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In vitro cytochrome P450 activity: Development and validation of a sensitive high performance liquid chromatography–tandem mass spectrometry method for the quantification of six probe metabolites after derivatization with pyridine-3-sulfonyl chloride in an aqueous environment

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ABSTRACT

For the determination of the *in vitro* cytochrome P450 activity in microsomes, a quantification method for the probe metabolites, formed during incubation, is required. Due to insufficient sensitivity of a previously developed high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for some of the metabolites, a fast and easy derivatization method with pyridine-3-sulfonyl chloride (PS) is described. Acetaminophen (CYP1A2), dextrorphan (CYP2D6), hydroxy-chlorzoxazone (CYP2E1) and hydroxy-mephenytoin (CYP2C19) can be derivatized because of the presence of a phenolic OH, whereas hydroxy-midazolam (CYP3A4) and hydroxy-tolbutamide (CYP2C9) remain unchanged. As PS improves the ionization efficiency in the positive electrospray ionization (ESI) mode, the sensitivity of the detection is improved significantly and meets requirements for the activity determination. Native negative electrospray type molecules, moreover, become positive ESI candidates. The direct derivatization in the aqueous incubation medium, without any other sample pre-treatment steps, such as evaporation or extraction, makes this procedure easy to perform. The method using 20 s microwave irradiation was shown to equal a 10 min reaction in a 60 °C heating block, consequently simplifying and shortening the process. Collision induced fragmentation of the derivatives resulted in at least one native compound, rather than derivative, specific product ion, thereby improving the selectivity of the method in the multiple reaction monitoring mode. The HPLC–MS/MS method was validated, and was demonstrated to be sensitive, selective, precise and accurate. The absence of a relative matrix effect was established, notwithstanding that an absolute matrix effect was observed. The analysis of a sample after microsomal incubation, from which some of the metabolites could not be quantified using the method without derivatization, proved the usefulness of the method.

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1. Introduction

Many pre-clinical pharmacokinetic experiments determine the *in vitro* activity of the cytochrome P450 system, as these enzymes play a major role in the metabolism of many drugs. The assessment of the metabolic fate of a new drug, metabolic profiling, potential drug–drug interactions, or the activity in a specific patient population, requires the incubation of a (sub)cellular system, such as hepatocytes or microsomes, with specific probe substrates [1]. Microsomes contain 96% of the total hepatic CYP content [2] and they are easy to use and store, which makes them a valuable tool in these experiments. During the incubation, the substrates are metabolized by a specific isoform into a specific metabolite. The appropriate substrates for the phenotyping of the six clinically most important isoforms 1A2, 2C19, 2C9, 2D6, 2E1 and 3A4 are phenacetin, S-mephenytoin, tolbutamide, dextromethorphan, chlorzoxazone and midazolam, respectively [3]. The amount of metabolite formed reflects the intrinsic (hepatic) enzymatic activity of the patient. LC–MS/MS has been used in many studies for the quantification of these metabolites. The literature based HPLC–MS/MS method for the simultaneous quantification of the six metabolites, implemented in our laboratory, showed insufficient sensitivity for the intended applications, i.e. the influence of distinct liver pathologies on the CYP activity. Moreover, not all metabolites could be detected in the same electrospray ionization method. Hence, the development of another analytical method was recommendable.

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Even though electrospray ionization mass spectrometry (ESI-MS) is a valuable tool for the analysis of a wide range of molecules, not all compounds can be analyzed with this technique. Weakly ionizable compounds, such as neutral, nonpolar molecules, are not detected with ESI-MS. The ESI behaviour of these compounds can be altered by the attachment of a moiety carrying a formal charge or with high proton affinity, consequently improving the detectability of the analytes with ESI-MS and adding a degree of analyte selectivity. Furthermore, derivatization may allow detection of a native negative charge type analyte in the positive ion mode [4]. The potential of chemical derivatization has been proven in previously published reports, where a wide range of molecules are being derivatized using several reagents.

