or toxicological activities is very important during the drug discovery and development process. Since the analyte response can change due to small variations in chemical structure, metabolite standards are required to construct standard curves for quantitation using mass spectrometry, or alternatively [¹⁴C], [³H] labeled compounds are needed. However, at early stages in drug discovery, metabolite standards and/or radiolabeled compounds are usually not available. Therefore,

alternative approaches are urgently needed. Since most drugs contain at least one nitrogen atom, a possible alternative is to use the chemiluminescent nitrogen detector (CLND) which gives a response corresponding to the number of nitrogens in the molecule independent of the compound structure, with a few exceptions e.g. compounds with nitrogens adjacent to each other like hydrazines may eliminate nitrogen gas during combustion in the detector which results in a lower response [36]. Analytes from HPLC are introduced into CLND detector *via* a nebulizer, with nebulizing gas (mixture of oxygen and argon). Oxygen at high temperature in the detector oxidizes the analytes to nitric oxide, water and carbon dioxide. The gases are dried and drawn into the reaction chamber, where nitric oxide in the presence of ozone reacts to form nitrogen dioxide in the excited state (*NO₂). The *NO₂ emits photons, which are detected using a photomultiplier tube and are converted to a signal recorded by an integrator [36]. Thus the metabolites can be generated *in vitro* and quantified by CLND detection to establish a known standard solution. This standard solution can then be used to develop a LC/MS/MS method on a triple quadrupole instrument and the standard can be used by establishing a calibration factor relative to the parent compound. Using the response factor ratio obtained from the CLND standard, the metabolite can then be quantified with LC/MS/MS using the response obtained from the parent drug's standard curve.

The usefulness of the CLND detector has been shown for both *in vitro* as well as *in vivo* experiments in previous studies [10, 37]. It should be noted that it is important to exclude the use of acetonitrile during sample preparation and that methanol should be used instead in order to avoid saturation of the nitrogen detector.

Chemically reactive metabolites

According to the "covalent binding theory", metabolic activation of a chemical compound can result in the formation of electrophilic, chemically reactive metabolites, which can bind covalently to cellular proteins and/or DNA and cause toxic, adverse effects [12, 23]. The mechanisms of formation and action as well as toxicological significance of reactive metabolites are not fully understood today. But even so, many pharmaceutical companies prefer to screen

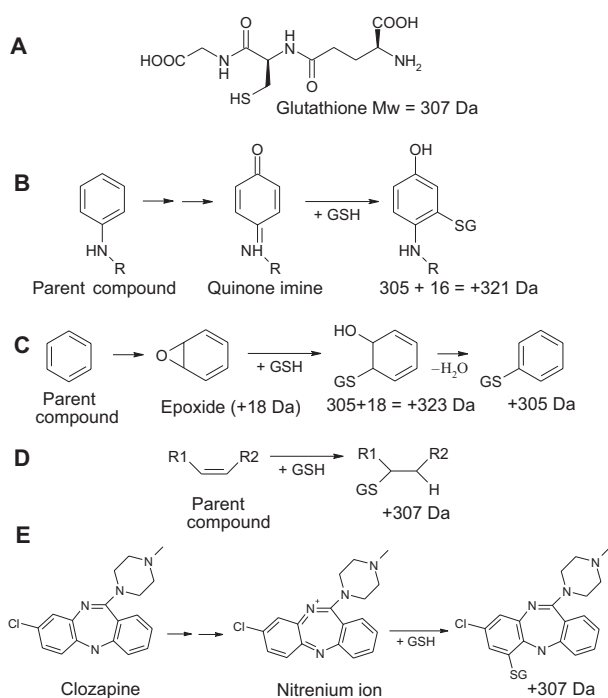


Fig. 3. **A** – Structure of glutathione (GSH) and mass differences due to conjugation of L-glutathione (GSH) with different electrophiles; **B** – quinone imine, **C** – epoxide, **D** – activated double bond and **E** – nitrenium ion

NCEs for such metabolites early in the drug discovery process in order to eliminate this kind of reactive species [1]. Usually this type of experiment is performed concomitantly with metabolite identification studies for NCEs using liver microsomes as the metabolic activation system supplemented with L-glutathione (GSH) as a trapping agent [3]. GSH is a tripeptide: γ -L-glutamyl-L-cysteinylglycine, commonly present in mammalian cells, at physiological concentration as high as 10 mM (Fig. 3A). Its physiological role is multifunctional: it acts as a cofactor of enzymes, e.g. glutathione peroxidase (GPX) and glutathione-S-transferases (GST), affords protection against electrophilic damage and oxidative stress and helps maintain the redox balance of the cell [3, 28].

