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Development and validation of a fast and sensitive UPLC–MS/MS method for the quantification of six probe metabolites for the in vitro determination of cytochrome P450 activity

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ABSTRACT

A fast and sensitive UPLC–MS/MS method was developed and validated for the simultaneous quantification of six probe metabolites for the in vitro cytochrome P450 activity determination in hepatic microsomes from patients with hepatic impairment. The metabolites acetaminophen (CYP1A2), 4′-hydroxy-mephenytoin (CYP2C19), 4-hydroxy-tolbutamide (CYP2C9), dextrorphan (CYP2D6), 6-hydroxy-chlorzoxazone (CYP2E1) and 1-hydroxy-midazolam (CYP3A4), together with the internal standard chlorpropamide, were separated on a Waters Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm particle size) with VanGuard pre-column (5 mm × 2.1 mm, 1.7 μm particle size). A short gradient elution (total run time of 5.25 min), using water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B) at a flow rate of 400 μl/min, was used. The metabolites were detected with a triple quadrupole mass spectrometer in the multiple reaction monitoring mode. Two runs, one in the positive ionization mode and one in the negative mode, were necessary for the detection of all metabolites. The method was selective and showed good accuracy (84.59–109.83%) and between-day (RSD% < 5.13%) and within-day (RSD% < 9.60%) precision. The LOQ was in full accordance with the intended application, and no relative matrix effects were observed. Also, the sample incubation extracts were stable after three freeze–thaw cycles. The usability of the method was demonstrated by the incubation of pediatric microsomes with subsequent quantification of the formed metabolites and CYP activity calculation.

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1. Introduction[1]

The activity of the cytochrome P450 enzyme system may be compromised in patients with hepatic pathologies. As this enzyme system is responsible for the metabolism of the vast majority of drugs, these patients often show altered drug pharmacokinetics, which may lead to inefficient therapy or adverse reactions [1]. In order to study the differential alterations in activity of the six most important isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4), in vitro CYP activity can be investigated in hepatic microsomes. For the determination of the enzyme activity, these assays use the incubation of the microsomes with well established isoform-specific probe substrates, and the quantification of the resulting metabolite levels. Many different combinations of probe substrates have been used. The most commonly used and preferred in vitro probe substrates are phenacetin (PH; CYP1A2), S-mephenytoin (ME; CYP2C19), tolbutamide (TB; CYP2C9), dextromethorphan (DM; CYP2D6), chlorzoxazone (CZ, CYP2E1) and midazolam (MDZ; CYP3A4) [2].

Liquid chromatography, coupled to mass spectrometry (LC–MS) has been shown to be a valuable tool in CYP450 assays for the quantification of the metabolites, as reviewed by Youdim and Saunders [3] and Lahoz et al. [4]. Different LC–MS(MS) methods for simultaneous quantification of metabolites have been described [5–9]. However, to our knowledge, only two methods used a combination of probes comparable to those mentioned above [5,6]. Li et al. [6] used two different HPLC systems with isocratic elution, one coupled to an MS with a positive atmospheric pressure chemical ionization interface, and one using the negative electrospray ionization mode. In the method of Kim et al. [5], gradient elution was performed, followed by detection of most of the metabolites in the positive electrospray ionization mode, and one (HCZ) in the negative mode. Both methods showed sufficient sensitivity for the intended applications.

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[1] *Non-standard abbreviations*: Acetaminophen (AP); Chlorpropamide (CP); Cytochrome P450 (CYP); Dextrorphan (DX); 6-hydroxy-chlorzoxazone (HCZ); 1-hydroxy-midazolam (HMDZ); 4′-hydroxy-mephenytoin (HME); 4-hydroxy-tolbutamide (HTB); Internal standard (IS); Matrix factor (MF).

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A marked reduction in the time of analysis of LC separations has been established by using columns with sub-2 μm particles [10]. The combination of small particles and higher solvent flows in ultra-high performance liquid chromatography (UPLC), enables a fast elution and improved resolution in the chromatographic separation. This technology therefore offers a powerful tool for a high throughput analysis of samples. Recently, a UPLC–MS/MS method for the quantification of six probe metabolites was published. However, this study lacked the inclusion of a probe for the CYP2E1 isoform activity determination [11,12]. As this isoform has been shown to be affected by liver disease [13], a probe to evaluate the activity of CYP2E1 is essential in the intended application as described above.