Most of the metabolites formed during the microsomal incubation contain a phenolic OH-function. A derivatization process commonly used for the derivatization of phenols, is 5-(dimethylamino)naphthalene-1-sulfonyl chloride (or dansyl chloride) [5–10]. Recently, Xu and Spink described the use of other sulfonyl chlorides for the derivatization of weakly ionizable estrogens [11,12]. The formed sulfonate can be protonated in acidic solutions, consequently improving the ionization efficiency significantly. The use of these derivatives was suggested for other phenolic compounds [12].

A major strength of the previously published methods for the detection of (underivatized) metabolites is the lack of sample preparation: after incubation, the samples are centrifuged and the supernatant is injected as such. Therefore, a derivatization method to improve sensitivity should be as short and easy as possible. Most of the described derivatization processes consist of several evaporation, reconstitution or extraction steps. Furthermore, the reaction is performed in organic solvents. A derivatization reaction directly in the incubation medium would allow competitive comparison with non-derivatized methods. Also, as liquid chromatography is an aqueous system, the injection of an aqueous sample is preferred.

This article describes the development of a derivatization procedure of the metabolites formed during the activity assessment of the six most important CYP isoforms 1A2, 2C19, 2C9, 2D6, 2E1 and 3A4, using phenacetin, S-mephenytoin, tolbutamide, dextromethorphan, chlorzoxazone and midazolam, respectively, as probe substrates. The pyridine-3-sulfonyl derivatives were formed directly in the aqueous incubation medium during the easy and short derivatization process. The LC–MS/MS method was optimized and validated, and the complete procedure was assessed through the analysis of a rat microsomal incubation sample.

2. Experimental

2.1. Chemicals

Phenacetin, acetaminophen (AP), tolbutamide, 4-OH-tolbutamide (HTB), S-mephenytoin, 4′-OH-mephenytoin (HME), dextromethorphan, dextrorphan (DX), chlorzoxazone, 6-OH-chlorzoxazone (HCZ), levallorphan (LA), and chlorpropamide (CP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Midazolam and 1-OH-midazolam (HMDZ) were kindly donated by Roche (Basel, Switzerland). Pyridine-3-sulfonyl chloride hydrochloride (PS) and 4-(1H-pyrazol-1-yl)benzenesulfonyl chloride (PBS) were purchased from Maybridge (Cambridge, UK). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Biopredic International (Rennes, France). All other chemicals were of analytical grade. Commercial rat microsomes were acquired from BD Biosciences (Wistar Han, male rat, BD Gentest, BD Biosciences, Erembodegem, Belgium). Human microsomes were prepared from human liver tissue (approved by the Ethics Committee of Ghent University Hospital, B67020084281).

2.2. Preparation of standards and quality controls

Primary stock standards of all metabolites and the internal standard of 1 mg/ml in methanol 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 stop reagent ($H_2O/CH_3CN/HCOOH$; 42:55:3). After vortex mixing and cool down on ice for protein denaturation, a specific amount of the stock solution mix 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, supernatant was maintained and stored at 4 °C.

2.3. Chromatographic conditions

The chromatographic system consisted of a Kontron instrument (Zurich, Switzerland). The separation was carried out using a Luna C18 column (50 mm × 2.0 mm, particle size 3 μm; Phenomenex, Torrance, CA, USA) with an Alltima C18 guard column (7.5 mm × 2.1 mm, particle size 5 μm; Grace, Columbia, MD, USA). The injection volume was 10 μl. Gradient elution was performed with a flow rate of 0.2 ml/min, starting at 80% eluent A (water containing 0.1% formic acid). Eluent B (acetonitrile containing 0.1% formic acid) was linearly increased from 20% to 90% during 9 min. The initial conditions were regained over a 0.1 min time interval, followed by a 5 min equilibration time prior to the next injection. This resulted in an overall run time of 14 min.

