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VALIDATION OF LC/MS/MS METHOD FOR ASSESSMENT OF THE *IN VITRO* ACTIVITY OF SELECTED RAT CYTOCHROME P450 ISOENZYMES - APPLICATION TO EARLY DRUG METABOLISM SCREENING

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Abstract: A sensitive and specific liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for simultaneous determination of seven metabolites of CYP450 model substrates (acetaminophen, 4-hydroxytolbutamide, 4'-hydroxymephenytoin, 1-hydroxybufuralol, 6-hydroxychlorzoxazone, 1'- and 4'-hydroxymidazolam) in rat liver microsomes was developed. The assay used Kinetex analytical column and a gradient mobile phase consistent of acetonitrile and water with addition of 0.1% formic acid. The analysis was performed in selected reaction monitoring (SRM) mode both in positive and negative (for 6-hydroxychlorzoxazone) mode. The method was validated over the concentration ranges of 10-2000 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide, 50-2000 ng/mL for 1-hydroxybufuralol and 25-2000 ng/mL for the rest of the analytes. The intra- and inter-day precision (2-12%) and accuracy (93-119%) were within the limits set by the FDA and EMA guidelines. The developed method was successfully applied to assess the activity of selected CYP450 isoenzymes in rat liver microsomes after addition of ketoconazole.

Keywords: rat liver microsomes, CYP450 isoenzymes, cocktail method, LC/MS/MS, ketoconazole

The development of new drugs is determined largely by their ADME properties of which metabolism is an important factor affecting e.g., bioavailability, detoxification, drug-drug interactions and individual pharmacokinetics and pharmacodynamics variability. Drugs and xenobiotics are mainly metabolized by different oxidation and conjugation enzymes to more polar and better soluble metabolites to facilitate their excretion (1).

Cytochrome P450 system represents drug-metabolizing enzymes from a superfamily of hemoproteins, engaged in phase I metabolism. Among the CYP isoforms, family 1 to 4 are involved in metabolism of different xenobiotics, and in human more than 90% of drugs that undergo biotransformation are metabolized by CYP 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 (2, 3). In the development of new chemical entity, drug candidate, preclinical screening of metabolism rates and assessment of elimination routes are considered to be essential for further development.

In the field of drug discovery, a demand has risen for efficient and rapid drug metabolism screening techniques. To speed up the selection of new drug candidates, to identify the metabolic profile of new molecules, to indicate likely drug interactions or to assess the role of polymorphic enzymes, different *in vitro* systems have been developed.

LC/MS/MS technique provides a powerful solution for investigating the biotransformation of xenobiotics and is used at various stages of drug discovery and development (4). Because of the need to test a large number of compounds, recently a high-throughput screening methods were created. One of the application includes the use of the cocktail of substrates added to the microsomal incubations for simultaneous evaluation of the activities of CYP450 isoenzymes (5). Due to this approach multiple enzymes may be assessed in the same study and the effect of intra-individual variability is minimized. Recently, numerous of drug metabolism cocktails have been proposed and developed (6–15).

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According to FDA guidelines, preclinical metabolism studies of new drug candidates should be conducted using human microsomes (16), while there are numerous *in vitro* works for the assessment of preliminary metabolic pathway of new compounds using rat liver microsomes, cytosol and primary hepatocytes, because rats are the most commonly used model in the field of preclinical studies (17–20). Among the frequent examples, work of Xiao Hu et al. (21) demonstrated the variance analysis of V_{max}, K_m and Cl_{int} values from the study of enzyme kinetics of osthole and imperatorin, and showed no significant difference between these values in rat and human liver microsomes. The study demonstrated that the rat liver microsomes were reliable to analyze drug metabolism *in vitro*. The study of De Bock et al. (22) described the validation of the method for determination of metabolites of model substrates in rat microsomal matrix using earlier derivatization of metabolites. The metabolic fate of sublethal exposure of different herbicides in rat microsomes was studied in the work of Larsen et al. (23). On the basis of the results of Xue-Jin Zhao et al. (24) one may assume that from a qualitative point of view, the dog, rat and even mouse could be an appropriate species for conducting an *in vitro* study on the metabolism of quinine. However, using rodent data to estimate human risk should be aware of interspecies variability both in expression and catalytic activity of relevant CYP450 isoforms (25).

The present study aims to evaluate a simple and an accurate LC/MS/MS method for simultaneous determination of specific metabolites of six model substrates metabolized by CYP450 isoenzymes after incubation with rat liver microsomes. In the next step, the optimized method was used to establish the strength of enzyme inhibition by ketoconazole on selected CYP450 isoenzymes.