Matrix effect is a major issue in LC–MS method development [14]. Despite the specificity of MS/MS, co-eluting compounds may cause suppression or enhancement of the analyte response [15]. Improved chromatographic separation of the analytes or extended sample preparation has been suggested to minimize these matrix effects [16]. Therefore, base line separation of the chromatographic peaks should be the aim, rather early than late in the method development. Furthermore, base line separation also contributes to an increased selectivity of the detection method. In the previously cited studies using the same combination of probes [5,6], no complete base line separation of the metabolites was accomplished. Nevertheless, Li et al. [5,6] concluded that matrix effects were absent, whereas in the method described by Kim et al. [5], matrix effects were not evaluated. UPLC could be a favourable approach to yield full chromatographic separation without unacceptably prolonging sample turn over times.

This study aimed for the development of a generic fast chromatographic method that could be used for the detection of those metabolites most interesting for CYP activity evaluation (acetaminophen (AP), 4′-hydroxy-mephenytoin (HME), 4-hydroxy-tolbutamide (HTB), dextrorphan (DX), 6-hydroxy-chlorzoxazone (HCZ) and 1-hydroxy-midazolam (HMDZ)). This method is useful in either positive or negative electrospray ionization mode. Furthermore, an extensive analytical validation was performed prior to the implementation of the method. The developed and validated method was tested for its usability in incubation experiments with microsomes of pathological origin.

2. Materials and methods

2.1. Chemicals and biological samples

Phenacetin, acetaminophen, tolbutamide, 4-OH-tolbutamide, S-mephenytoin, 4′-OH-mephenytoin, dextromethorphan, dextrorphan, chlorzoxazone, 6-OH-chlorzoxazone and chlorpropamide (CP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Midazolam and 1-OH-midazolam were kindly donated by Roche (Basel, Switzerland). NADPH was obtained from Biopredic International (Rennes, France). Potassium chloride, potassium dihydrogenphosphate and dipotassium hydrogenphosphate were purchased from VWR (Leuven, Belgium). All other chemicals were of analytical grade.

Microsomes were prepared from liver samples [17], collected from the diseased liver from children undergoing a liver transplantation (approved by the Ethics Committee of Ghent University Hospital, B67020084281).

2.2. Standard solutions and calibrators

Primary stock standards of 1 mg/ml in methanol of all metabolites and the internal standard were prepared and stored at −20 °C. Stock standards were mixed in the appropriate proportions and serially diluted in methanol. Microsomal calibration standards consisted of metabolites spiked to the microsomal incubation medium. This incubation medium was prepared by mixing 1 ml 5 mM NADPH, 1 ml 1.25 mg/ml microsomal protein, 1 ml 1.15% KCl, 1 ml 0.2 M potassium phosphate buffer (pH 7.4) and 0.5 ml stopreagent ($H_2O/CH_3CN/HCOOH$; 42:55:3 (v:v)) containing 220 ng/ml internal standard (IS; chlorpropamide). After mixing and protein denaturation, a specific amount of stock solution was spiked to the incubation medium. Water was added to obtain a final volume of 5.5 ml per calibrator. Calibrators were centrifuged at 20,000 × *g* for 15 min at 4 °C, supernatant was separated and stored at 4 °C.

2.3. Chromatographic conditions

UPLC was performed on a Waters Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm particle size) with VanGuard pre-column (5 mm × 2.1 mm, 1.7 μm particle size) using a Acquity UPLC system (Waters, Manchester, UK). The column was kept at 35 °C. An aliquot of 20 μl was injected using full loop injection. The mobile phase consisted of water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). At a flow rate of 400 μl/min, the amount of eluent B was increased linearly from 5% to 80% in 4 min, kept at 80% B for 0.18 min, and then the column was left to re-equilibrate at initial conditions for 1.8 min, resulting in a total turnover time of 5.25 min. Due to the presence of the potassium phosphate buffer in the sample, strong wash contained 95/5 (v/v) water/methanol, and weak wash and needle wash 50/50 (v/v) water/methanol.

2.4. MS conditions

Eluting compounds were detected using a Waters Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK). The electrospray source (orthogonal Z-spray®) used a standard 120 μm capillary. Optimal source temperature and desolvation temperature were 150 and 400 °C, respectively. Cone gas flow and desolvation gas flow (both nitrogen) were set at 175 and 575 l/min. Argon was used for the collision-induced fragmentation. Due to inadequate ionization for some compounds indebted to their particular chemical structure, both the positive and negative electrospray ionization mode were used as interface. As the instrument is not capable of effective in-run polarity switching, two runs were necessary for each cocktail sample. Data were collected and processed using the MassLynx® and QuanLynx® software (Micromass Waters).