2.4. MS conditions

Detection was performed using a Quattro II triple quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source in the electrospray positive ion mode (ESI+). Nitrogen was used as both drying and nebulising gas. Product ions were detected using the multiple reaction monitoring (MRM) mode, using argon as collision gas. The capillary voltage and source temperature were optimized at 3.6 kV and 120 °C, respectively. The collision energy and cone voltage were optimized for each compound individually. The collision energy varied from 17 eV to 30 eV and the cone voltage varied from 35 V to 50 V (see Table 1). Data were collected and processed using the MassLynx and QuanLynx software 4.0 (Waters, Manchester, UK). Table 1 illustrates the individual parameters and MRM transitions used for detection and quantification.

2.5. Derivatization

The first experiments were conducted following the method described by Xu and Spink [12]. Shortly, a solution of the metabolites in 100% MeOH was evaporated to dryness under N_2. Eighty μl of 0.1 M sodium bicarbonate (pH 10) and 80 μl of a 1 mg/ml PS or PBS solution in acetone were added to the vial. After mixing, the vials were placed in a heating block (Multi-Blok Heater, Lab-Line Instruments Inc., Melrose Park, Ill, USA) at 60 °C for 15 min for PS and 30 min for PBS. Subsequently, the vials were cooled on ice for 10 min. The reaction mixture was transferred to a 250 μl insert and analyzed using an LC–MS/MS system. As described in Section 3, this basic protocol was investigated for its performance characteristics, where appropriate optimized and adapted to the needs of our specific application, i.e. a biological medium containing microsomes.

After selection of the most useful approach (i.e. derivatization reagent type, solvent medium, etc.) and optimization of

Table 1
Instrument parameters for the detection of the (derivatized) metabolites. PS: pyridine-3-sulfonyl chloride; HMDZ: hydroxy-midazolam; DX-PS: PS-derivatized dextrorphan; LA-PS: PS-derivatized levallorphan; HTB: hydroxy-tolbutamide; AP-PS: PS-derivatized acetaminophen; HME-PS: PS-derivatized hydroxy-mephenytoin; HCZ-PS: PS-derivatized hydroxy-chlorzoxazone; IS: internal standard.

	HMDZ	DX-PS	LA-PS	HTB	AP-PS	HME-PS	HCZ-PS
Targeted CYP450 isoform	CYP3A4	CYP2D6	IS	CYP2C9	CYP1A2	CYP2C19	CYP2E1
Molecular mass metabolite	341.77	257.37	283.41	286.35	151.16	234.25	185.56
t_R (min)	3.75	3.9	4.34	4.46	5.05	5.95	6.03
Cone voltage (V)	40	50	45	35	40	45	35
Collision energy (eV)	20	30	30	18	18	23	17
Precursor ion	342.6	399.3	425.5	287.05	293.3	376.1	328.3
Product ion 1 (quantifier)	324.2	257.5	283.3	171.00	151.4	274.1	264.56
Product ion 2 (qualifier)	203.2	199.02	199.00	188.00	109.1	132.1	144.25
Calibration curve range (ng/ml)	2.3–660	1.1–245		25–1500	5.6–1260	6.9–1940	128.1–2560

the different key process parameters (time, reaction temperature and heating method), the final protocol was: 10 μl 1.75 N NaOH, 10 μl 1.25 μg/ml levallorphan (LA) and 70 μl 1 mg/ml pyridine-3-sulfonyl chloride (PS) hydrochloride in acetonitrile were added to 200 μl of incubation medium/calibrator and were mixed by vortexing. The derivatization was catalyzed by heating the vial for 20 s in an 850 W microwave oven (domestic, Daewoo, KOG-376T, Korea). In order to absorb the excess microwave energy, a glass container with 60 ml water was placed next to the vials [13]. After mixing, the vial was cooled on ice for 10 min, and after transfer of the sample to a 250 μl insert, the sample could be readily injected.