MATERIALS AND METHODS

Chemicals and reagents

Midazolam, 1'-hydroxymidazolam (1-OH-MZ), 4'-hydroxymidazolam (4-OH-MZ), phenacetin, acetaminophen (ACP), tolbutamide, 4-hydroxytolbutamide (4-OH-TB), bufuralol, 1-hydroxybufuralol (1-OH-BF), S-mephenytoin, 4'-hydroxymephenytoin (4-OH-MP), chlorzoxazone, 6-hydroxychlorzoxazone (6-OH-CH), dextrophan, magnesium chloride, NADPH, Folin & Ciocalteu's phenol reagent, potassium sodium tartrate tetrahydrate, cooper sulfate, sucrose, TRIS, sodium phosphate dibasic and potassium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was obtained from Fluka (Buchs, Switzerland). Potassium dihydrogen phosphate, sodium chloride, acetone and acetonitrile were purchased from J.T. Baker (Phillipsburg, PA, USA). Purified water (18.2 MΩ) used throughout the study was delivered by a Milli-Q water system (Millipore, Billerica MA, USA).

Isolation of hepatic microsomes

Rat liver microsomes were prepared using a differrential centrifugation method. Liver fragments were minced with scissors, washed with TRIS/KCl (pH 7.4) buffer and homogenized. The homogenate was centrifuged (Sorval WX Ultra Series, Thermo Scientific) at approximately 11 500 × g for 20 min at 4°C. The supernatant (S9 fraction) was transferred to new centrifuge tubes and then ultracentrifuged at 100 000 × g for 60 min at 4°C. The pellet was resuspended in 0.15 M KCl with Ultra Turrax IKA T10 basic homogenizer (IKA-Werke GmbH & Co. KG Staufen, Germany) and ultracentrifuged again at 100 000 × g for 60 min at 4°C. The obtained pellet was dispersed in TRIS/sucrose buffer and stored at -80°C until use.

Protein concentration in microsomal fraction was determined by Lowry protein assay (26).

Chromatographic conditions

Chromatographic separation was performed on a Kinetex (2.6 µm, PFP, 100 Å, 100 × 3 mm, Phenomenex, Torrance, CA, USA) analytical column using UltiMate 3000 UPLC system (Dionex, USA). The mobile phase consisted of acetonitrile containing 0.1% formic acid (eluent A) and water containing 0.1% formic acid (eluent B). At the flow rate of 500 µL/min, the amount of eluent A increased lineary from 20 to 95% in 2 min, maintained by 95% for 6 min, then returned to 20% in 3 min, and was left to equilibrate for 6 min. The total run time was 15 min.

LC/MS/MS conditions

The analyzed compounds were detected using TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA). Heated electrospray ionization (H-ESI) was used both in the positive and negative (for 6-hydroxychlorzoxazone) modes. The selected reaction monitoring transitions and collision energies optimized for each metabolite and internal standard are listed in Table 1. The mass spectrometric conditions were as follows: needle voltage 4020 V, vaporizer temperature 250°C, sheath gas (nitrogen) pressure 30 psi, auxiliary gas (nitrogen) pressure 10 psi, capil-

lary temperature 370°C. The argon pressure in the collision cell was approximately 2 mTorr.

Standard and quality control samples

Stock solutions of metabolites of model substrates and dextrophan used as an internal standard (IS) were prepared at concentration of 0.5 mg/mL in methanol. Working solutions for calibration standards were prepared by diluting the stocks in water to the concentrations of 0.125, 0.3125, 0.625, 1.25, 3.25, 6.25, 9.375, 12.5 and 25 µg/mL of each analyte in cocktail, and 0.125, 0.375, 1.125, 10, 22.5 µg/mL for quality control samples. Dextrophan was diluted in acetone : acetonitrile mixture (1 : 1, v/v) to a final concentration of 250 ng/mL.

Calibration samples were made at 10, 25, 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide; 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for 1-hydoxybufuralol and 25, 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for the rest of analyzed metabolites.

Quality control (QC) samples were prepared at 30, 800 and 1800 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide, and 90, 800 and 1800 ng/mL for other substances. Calibration standards and quality control samples were prepared by spiking 40 µL of working solution of cocktail of analytes with blank microsomal medium (without NADPH). In the next step, to stop the reaction, the mixture of acetonitrile : acetone (1 : 1, v/v) with internal standard was added. After vortex mixing and cooling down on ice, samples were centrifuged at 15 000 × g for 15 min, and supernatant was analyzed using LC/MS/MS method.

Method validation

Validation of the method was carried out according to the FDA and EMA bioanalytical method validation guidelines (27, 28) with respect to the linearity, selectivity, precision, accuracy, recovery, limit of detection, limit of quantification, matrix effect and post-preparative stability.

Selectivity is the ability of the analytical method for separation and quantification of the analyte in the presence of other compounds in the sample. It was assessed by studying peak interference from six independent sources of microsomes.