The major problem in analyzing the possible formation of reactive metabolites is to understand the mechanism for their formation and to identify the molecular moiety at which this alteration is formed. To solve this problem mass spectrometry is commonly used in the analysis of glutathione conjugates [2]. Several characteristic neutral losses occur when glutathione conjugates are analyzed by LC/MS/MS (Table 1), e.g. $[M+H^+]-307$ Da (aliphatic adduct),

$[M+H^+]-273$ Da (aromatic adduct) and $[M+H^+]-129$ Da (common adduct). The most common mechanisms of formation of reactive metabolites as well as mass differences due to conjugation of GSH with different electrophiles are presented in Figure 3 (Fig. 3B–E). In order to obtain additional structural information about NCE adducts, the compounds may be tested for formation of reactive metabolites in the presence of other trapping agents, e.g. L-cysteine and/or $[^{13}\text{C}]$ -cysteine.

Another question is how high levels of glutathione conjugates can be accepted for further development of NCE. Most probably, many pharmaceutical companies have their own criteria, but to our knowledge the only published policy available so far is Merck's approach [1]. This strategy is based on data from *in vivo* experiments using acetaminophen as a source of covalent conjugates after treatment of animals with a dose sufficient to cause hepatic necrosis, and the use of a 20-fold safety margin. Under these conditions the "safe level" of conjugates was set to 50 pmol of drug equivalents per mg of total liver protein [1]. However, in some cases even higher level of glutathione conjugates, up to 200 pmol/mg of protein may be accepted, depending on several factors like intended therapeutic indication, availability of existing treatments, predicted clinical dose etc. [1].

It can be concluded that progress in analytical techniques, especially mass spectrometry significantly contributes to new approaches for detection, identification and understanding of the reactive metabolite phenomena [17, 40]. However, further studies are needed in order to better characterize the mechanisms of formation and action of chemically reactive metabolites.

Conclusions

Metabolite identification is an important and critical part of drug metabolism studies during the drug discovery and development process. Early identification of "metabolic soft spots", chemically reactive metabolites and/or pharmacologically active metabolites can be a useful information for chemists in order to modify existing structures of NCEs. Introduction of soft ionization techniques e.g. ESI and APCI and accurate mass measurement with Q-Tof instruments allow metabolites to be identified at early lead generation and optimization stage of the drug discovery pro-

cess. Additionally, the quality of the data as well as time needed for these studies can be significantly reduced, and the number of NCEs tested has increased. Today, the LC/MS technique is the method of choice for metabolite identification studies, and most of the quantitative analysis is performed with triple quadrupole instruments while ion traps, Q-ToF and Q-trap instruments are used for qualitative work due to higher sensitivity in the full scan mode [21]. However, even this technique has some limitations and often chemical structure of metabolites can only be confirmed with nuclear magnetic resonance spectroscopy (NMR) [7]. The NMR technique and recently introduced LC-NMR-MS technology can provide a wide range of structural information e.g. position of hydroxylation at the aromatic and/or aliphatic rings. The major disadvantage of NMR technique is the lack of sensitivity and demand for a pure compound, which is not sufficient for studies of trace amounts of material in complex samples and the sensitivity of NMR is not comparable with the sensitivity of mass spectrometry. However, the sensitivity of NMR is continuously improving [7]. Another limitation of the LC/MS technique is need of standards and/or labeled compounds for quantitative analysis. Introduction of new detectors, like CLND offers new possibilities to quantify metabolites, and further improvements are expected in the future. Even if LC/MS is suitable for automated analysis and metabolites can be detected in an automated fashion with a suitable computer program [26] searching for the expected mass shifts given in table 1 or by an unbiased chemometric approach [16] much of the interpretation of the mass spectra still has to be done manually. Recent progress in separation technologies, miniaturization and development of new techniques and new software will soon open new possibilities. One example can be a new soft ionization technique recently introduced – APPI, which opens new possibilities for analysis of non-polar metabolites [6, 19, 33]. An often overlooked alternative could be to use alkylamines as adduct-forming agents for electrospray ionization of neutral and unstable compounds [24, 35].

To summarize, the introduction and continuous development of liquid chromatography and mass spectrometry are responsible for an enormous progress in metabolite identification studies in the past ten years. The studies are performed significantly earlier during the drug discovery and development process, the quality of the data is sufficient and further improve-

ments are under way. It is assumed that this progress in metabolite identification studies is to some degree responsible for a decreased attrition of NCEs during the drug discovery and development process.

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