2.6. Method validation

The analytical method was validated according to the FDA Guidance [14]. The following parameters were evaluated: selectivity, LLOQ, calibration model, accuracy, precision, and stability. Selectivity was evaluated by the analysis of blank microsomes from six different sources. The lower limit of quantification was defined as the lowest concentration for which the accuracy was 80–120% and precision (RSD%) < 20%, and was used as the lowest point of the calibration curve. Furthermore, the signal-to-noise (S/N) ratio of the LLOQ should be at least 9. Calibration models were evaluated statistically using StatGraphics 4.1 (Warrenton, Virginia, US). Accuracy of the analysis of six QC samples on three concentration levels should be within 85–115% of the nominal concentration, and within-run (n = 6) and between-run (n = 6) precision should not exceed 15% (RSD%).

Stability of the (derivatized) metabolites in the microsomal incubation medium was evaluated after three freeze-thaw cycles, after 10 days storage at 4 °C and after 24 h of residence in the autosampler. Matrix effect was evaluated through the calculation of the internal standard (IS)-normalized matrix factor (MF) [=peak ratio (analyte/IS) in presence of matrix ions/peak ratio (analyte/IS) in absence of matrix ions] [15] in five different matrix sources (microsomes from five different patient samples; matrices A–E) and at three concentration levels (except for HTB and HCZ-PS, 2 levels). The coefficient of variation at each concentration level should not exceed 15%. The relative intensities of the qualifier and quantifier ion were identified by the analysis of samples in absence of matrix. The maximum permitted tolerances of the ion ratios in presence of matrix were used from the EU Guidelines [16].

2.7. Application of the method

Rat microsomes were incubated with each probe substrate individually in a concentration near their apparent K_m [17]. Shortly, the probe substrate was added to 1.25 mg microsomal protein/ml, 1.15% KCl and potassium phosphate buffer (pH 7.25). After pre-incubation for 3 min at 37 °C, the addition of NADPH initiated the reaction. After exactly 15 min, the reaction was stopped with a reagent containing formic acid and acetonitrile (H_2O/CH_3CN/HCOOH; 42:55:3). Samples were frozen and stored at −20 °C until analysis. After thawing, samples were derivatized following the previously described protocol, and were analyzed using the validated method.

3. Results and discussion

Due to its major role in the pharmacokinetics of many drugs, the *in vitro* activity determination of the cytochrome P450 enzyme system shows up in many different experiments during drug development. *In vitro* systems are used in the metabolic profiling of new drugs and in the evaluation of drug–drug interactions. Furthermore, changes in CYP activity in specific patient populations can be examined. In some cases, the amount of metabolite formed is (very) low (in inhibition experiments or in patients with liver disease). These experiments necessitate a sensitive quantification method for the metabolites formed.

3.1. Derivatization with PS and PBS

As our developed HPLC–MS/MS method showed insufficient sensitivity, chemical derivatization in order to improve ionization efficiency was used. Derivatization of the phenolic metabolites with commercially available pyridine-3-sulfonyl chloride (PS) and 4-(1H-pyrazol-1-yl)benzenesulfonyl chloride (PBS) was assessed. Fig. 1 depicts the derivatization reaction with PS. As HTB and HMDZ lack a phenolic OH-function, they will not be derivatized by PS or PBS. However, the sensitivity of detection of these components already proved sufficient for the application and they can be detected in the positive electrospray ionization mode. Thus, derivatization is not essential for these components. In contrast, the derivatization of HME is strongly desired, as there is very poor sensitivity. Also, as HCZ needs to be detected in the negative ESI mode, derivatization may lead to detection in the positive mode. This obviates the need for within-run polarity switching (when possible) or double analysis at opposite polarities. The phenolic OH-function of DX and AP will also be derivatized.