Calibration curves were constructed using seven to nine calibration standards and fitted by a weighted least-squares linear regression. To assess linearity, deviations of the back calculated concen-

Table 1. Selected reaction monitoring parameters for quantified analytes.

Analyte	Q1 [m/z]	Q3 [m/z]	CE [V]
Acetaminophen	152	110	14
4-Hydroxytolbutamide	287	89	38
4'-Hydroxymephenytoin	235	150	19
1-Hydroxybufuralol	278	186	14
6–Hydroxychlorzoksazone	180	120	20
1'-Hydroksymidazolam	342	324	21
4'-Hydroxymidazolam	342	297	24
Dextrophan (IS)	258	157	38

Table 2. Linear ranges, determination coefficients, limits of detection and limits of quantification for selected compounds.

	ACP	6-OH-CH	4-OH-MP	1-OH-BF	4-OH-TB	1-OH-MZ	4-OH-MZ
Liner range [ng/mL]	25-2000	25-2000	10-2000	50-2000	10-2000	25-2000	25-2000
Determination coefficient (r^2)	0.9994	0.9995	0.9916	0.9908	0.9965	0.9974	0.9983
LOD [ng/mL]	0.1	1	0.05	0.01	0.01	0.025	0.01
LLOQ [ng/mL]	25 ± 2.8	25 ± 2.8	10 ± 1.3	50 ± 5.9	10 ± 0.5	25 ± 3.0	25 ± 3.1

ACP - acetaminophen; 6-OH-CH - 6-hydroxychlorzoxazone; 4-OH-MP - 4'-hydroxymephenytoin; 1-OH-BF - 1-hydroxybufuralol; 4-OH-TB - 4-hydroxytolbutamide; 1-OH-MZ - 1'-hydroxymidazolam; 4-OH-MZ - 4'-hydroxymidazolam.