2.5. Microsomal incubations

Microsomes were incubated with each probe substrate in a concentration near their apparent Km (see Table 1) [18]. In short, 50 μl of the probe was added to 50 μl 1.25 mg microsomal protein/ml (final concentration of 0.25 mg protein/ml), 50 μl 1.15% KCl and 50 μl 0.2 M potassium phosphate buffer (pH 7.25). To initiate the reaction, 50 μl of 5 mM NADPH was added after preincubation of 3 min at 37 °C (total reaction volume of 250 μl). The reaction was terminated after exactly 15 min (40 min for incubation with ME) by adding 25 μl of the stopreagent ($H_2O/CH_3CN/HCOOH$; 42:55:3) containing the internal standard, and cooling the mixture on ice. Subsequently, the samples were centrifuged for 10 min at 20,000 × *g* (4 °C), 200 μl of the supernatant was transferred to a 250 μl insert, and samples were frozen at −20 °C until analysis.

2.6. Method validation

The method was validated according to the "Guidance for Industry – Bioanalytical Method Validation" recommended by the FDA [19].

Table 1
Incubation conditions and analytical parameters for the individual metabolites and internal standard.

	CYP1A2	CYP2C9	CYP2C19	CYP2D6
Substrate	Phenacetin	Tolbutamide	S-mephenytoin	Dextromethorphan
Km (μM)	50	100	100	5
Metabolite	Acetaminophen (AP)	4-OH-tolbutamide (HTB)	4′-OH-mephenytoin (HME)	Dextrorphan (DX)
t_R (min)	0.99	2.02	1.64	1.48
Ionization mode	ESI+	ESI−	ESI+	ESI+
Capillary voltage (kV)	+3.25	−2.80	+3.25	+3.25
Cone voltage (V)	12	24	24	22
Collision energy (eV)	28	12	14	28
Precursor ion (m/z)	152.10	285.09	235.41	258.00
Quantifier (m/z)	110.00	185.60	150.00	156.70
Qualifier (m/z)	93.00		133.00	132.80
Range (nM)	18.52–8333	2.79–5238.34	18.43–8293.56	2.13–959.29
QC low (nM)	52.09	21.83	51.83	6.00
QC medium (nM)	1111.20	698.45	1105.81	127.91
QC high (nM)	4166.98	2619.17	4146.78	479.65

	CYP2E1	CYP3A4	Internal standard	
Substrate	Chlorzoxazone	Midazolam	Chlorpropamide (CP)	
Km (μM)	50	5		
Metabolite	6-OH-chlorzoxazone (HCZ)	1-OH-midazolam (HMDZ)		
t_R (min)	1.44	2.08	2.75	
Ionization mode	ESI−	ESI+	ESI+	ESI−
Capillary voltage (kV)	−2.80	+3.25	+3.25	−2.80
Cone voltage (V)	25	22	25	20
Collision energy (eV)	14	19	11	11
Precursor ion (m/z)	183.83	342.04	276.86	274.89
Quantifier (m/z)	119.80	323.70	174.69	189.57
Qualifier (m/z)	147.70	202.80	191.65	125.85
Range (nM)	49.09–5522.79	4.29–1929.60		
QC low (nM)	86.29	12.06		
QC medium (nM)	1840.93	257.28		
QC high (nM)	3451.77	964.80		

2.7. Calibration curve

Calibration curves were constructed over a specific range for each compound (based on Walsky and Obach [20]). An analysis of variance with lack of fit test (StatGraphics 4.1, Warrenton, VA, US) was used to determine whether the selected model of the relation between analyte-to-IS ratio and concentration was adequate to describe the observed data ($p > 0.10$), or whether a more complicated model was required. If necessary, a weighting factor was used to increase the accuracy. The statistical significance of the terms of the model (slope and intercept) was assessed by the comparison of calibration curves analyzed in 5 independent runs using an ANOVA for variables in the order fitted ($p > 0.10$).

Quality control (QC) samples at three different concentration levels (replicate analysis; in total 6 samples) were used to either accept or reject the analytical run. At least four out of six of the QC samples should be within 15% of their nominal value, whereas two out of six QC samples (not all replicates at the same concentration level) may be outside 15% of the nominal value. Table 1 shows the concentration ranges of the different calibration curves and the concentration levels of the QC samples.

2.8. Precision, accuracy and LOQ

Within-day precision and accuracy were determined by analyzing six aliquots of each QC sample on the same day. Between-day precision was evaluated by analyzing the QC samples in duplicate for five days. Precision was expressed as the relative standard deviation (RSD%) of the measured QC samples and accuracy was calculated as trueness.

2.9. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) was calculated following the IUPAC definition [21] and was defined as 3 times the standard deviation of the intercept, divided by the slope or analytical sensitivity. However, as this equation only counts for linear calibration curves, the LOD of the metabolites using a quadratic calibration curve was calculated following Burkart [22].

The limit of quantification (LOQ) was defined as the lowest concentration, which could be measured ($n = 6$) with a precision not exceeding 20% (RSD%) and with an accuracy between 80% and 120%. The lowest calibrator of the calibration curve was targeted as LOQ.