In the first series of experiments, mixtures of standard solution (in methanol) of the metabolites were used. The metabolites were derivatized following the procedure described by Xu and Spink [12]. After the derivatization reaction, analysis was performed by using the multiple reaction monitoring (MRM) mode. All derivatives showed a precursor ion on $[M+143]^+$ or $[M+207]^+$, with M being the molecular mass of the original metabolite minus one hydrogen, and 143 and 207 representing the protonated sulfonate moiety of PS and PBS, respectively. The collision induced fragmentation of the precursor ions of the PS derivatives resulted in specific product ions. It is important for the qualitative properties of the method that there are other diagnostic fragments rather than only the original metabolite after removal of the derivatization moiety. The product ion spectrum of the (derivatized) metabolites always

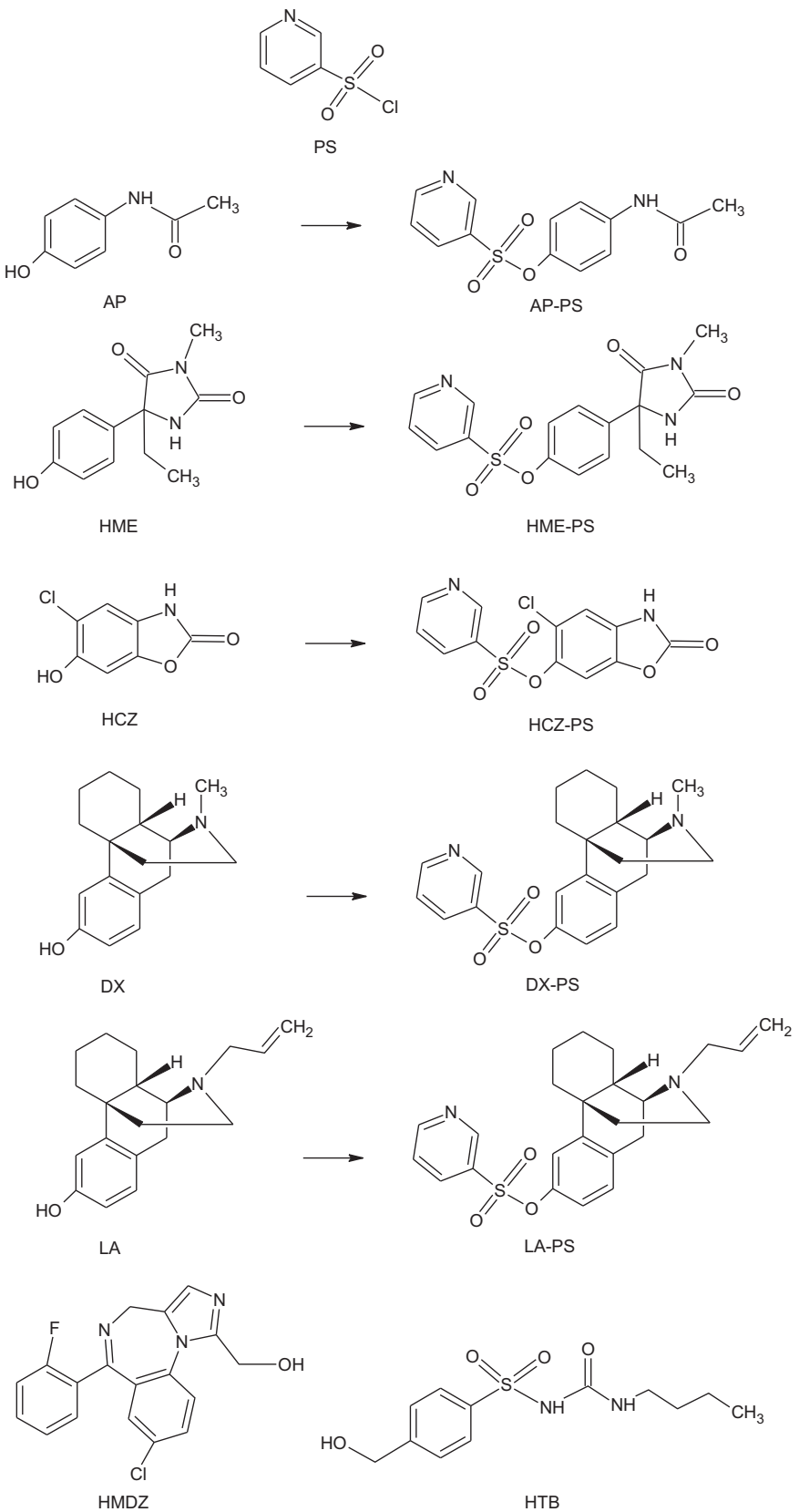

Fig. 1. Molecular structures of the (derivatized) metabolites. PS: pyridine-3-sulfonyl chloride; HMDZ: hydroxy-midazolam; DX: dextrorphan; DX-PS: PS-derivatized dextrorphan; LA: levallorphan; LA-PS: PS-derivatized levallorphan; HTB: hydroxy-tolbutamide; AP: acetaminophen; AP-PS: PS-derivatized acetaminophen; HME: hydroxy-mephenytoin; HME-PS: PS-derivatized hydroxy-mephenytoin; HCZ: hydroxy-chlorzoxazone; HCZ-PS: PS-derivatized hydroxy-chlorzoxazone.