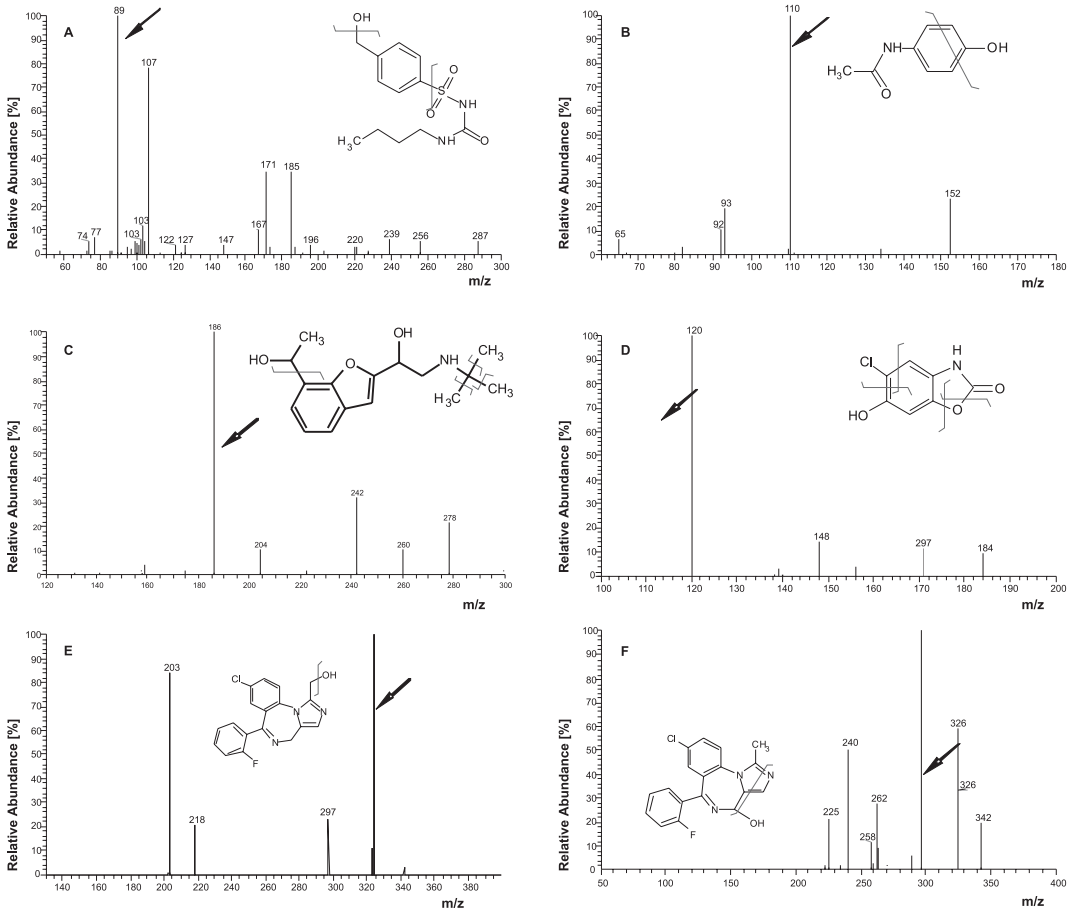

Figure 1. Fragmentation mass spectra of selected metabolites with purposed fragmentation pathways: 4-hydroxytolbutamide (A), acetaminophen (B), 1-hydroxybufuralol (C), 6-hydroxychlorzoxasone (D), 1-hydroxymidazolam (E) and 4-hydroxymidazolam (F)

trations were set at ±15% of the nominal ones, except of the LLOQ, when a deviation of ±20% was permitted.

Inter- and intra-batch precision and accuracy of the assay were verified for three concentrations of QC samples. Six replicates of sample were analyzed on the same day to determine the intra-day precision and accuracy, and on each of two separate days to determine inter-day precision and accuracy. The mean values of precision should be within 15% of the actual value, except of LLOQ, where it should not deviate more than 20%. The accuracy determined at each concentration level should not exceed 85-115%, except of LLOQ, where it should not exceed 80-120%.

The limit of detection (LOD) was defined as the concentration that generates a signal-to-noise ratio of 3, whereas the limit of quantification (LLOQ) was determined as the lowest concentration of the analytes in the sample, which can be quantified with precision lower than 20%, and an accuracy between 80% and 120%.

The stability of samples in autosampler batch was assessed by reanalyzing extracted QC samples kept under the autosampler conditions (10^{o}C) for 24 h. An acceptable stability was defined if the initial loss of concentration was below 15%.

Recovery and absolute matrix effect were assessed at three concentrations of QC samples by comparing the peak areas of 6 runs at each concentration for the neat solutions of analytes and the standard solutions spiked before and after protein precipitation. Recovery was calculated by dividing the mean peak area of standards spiked before extraction by mean peak area of standards spiked after extraction.

The matrix effect was evaluated by comparing the mean peak area of the analyte spiked post extraction to the mean peak of its neat solutions. The absolute matrix effect was defined as (1 - signal of post-extracted spiked sample/signal of pure solution) × 100%. The relative matrix effect was also estimated according to the work of Matuszewski (29) by assessing the variability of standard line slopes expressed as coefficient of variation. The precision of the method should not exceed 4%, and then is considered to be reliable and free from the relative matrix effect.

Inhibition study

To the total volume (0.5 mL) of incubation mixture of rat liver microsomes (1 mg/mL) added 0.1 M phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM NADPH and substrates cocktail in concentrations near their K_m values: phenacetin - 7.5 µg/mL, tolbutamide - 2.5 µg/mL, bufuralol - 12.5 µg/mL, chlorzoxazone - 15 µg/mL and midazolam - 7.5 µg/mL. To the incubation mixture ketoconazole was added in the concentration range of 0.3 to 37.5 µM. Mixtures were preincubated for 5 min at +37°C in a shaking water bath (OLS 200, Grant Instruments, Cambridge, UK). The reaction was initiated by addition of NADPH (cofactor of metabolic reaction). Following a 10 min incubation, the reaction was terminated with 500 µL of ice cold mixture of acetonitrile : acetone (1 : 1, v/v) containing dextrophan as an internal standard (250 ng/mL). Samples were subsequently cooled on ice for 20 min to precipitate the proteins, and then centrifuged at approximately 15 000 × g for 15 min at 4°C. The supernatant (200 µL) was transferred to vials, and a volume of 20 µL was injected onto the analytical column.

CYP-mediated activities in the presence of ketoconazole were expressed as a percentage of the corresponding control values. A sigmoidal shaped curve were fitted to the data and the IC_{50} values for ketoconazole were calculated by fitting the Hill equation to the data using non-linear regression of the plot of percent control activity *versus* concentration of ketoconazole using Prism (GraphPad Software, Version 6). The enzymes activities in the presence of ketoconazole were compared with the control samples without addition of ketoconazole.