2.10. Selectivity and stability

Selectivity was assessed by examining peak interference (signal-to-noise ratio <9) from six independent sources of microsomes. Stability of the sample incubation extracts was tested after storage in the autosampler (15 °C) for 14 h and after three freeze–thaw cycles. The autosampler stability was tested by the analysis of 3 aliquots of a middle standard in the beginning of an analytical run, and 3 aliquots 14 h (average total run time) later. Stability was determined by statistical comparison of the calculated concentrations (t-test, 95% significance level). Freeze–thaw stability was assessed using the commonly accepted procedure: after three freeze–thaw cycles, samples were compared with regularly analyzed samples with the same amount of analyte (t-test, 95% significance level).

2.11. Matrix effect

Matrix effect was evaluated following Viswanathan et al. [23], through the calculation of the internal standard-normalized (IS-normalized) matrix factor (MF), using Eq. (1).

$$\text{IS-normalized Matrix Factor} = \frac{\text{peak ratio (analyte/IS) in presence of matrix ions}}{\text{peak ratio (analyte/IS) in absence of matrix ions}} \quad (1)$$

A value different from 1, indicated an absolute matrix effect. The relative matrix effect was evaluated through the determination of the coefficient of variation (CV%) of the IS-normalized MF of five different matrix sources. This CV% should not exceed 15%.

2.12. Application of the method

The above described validated analytical method was used for the analysis of samples from a pharmacokinetic study. CYP450 enzyme activities were determined in three liver samples obtained from explanted livers from children undergoing liver transplantation for various reasons (biliary atresia, cystic fibrosis, progressive familial intrahepatic cholestasis). The pediatric microsomal samples were incubated with the probe substrates, and the formed metabolites were quantified.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

Sample preparation is of major importance in LC–MS/MS analysis. In order to reduce matrix interference, complex matrix ions, such as proteins, have to be removed from the sample. Protein precipitation is the most widely used and easiest technique for this purpose. In CYP450 assays this is typically achieved through the addition of an equal volume of organic solvent (mostly acetonitrile), or through acidification. Subsequently, the sample can be filtered [20] or centrifuged [5,24] in order to remove the proteins. The use of large volumes of organic solvent was deemed highly undesirable in this method, as this dilutes the sample at least three times, moreover providing an injection organic solvent composition incompatible with good reversed phase chromatography. In CYP450 assays, protein denaturing can also serve to end the enzymatic incubation, i.e. as a stopreagent. Taking all of this into consideration, a small volume (25 μl in 250 μl incubation volume) of a reagent containing a combination of acetonitrile and a strong acid (formic acid) was used in order to stop the enzymatic reaction and at the same time provide adequate conditions to pellet the incubation mix proteins. Addition of a small volume of 60% perchloric acid was also evaluated, but as this significantly affected peak shape, this method for sample preparation was not retained. Besides acetonitrile and formic acid, the stopreagent contained the internal standard, chlorpropamide. The terminated incubation medium was vortex mixed and placed on ice for further protein precipitation. After centrifugation for 10 min at 20,000 × *g* and 4 °C, supernatant was collected and could be readily injected. As the sample preparation step is incorporated in the in vitro protocol (termination step of the incubation), sample handling is minimized and unduly dilution of the resulting extract is avoided. This in combination with a selective and sensitive MS method thus increases the quality of the result, as well as the throughput of the method [4].

3.2. Optimization of the mass spectrometric (MS) detection

The individual parameters for the detection with the triple quadrupole MS were optimized by the infusion of a standard solution of the metabolites (1 μg/ml (100 ng/ml for DX) in methanol/water 50/50 (v/v) + 0.1% formic acid). Detection of all metabolites (depicted in Fig. 1) was evaluated in the positive electrospray ionization (ESI+) mode. No or poor MS responses were observed for HCZ and HTB in the positive mode. In contrast, intense MS signals were observed for AP, DX and HMDZ. Infusion of HCZ and HTB in the negative ESI (ESI−) mode showed strong responses. Therefore, detection of AP, DX and HMDZ was performed using the ESI+ mode, whereas HCZ and HTB need the ESI− mode. As HME showed poor MS response in both ESI+ and ESI− mode during infusion, this compound was initially detected in both modes. The MS parameters were optimized in order to achieve the highest MS response. The optimal cone voltages were determined, and an acceptable signal for the detection of HME in ESI+ was obtained by an increased source (150 °C) and desolvation temperature (400 °C). Consequently, the preferred detection mode of HME was finally ESI+. The collision energy (using argon gas) was optimized for each metabolite, based on the product ion mass spectra. Collision energy was varied until the intensity of the precursor ion $[M+H]^+$ was 10–20% of the intensity of the quantifier ion (i.e. the product ion with the highest response).