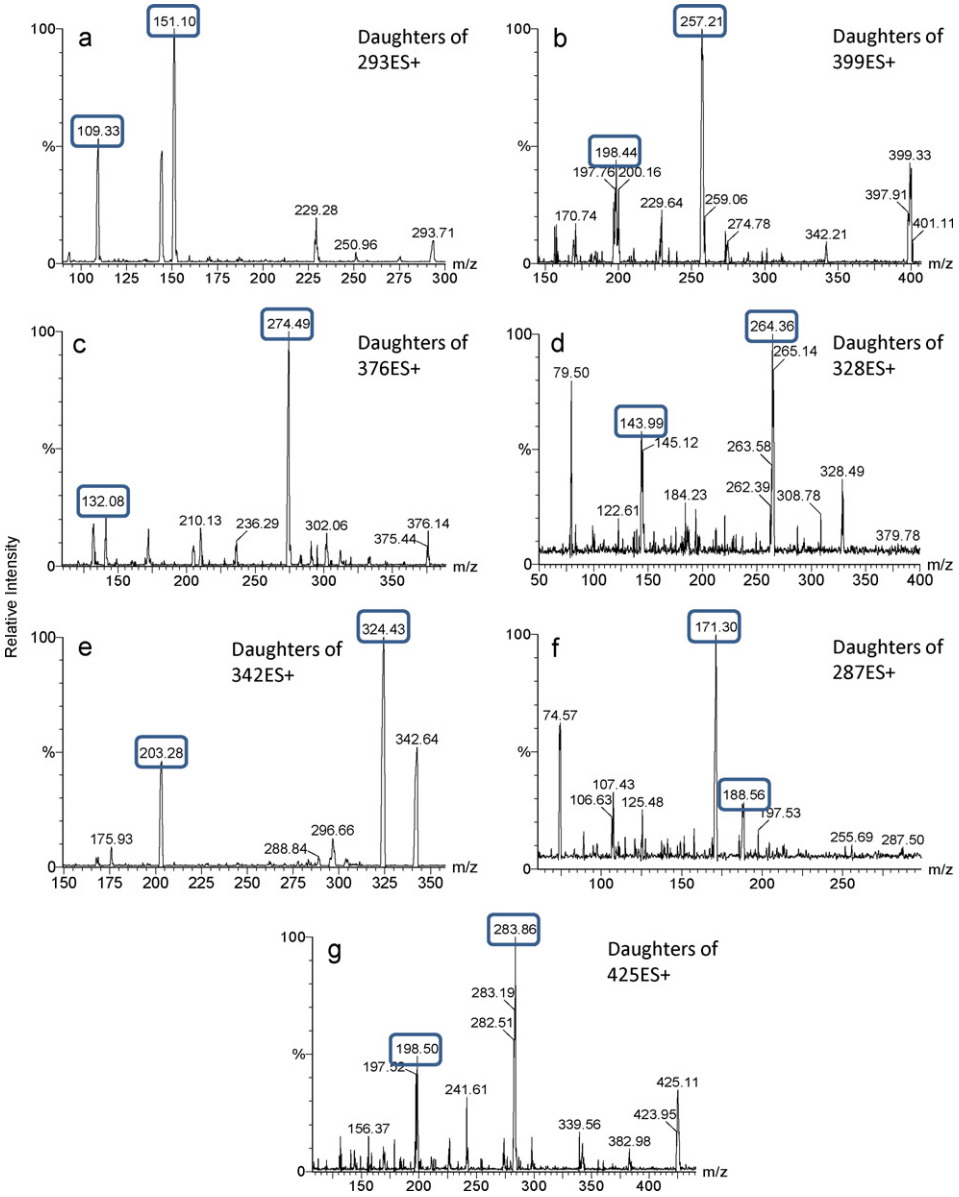

Fig. 2. MS/MS spectra of (a) PS-derivatized acetaminophen (AP-PS); (b) PS-derivatized dextrorphan (DX-PS); (c) PS-derivatized hydroxy-mephenytoin (HME-PS); (d) PS-derivatized hydroxy-chlorzoxazone (HCZ-PS); (e) hydroxy-midazolam (HMDZ); (f) hydroxy-tolbutamide (HTB); and (g) PS-derivatized levallorphan (LA-PS). Highlighted ions indicate the selected product ions used in further analysis.

showed at least one specific fragment from the original metabolite. To illustrate this, the fragment ions of AP-PS, as an example, are annotated in Fig. 3. The cleavage of the S–O bond between the PS and the AP moiety leads to two fragments with m/z of 144 and 151.4. The m/z 109.1 fragment corresponds to the $(NH_3{-}C_6H_4{-}OH)^+$ ion obtained after simultaneous $CO{=}CH_2$ and PS loss. In contrast, the fragments from the PBS derivatives originated mainly from the fragmentation of the PBS moiety. For the quantification in the MRM mode, the most abundant product ion was used as quantifier. The specific fragment ion (or qualifier) was monitored in order to evaluate possible interference, as the ratio of the areas of the quantifier and qualifier should be constant. Fig. 2 shows the MS/MS spectra of the (PS-derivatized) metabolites.

Prior to the MS detection, the metabolites were separated on a C18 chromatographic column. CYP enzymes are responsible for the phase I metabolism of drugs, leading to a more polar compound for renal excretion. Due to the derivatization, the polar metabolites are turned into less polar molecules, showing more retention on the reversed-phase (C18) column than prior to derivatization, and thus favourably influencing the chromatographic separation. Due to the less hydrophobic properties of PS, the PS derivatives show a lower retention time than the PBS derivatives.

Evaluation of the ionization efficiency showed a significant improvement of the detectability of both the PS and PBS derivatives compared to the non-derivatized molecules. The sensitivity of HME increased significantly. Moreover, all compounds, including HCZ, could be detected in the positive ESI mode. The decision to select PS as reagent to perform the analysis was based on several observations. Due to the more favorable fragmentation pattern, a more specific detection is possible. Also, the shorter retention times of the derivatives may decrease the total time of analysis.

3.2. Derivatization with PS in aqueous medium: optimization

Many of the previously published methods for the quantification of the metabolites after microsomal incubation only require a centrifugation step prior to injection. In order to keep the method with derivatization as simple as those methods, the evaporation