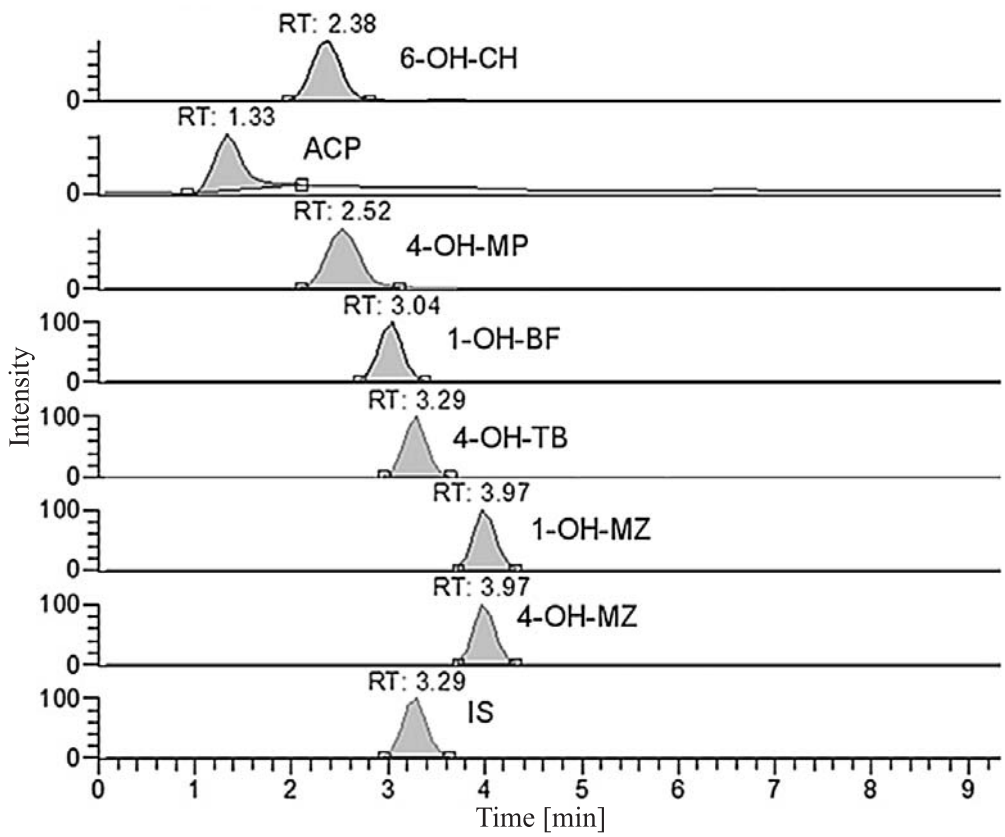

Figure 2. Extracted SRM chromatograms of simultaneous analysis of seven metabolites of model substrates and IS in calibration standard sample. Abbreviations see Table 2

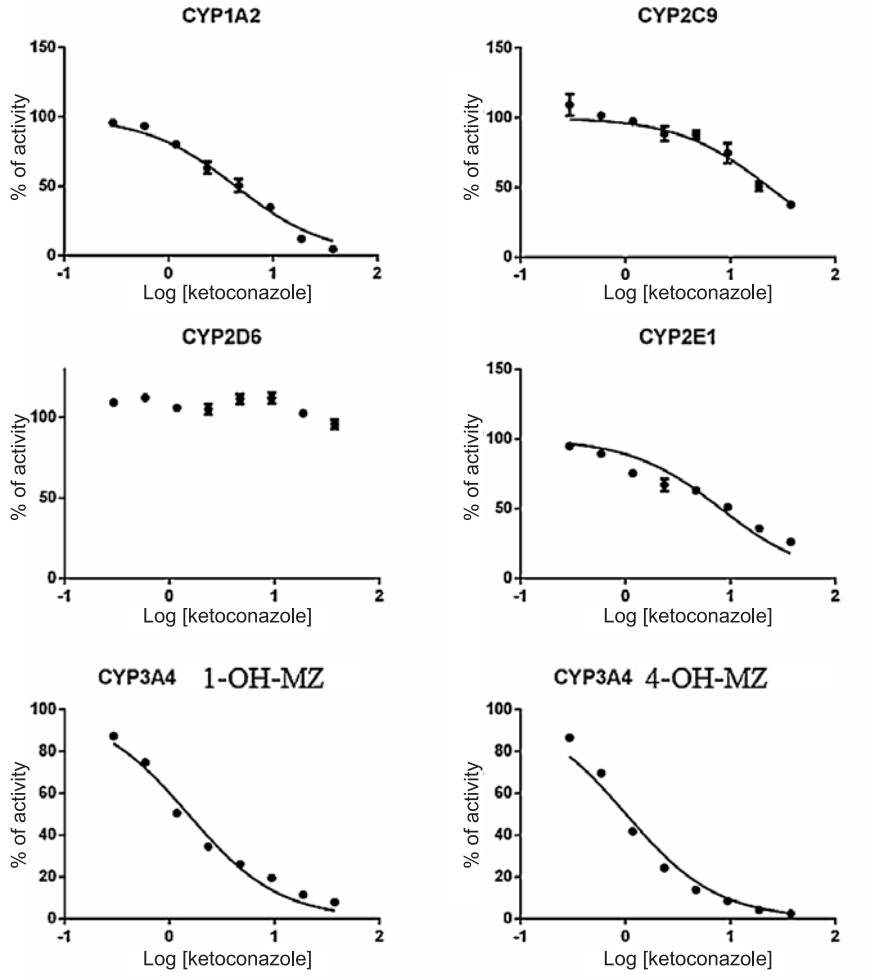

Figure 3. The activity of selected isoenzymes after incubation of model substrates of CYP450 with ketoconazole in rat liver microsomes. Data are shown as the mean ± SD, n = 3. 1-OH-MZ - 1'-hydroxymidazolam; 4-OH-MZ - 4'-hydroxymidazolam