For the actual detection using multiple reaction monitoring (MRM), the two most abundant product ions were selected for each analyte; except for HTB, where only one product ion was formed during fragmentation. The most abundant product ion served as quantifier (for the quantification), the other as qualifier (confirmation of the identification).

Based on Kim et al. [5], chlorpropamide was selected as internal standard, as this molecule can be detected in both ESI+ and ESI− mode. The internal standard was added to the sample at the end of the incubation reaction (in the stopreagent) and was used to correct for variability of the analytical system. Table 1 depicts the MS parameters of all the metabolites and the internal standard.

3.3. Optimization of the chromatographic separation

In order to obtain a short analysis time, a sub 2-μm particle size UPLC column was selected. Considering the chemical diversity in structures of the metabolites, gradient elution was applied, using water containing 0.1% formic acid as eluent A. Eluent B (acetonitrile + 0.1% formic acid) was increased gradually from 5% to 80% during 4 min using a 0.4 ml/min flow. A chromatogram in both ionization modes is shown in Fig. 2. All peaks were base line separated.

An increase in flow rate (>0.4 ml/min) also allowed good separation of the peaks. However, quantification with the MS in MRM was no longer adequate, as the MS system could not switch masses as fast as needed to obtain sufficient scans per peak (i.e. a minimum of 10 points across each peak). This led to a decreased sensitivity, and thus, a flow rate of 0.4 ml/min was defined to be optimal. The final gradient already enabled an analysis in only 5.25 min, including the equilibration time required for the next run.

3.4. Method validation

3.4.1. Calibration curves

Lack of fit analysis of the model describing the relation between the analyte-to-IS ratio and concentration demonstrated a linear correlation for AP, DX and HMDZ, and a quadratic correlation for HTB, HME and HCZ ($p < 0.10$). Due to the large concentration range, a weighting factor of $1/x^2$ and $1/x$, was necessary for the linear and the quadratic models, respectively, to obtain the best residuals, and consequently the best accuracy. When five independent calibration