RESULTS AND DISCUSSION

Method development

Metabolism studies of new compounds that occur with the participation of enzymes require simple, quick, sensitive and reproducible analytical methods. The aim of this work was to develop and validate a simple and reliable LC/MS/MS method, which can be used to determine simultaneously seven metabolites of specific substrates of selected CYP450 isoenzymes in rat liver microsomes.

Animal models are widely used to predict kinetics and toxicity of drugs in human, and cytochrome P450 enzymes are mainly involved in the metabolism of drugs both in human and rodents. The three families: CYP1, CYP2 and CYP3 play a significant role in drug biotransformation (30). Differences between e.g., human and rat enzymes concern mostly individual genes, while not only families but also subfamilies are common. Major isoenzymes of CYP450 in human liver are: CYP 1A2, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2D6 and CYP 3A4/5, while in rat are: CYP 1A1, CYP 1A2, CYP 2B1, CYP 2C6, CYP 2C11, CYP 2D1/2, CYP 3A1/2 and CYP 4A1. Enzymes CYP 2C and CYP 3A appear to be the most abundant cytochromes P450 in rat and human liver, respectively (31).

Most of the described in the literature experiments deal with human microsomes, while there are a lot of work performed on rat microsomes, because rats are the most commonly used model in preclinical studies (6, 32-34). Zhang et al. (6) studied phenacetin, tolbutamide, mephenytoin, dextromethorphan and midazolam metabolism incubated

individually and in cocktail form with rat microsomes and no significant differences were observed. Phenacetin and tolbutamide metabolism in rat liver microsomes were studied by Jurica et al. (32). Coctail assay was used to study herb-drug interaction in rat microsomes by Xia et al. (33). Recently, comparison between human and rat microsomal CIME cocktail assay was verified by Videau et al. (34).

In presented work, the model substrates of selected isoenzymes of CYP450 were added in the form of cocktail to rat microsomes. Substrates were selected on the basis of a number of literature reports describing the metabolism of drugs in rats (30, 31, 35–37) and the EMA guideline describes probe substances, which can be used for evaluation of cytochrome P450 activity in human (38). The following model substrates were selected: phenacetin for CYP 1A2, tolbutamide for CYP 2C9, S-mephenytoin for CYP 2C19, bufuralol for CYP 2D6, chlorzoxazone for CYP 2E1 and midazolam for CYP 3A4. Fragmentation mass spectra of selected metabolites and proposed fragmentation pathways are shown in Figure 1.

The analytes were at first detected in full scan mass spectrum and then fragmented to product ions to ascertain their pseudo-molecular ions and to select product ions used in SRM mode. Except of chlorzoxazone and its metabolite, which responded better in negative ionization mode, all other substances were quantified in positive ion mode.

A simple protein precipitation method was used for sample purification and different precipitation mixtures were verified. The results showed that the most efficient way was precipitation using a mixture of acetonitile and acetone (1 : 1, v/v).

Table 3. Intra- and inter-day precision and accuracy of the method.

		ACP	6-OH-CH	4-OH-MP	1-OH-BF	4-OH-TB	1-OH-MZ	4-OH-MZ
Intra-day precision (RSD%)	LLOQ	11.1	10.4	13.0	19.5	4.5	12.6	13.0
	QC low	3.2	8.8	4.0	7.5	4.4	7.1	5.1
	QC medium	11.0	7.6	3.4	2.7	2.5	1.7	1.0
	QC high	8.4	6.5	3.8	2.1	3.0	3.5	2.7
Inter-day precision (RSD%)	LLOQ	13.9	12.0	12.0	7.5	3.2	5.4	6.6
	QC low	5.6	7.5	7.4	14.9	6.1	6.3	4.1
	QC medium	11.7	5.9	11.0	4.0	7.5	4.7	3.0
	QC high	10.8	5.4	7.5	3.5	3.5	4.0	2.4
Accuracy (%)	LLOQ	118.7	106.3	103.1	83.7	107.0	97.2	96.5
	QC low	102.9	95.5	106.0	113.6	99.8	98.2	109.2
	QC medium	103.3	98.1	104.6	106.7	99.8	103.8	102.7
	QC high	107.4	93.0	99.7	99.6	97.6	105.4	98.2

Abbreviations see Table 2.