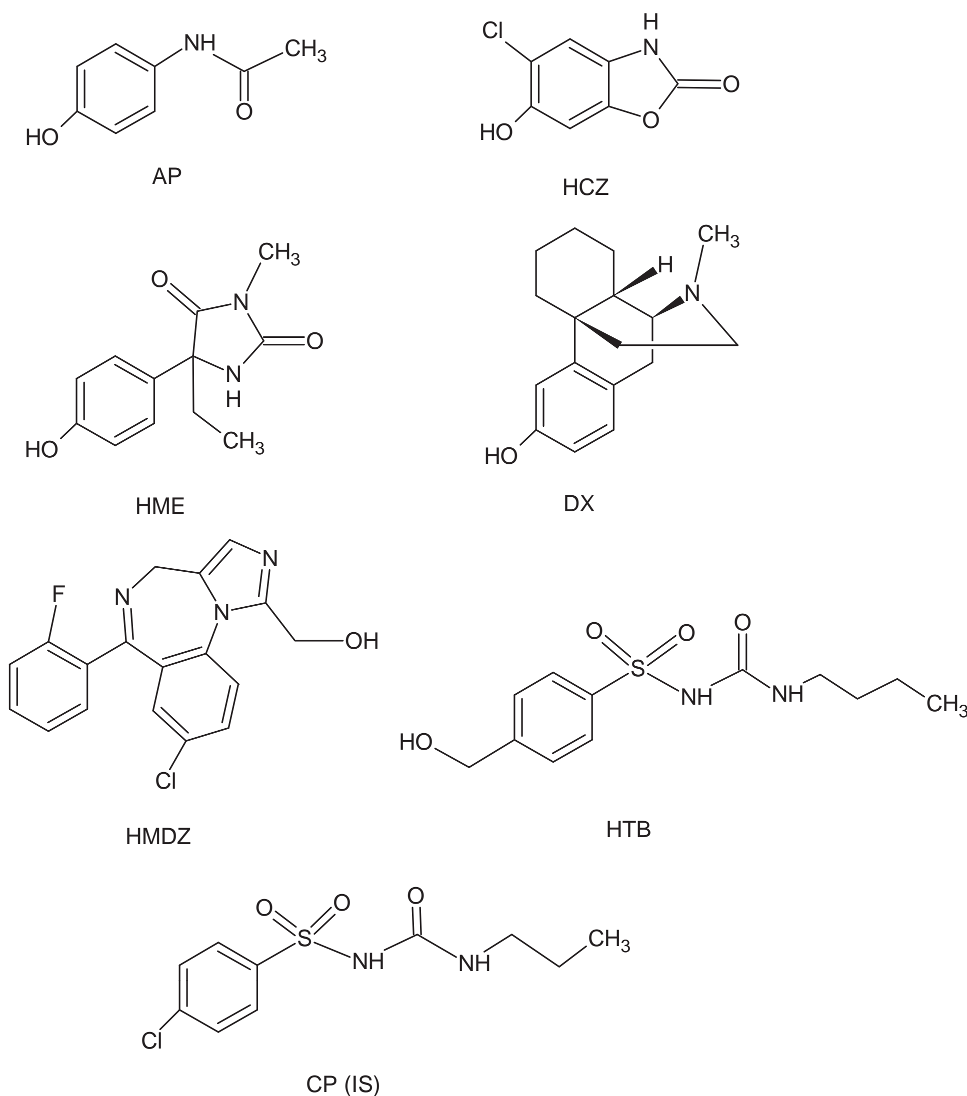

Fig. 1. Molecular structures of the metabolites. (AP: acetaminophen, HCZ: 6-OH-chlorzoxazone, HME: 4′-OH-mephenytoin, DX: dextrorphan, HMDZ: 1-OH-midazolam, HTB: 4-OH-tolbutamide, CP: chlorpropamide, IS: internal standard.)

curves were compared statistically, they were proven to have an equal slope and intercept ($p > 0.10$).

3.5. Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection (LOD) and quantification (LOQ) of all metabolites are depicted in Table 2. Precision and accuracy of the LOQs met the requirements of the FDA (RSD < 20%, and between 80% and 120%, respectively). The obtained LOQs are evaluated as adequate for the intended pharmacokinetic application(s), as very low CYP enzyme activities can still be detected, important in diseased liver situations.

The between-day and within-day precision were better than 5.13% and 9.60% (RSD%), respectively, and the accuracy ranged from 84.59 to 109.83% (see Table 3). Thus, the method proved to be precise and accurate.

3.6. Selectivity and stability

No interference was observed at the retention times of the analytes and the IS when analyzing blank microsomes from six independent sources (signal-to-noise ratio >9; data not shown).

Statistical analysis of the autosampler stability only showed significant differences of the HME and DX concentration after 14 h ($p < 0.05$). However, the mean of the calculated concentration is still within the 85–115% interval of the nominal value (DX: 88.48%, HME: 92.11%) and therefore acceptable. Consequently, sample incubation extracts may be placed in the autosampler for up to 14 h.

The evaluation of the freeze–thaw stability showed similar results: HME QC high, HMDZ QC low and high and HCZ QC mid showed statistically significant differences ($p < 0.05$), but the mean of the calculated concentration is within the 85–115% interval of the nominal value. This means that (large) batches of incubations can be stored temporarily upon final analysis.

3.7. Matrix effect

Co-eluting compounds may cause enhancement or suppression of the ionization of the analyte. Due to the scarce sample preparation of the biological matrix (only protein precipitation), matrix effects were expected. In order to try to reduce these matrix effects base line separation of the metabolites was pursued. As a quantitative measure of matrix effect, the IS-normalized matrix factor

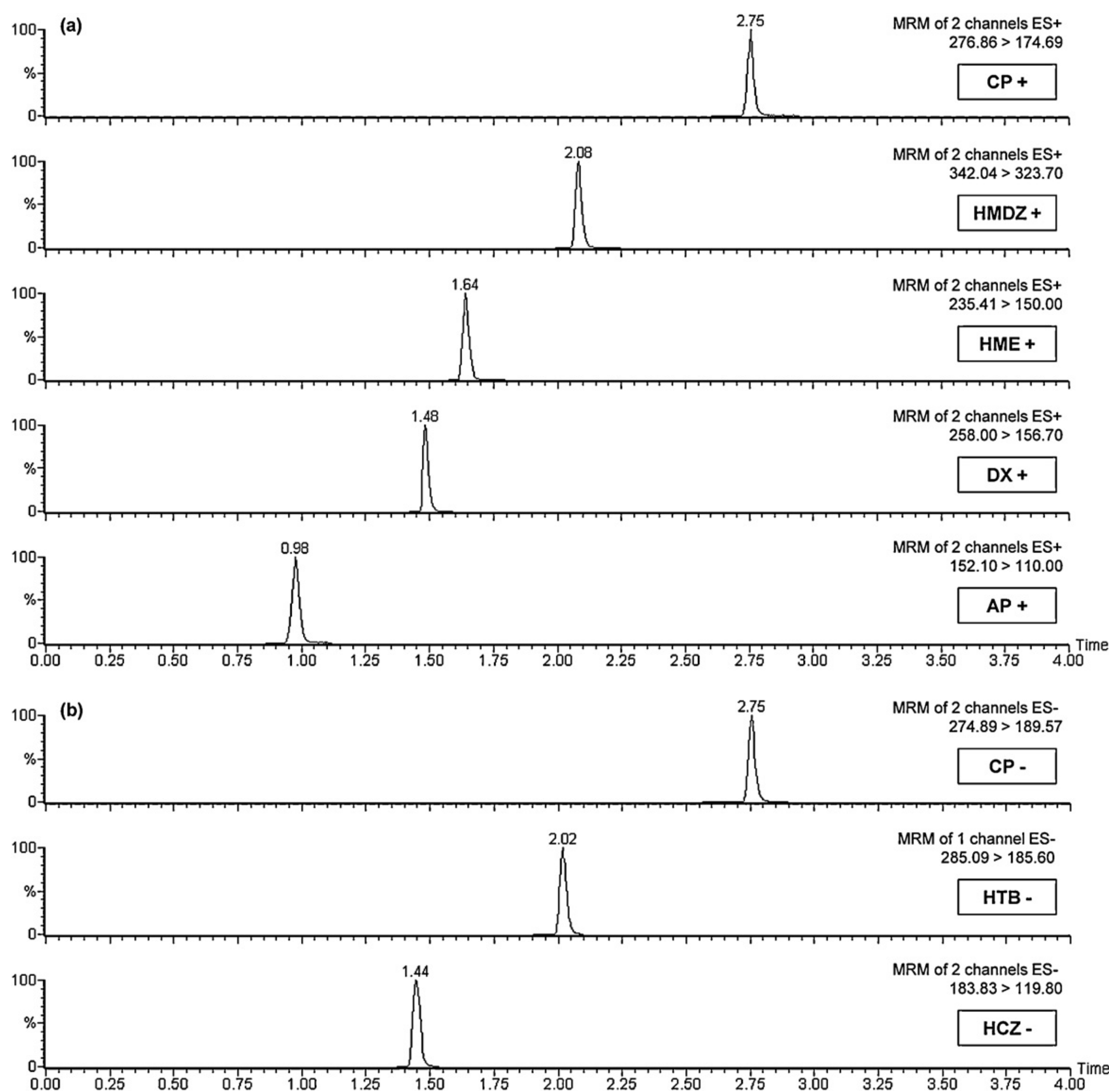

Fig. 2. Representative chromatogram of the metabolites in positive (a) and negative (b) electrospray ionization mode, obtained after the injection of QC medium (concentrations: see Table 1). Concentration of the internal standard: 20 ng/ml (compound abbreviation identification as in Fig. 1).

Table 2
Limit of detection (LOD) and limit of quantification (LOQ) of all metabolites. The precision and accuracy of the LOQs met requirements (<20%, and between 80% and 120%, respectively) (compound abbreviation identification as in Fig. 1).

		AP	DX	HME	HMDZ	HCZ	HTB
LOD (ng/ml)		0.134	0.088	0.912	0.095	1.871	0.173
LOQ (ng/ml)		2.8	0.55	4.32	1.47	9.11	0.8
Precision (RSD%)	$n=6$	5.13	2.64	4.50	5.33	4.13	3.27
Accuracy (%)	$n=6$	107.52	101.51	110.93	92.63	89.62	98.02

Table 3
Validation data: within-day precision, between-day precision and accuracy (compound abbreviation identification as in Fig. 1).

			AP	DX	HME	HMDZ	HCZ	HTB
Within-day precision (RSD%)	$n=6$	QC low	4.48	1.37	1.65	1.97	1.72	2.61
		QC medium	2.16	2.41	2.04	2.66	2.03	2.33
		QC high	5.13	2.37	1.14	4.61	1.10	1.47
Between-day precision (RSD%)	$n=6$	QC low	6.29	3.75	5.88	4.63	9.60	3.34
		QC medium	3.01	4.36	5.27	3.84	6.16	3.64
		QC high	2.86	2.69	3.27	4.82	4.29	3.84
Accuracy (%)	$n=6$	QC low	87.75	86.26	91.10	93.52	85.32	95.04
		QC medium	104.61	107.61	105.07	102.61	84.59	99.59
		QC high	107.39	107.46	107.93	97.04	106.83	109.83

Table 4
Internal standard-normalized matrix factors of the six metabolites at three concentration levels. Despite the absolute matrix effect observed for some of the metabolites (MF $\neq$ 1), no relative matrix effect was seen (CV% < 15%) (compound abbreviation identification as in Fig. 1).