Table 4. Extraction recovery and absolute matrix effect of the method.

		ACP	6-OH-CH	4-OH-MP	1-OH-BF	4-OH-TB	1-OH-MZ	4-OH-MZ
Recovery [%]	QC low	85.4	92.0	68.4	83.5	74.1	79.4	82.1
	QC medium	70.5	91.1	68.3	86.4	74.3	84.8	83.0
	QC high	75.3	94.8	71.4	89.0	78.9	91.1	90.6
	Mean ± SD	77.1 ± 7.6	92.6 ± 1.9	69.4 ± 1.8	86.3 ± 2.8	75.8 ± 2.7	85.1 ± 5.9	85.2 ± 4.7
Absolute matrix effect [%]	QC low	65.0	16.5	8.9	-23.7	20.2	12.3	-14.8%
	QC medium	61.9	17.9	14.0	5.2	22.7	-5.4	0.2
	QC high	60.1	10.2	10.1	4.0	20.8	-6.8	2.0
	Mean ± SD	62.3 ± 2.5	14.9 ± 4.1	11.0 ± 2.7	-4.8 ± 16.4	21.2 ± 1.3	0.03 ± 10.6	-4.2 ± 1.2

Abbreviations see Table 2.

Method validation

Selectivity and linearity

No interference was found at the retention time of the analytes and the IS during analysis of blank microsomes from six independent sources (signal-to-noise ratio < 9). Extracted SRM chromatograms of seven metabolites of model substrates and IS in calibration standard sample are shown in Figure 2.

The assay was linear over the concentration range of 10-2000 ng/mL for 4'-hydroxymephenytoin and 1-hydroxytolbutamide, 50-2000 ng/mL for 1-hydroxybufuralol, and 25-2000 ng/mL for the rest of the analyzed metabolites. Linear ranges, determination coefficients, LOD and LLOQ values are shown in Table 2. The best linear fit of the calibration curve for each analyte was obtained using a weighting factor 1/x. Precision and accuracy of the LLOQ met the requirements both of FDA and EMA guidelines, RSD < 20% and 80-120%, respectively.

Precission and accuracy

For all quantified metabolites, the intra-day coefficients of variation were less than 11% for low, medium and high QC concentrations, and less than 20% for LLOQ. The inter-day data were also reproducible with CV less than 12% for prepared QC samples, and less than 14% for LLOQ. The study shows good accuracy: 93-114% for low, medium and high QC concentrations, and 83-119% for LLOQ level. Precision and accuracy data for each of the analytes are summarized in Table 3.

Recovery and matrix effect

Table 4 shows the results of recovery and absolute matrix effect for all selected analytes. A simple precipitation method used in this study yielded a mean recovery greater than 69% for all analytes. The extraction recovery was noticed to be consistent for each analyte over the entire QC concentration range, what can indicate that extraction efficiency of the method is reliable over the studied concentration.

The mean values of absolute matrix effect ranged from 0.03 to 62.3%, and the higher matrix effect was observed for acetaminophen (62.3%) but even so, the calibration curve covered the expected concentration range. To confirm the reliability of this method, the relative matrix effect was studied. Coefficients of variation of calibration curve slopes for compounds studied were less than 1.8% (Table 5), and according to Matuszewski indications (29), the method was free from the relative matrix effect liability.

Stability study

Analyzed aliquots were stable in autosampler batch in 10°C for at least 24 h. The mean values of the calculated concentrations were within 94-113% and therefore acceptable (Table 6). Consequently, extracts from sample incubation may be placed in autosampler rack for up to 24 h.

Application of the method

The method was used to assess the strength of inhibition of ketoconazole on selected CYP450 isoenzymes. The incubation curves are shown in Figure 3 and determined IC_{50} values for ketoconazole are listed in Table 7. The results showed that ketoconazole had the most potent inhibitory effect on CYP 3A4 (IC_{50} = 1 μM), but also suppressed activity of CYP 1A2 (IC_{50} = 4.4 μM), CYP 2E1 (IC_{50} = 8.1 μM) and CYP 2C9 (IC_{50} = 23.5 μM), whereas the activity of CYP 2D6 was not changed.

Many researchers found that antifungal azoles can inhibit the activity of several cytochrome P450

Table 5. Individual and mean values of standard line slopes for analytes studied.

	Slope of the calibration curve						
	ACP	6-OH-CH	4-OH-MP	1-OH-BF	4-OH-TB	1-OH-MZ	4-OH-MZ
	0.00048	7.8309E-06	0.000155	0.00275	0.00043	0.0046	0.00147
	0.00048	7.8867 E-06	0.00015	0.00286	0.00044	0.0049	0.00148
	0.0005	8.2371 E-06	0.000153	0.00291	0.00043	0.00478	0.00152
	0.00049	8.3464 E-06	0.00015	0.00288	0.00043	0.00468	0.00153
	0.00051	8.4954 E-06	0.000149	0.00286	0.00047	0.00461	0.00147
	0.00052	8.2371 E-06	0.000139	0.00288	0.00042	0.00491	0.00148
Mean	0.000496	8.17227 E-06	0.000149	0.00285	0.000436	0.004747	0.001492
CV [%]	3.3	3.2	3.7	1.9	4.0	2.9	1.8

Abbreviations see Table 2.

Table 6. Autosampler stability data for analytes studied.

	Stability of analytes [%]						
	ACP	6-OH-CH	4-OH-MP	1-OH-BF	4-OH-TB	1-OH-MZ	4-OH-MZ
QC low	103.2	95.7	101.3	100.4	109.5	102.2	94.9
QC medium	111.6	106.4	105.7	106.3	112.6	105.0	94.4
QC high	111.2	102.7	104.7	103.6	104.4	105.2	95.7

Abbreviations see Table 2.

Table 7. The influence of ketoconazole on the inhibition of selected isoenzymes.

Isoenzyme	Substrate	Metabolite	Ketoconazole IC_{50} [μM]
CYP 1A2	Phenacetin	Acetaminophen	4.40 ± 0.29
CYP 2C9	Tolbutamide	4-Hydroxytolbutamide	23.5 ± 0.05
CYP 2D6	Bufuralol	1-Hydroxybufuralol	—-
CYP 2E1	Chlorzoxazone	6-Hydroxychlorzoxazone	8.10 ± 0.041
CYP 3A4	Midazolam	1'-Hydroxymidazolam	1.51 ± 0.024
	Midazolam	4'-Hydroxymidazolam	1.02 ± 0.027

isoenzymes. In the work (39), Paine et al. demonstrated the inhibition of intestinal CYP 1A2 by ketoconazole. Suppression of tolbutamide hydroxylase (reaction catalyzed by CYP 2C9) by azoles in *in vitro* experiments was studied by Back et al. (40). They found that ketoconazole inhibited CYP 2C9 in human microsomes with IC_{50} = 16.5 μM. The effect of non-selective cytochrome P450 inhibitors such as 1-aminobenzotriazole, SKF-525A and ketoconazole on human cytochrome P450 isoenzymes was studied by Emoto et al. (41). They found that ketoconazole exerts weak inhibitory potential on CYP 1A2 activity (Ki = 120 μM), CYP 2C competitive inhibition (Ki = 7.9 μM for CYP 2C9 and Ki = 6.9 μM for CYP 2C19), mixted inhibition on CYP 2D6 activity (Ki = 12 μM) and CYP 2E1 activity (Ki = 41 μM) and competitive inhibition on CYP 3A4 (Ki = 0.02 μM).

The influence of ketoconazole on amiodarone metabolism both in human and rat microsomes was studied by Elsherbiny et al. (42). Interestingly, in rat liver microsomes ketoconazole exerted higher inhibitory potential on CYP 2D enzymes than on CYP 3A family. The human isoenzymes most inhibited by ketoconazole were CYP 1A1, CYP 3A4, CYP 2D6 and CYP 1A2, respectively. The working groups of Kobayashi et al. (43) and Eagling et al. (44) studied the effect of different inhibitors on cytochrome P450 activity in rats and showed that ketoconazole is not specific CYP 3A inhibitor, but it can suppress the activity of other isoenzymes. Only in one *in vivo* work, it was shown that ketoconazole had no effect on cytochrome P450 activity in rats (45).

Despite the use in presented studies different substrates and different substrate concentrations it can be seen that ketoconazole has the strongest inhibitory potential on CYP3A4 but this drug is also strong inhibitor of CYP1A2 and CYP2E1 and moderate inhibitor of CYP2C9. Presented data are generally in good agreement with earlier cited studies and confirmed that ketoconazole should not be consider as a selective CYP 3A4 inhibitor in rat liver microsomes.

CONCLUSIONS

A sensitive and an accurate LC/MS/MS method has been developed for the simultaneous evaluation of the activities of six cytochrome P450 izoenzymes in rat liver microsomes. The method was successfully validated following FDA and EMA guidelines that ensures the acceptability of its performance and the reliability of analytical results. The applied screening cocktail was verified with ketoconazole, a known CYP450 enzyme inhibitor. The resulting method can be used as a first and rapid screening of new compounds, drug candidates using rat liver microsomes.

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Declaration of interest

The authors report no conflict and declaration of interest.

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