IS-normalized matrix factor							
		Mean (n = 5)	CV (%)			Mean (n = 5)	CV (%)
AP	Conc 1	0.90	6.89	HME	Conc 1	0.95	10.77
	Conc 2	0.94	4.49		Conc 2	1.00	3.94
	Conc 3	0.99	4.06		Conc 3	1.08	3.99
DX	Conc 1	0.92	6.84	HCZ	Conc 1	1.25	4.46
	Conc 2	1.01	3.33		Conc 2	0.96	5.14
	Conc 3	0.99	3.75		Conc 3	0.98	6.62
HMDZ	Conc 1	1.23	4.30	HTB	Conc 1	0.92	9.82
	Conc 2	1.25	5.11		Conc 2	0.91	5.34
	Conc 3	1.22	3.92		Conc 3	0.91	3.00

Table 5
Enzyme activities calculated after incubation of pediatric microsomes, with the following conditions: 0.25 mg microsomal protein/ml incubation mix, probe substrate near Km, reaction time: 15 min (40 min for incubation with ME), reaction temperature 37 °C (n = 3). SD: standard deviation (compound abbreviation identification as in Fig. 1). Mean (±SD).

	CYP	Metabolite concentration			Enzyme activity		
		Mean (±SD) in ng/ml			Mean (±SD) in pmol/mg/min		
		Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
AP	1A2	3.51 (±0.28)	3.84 (±0.22)	6.07 (±0.57)	27.23 (±2.15)	29.84 (±1.72)	47.09 (±4.41)
DX	2D6	13.46 (±0.82)	32.76 (±4.29)	70.52 (±3.74)	61.38 (±3.73)	149.3 (±19.54)	321.51 (±17.07)
HME	2C19	<LLOQ	31.13 (±1.93)	17.13 (±1.68)	<LLOQ	155.9 (±9.64)	85.81 (±8.40)
HMDZ	3A4	22.27 (±2.04)	65.50 (±5.21)	6.43 (±0.55)	76.47 (±7.02)	224.9 (±17.89)	22.07 (±1.87)
HCZ	2E1	57.35 (±4.89)	30.95 (±2.39)	214.7 (±15.90)	362.6 (±30.92)	195.7 (±15.11)	1358 (±100.5)
HTB	2C9	12.31 (±1.17)	38.62 (±3.12)	62.49 (±7.18)	50.4 (±4.81)	158.2 (±12.78)	256.1 (±29.43)

(MF) was determined at three different concentration levels for all six metabolites, following Viswanathan et al. [23]. The absence of an absolute matrix effect is not indispensable for a valid bioanalytical method. Variable matrix effects in individual subjects, however, would cause a problem of reproducibility of the method. As shown in Table 4, an absolute matrix effect (IS-normalized MF $\neq$ 1) was observed for some of the metabolites at some of the concentrations (HMDZ and HCZ). Nevertheless, no relative matrix effects were seen, as the coefficients of variation (CV%) at each concentration level were <15% for all compounds. The observed absolute matrix effects are in contrast with the study described by Li et al., where ion enhancement or suppression from the matrix was found negligible [6]. This is probably due to the more selective nature of the sample preparation, consisting of a liquid–liquid extraction, followed by evaporation and reconstitution in mobile phase. Despite the lack of matrix effects in their method, the sample preparation is much more extended than the fast method used in the study described in this article (protein precipitation followed by centrifugation).

These results indicate that the selection of an appropriate internal standard is essential for the analytical method to be valid. Also, as the FDA prescribes, calibrators and QC samples need to be prepared in the same matrix as the samples.

3.8. Application of the method

The incubations of the microsomes originating from diseased livers from children undergoing liver transplantation resulted in the formation of metabolite concentrations as depicted in Table 5. With these metabolite concentrations, enzyme activities were calculated. Some of the isoforms showed very low activities, but nevertheless, these activities could be determined. These results show that the quantification method can be used in the in vitro determination of the enzyme activity of the six most important CYP isoforms. Interpretation of the results, however, is not included in this manuscript in view of the ongoing nature of the study, as well as the scope of this publication.

4. Concluding remarks

This paper presents the development and validation of a fast and sensitive UPLC–MS/MS method for the determination of the in vitro CYP450 enzyme activity. Especially in populations with liver dysfunction, a sensitive quantification method is required, as these patients often show a reduced activity. The UPLC–MS/MS approach in addition allows short sample analysis turn over times, which is interesting for high sample loads. The presented method was validated for selectivity, precision and accuracy. Despite the observed absolute matrix effects, a relative matrix effect could be ruled out, thus corroborating the validity of the obtained quantitative measurements. The sensitivity of the method was shown to be adequate for the intended pharmacokinetic applications, i.e. the incubation experiments with microsomes originating from liver samples from children with severe hepatic dysfunction. The low activities of some of the CYP isoforms could still be calculated. This method will be used in pre-clinical pharmacokinetic experiments.

Conflict of interest

The authors declare no conflict of interest.

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