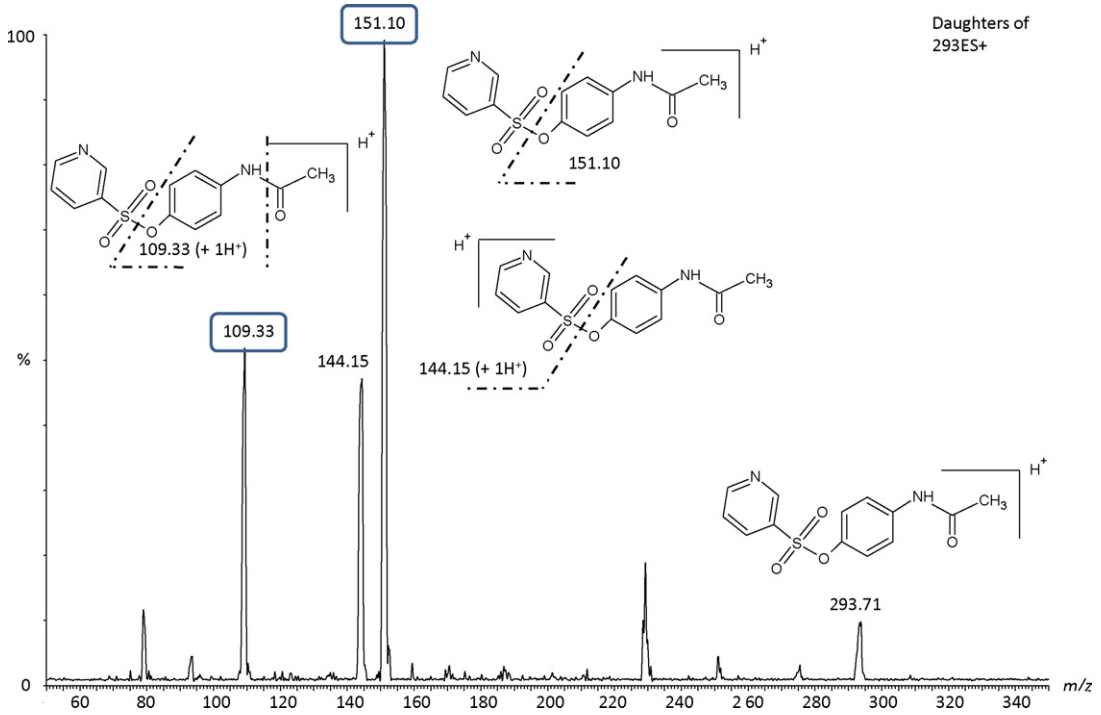

Fig. 3. Fragmentation of AP-PS. The product ions *m/z* 144 and 151.4 are formed by the cleavage of the S–O bond. The specific fragment *m/z* 109.1 is obtained after a CO=CH_2 neutral loss off the AP moiety concurrently with the elimination of PS.

to dryness under N_2 was omitted from the original procedure, and PS and sodium bicarbonate were directly added to the standard methanol solution of the metabolites. The derivatized metabolites could still be detected with sufficient sensitivity (data not shown).

In order to assess the possibility of direct derivatization in the aqueous medium of the incubation samples, metabolites were spiked to a mixture of the incubation buffer (potassium phosphate, pH 7.25) and 1.15% KCl (50/50 (v/v)). The addition of PS (dissolved in acetone) and sodium bicarbonate to this mixture resulted in precipitation. Two parameters of the protocol were changed. Firstly, PS was dissolved in acetonitrile instead of acetone. In accordance to the derivatization with dansyl chloride [5], an alkaline pH is important for the derivatization reaction with PS, as the nucleophilic phenolate ion reacting with the PS is formed at a pH 9–10. Consequently, another buffer was considered. Beaudry et al. [18] used 20 μl 100 mM NaOH in 350 μl sample and derivatization reagent in order to get a pH near 9. Due to the presence of a potassium phosphate buffer (pH 7.25), a greater amount of NaOH was required to obtain the correct pH. In order to avoid large dilution of the sample, a small volume (10 μl in 200 μl sample) of a higher concentrated (1.75 N) NaOH solution was added. These interventions were successful, as the solution remained clear after addition of these components and the PS derivatives could be detected.

As the FDA recommends the use of calibrators in the same matrix as the actual samples, calibrators prepared with microsomal suspensions (final concentration microsomal protein of 0.25 mg/ml) were used instead of the buffer mixture. After the confirmation that the derivatization reaction proceeded in this medium, the process was optimized with the intention to increase the yield. The optimal amount of derivatization reagent, the temperature of the heating block during reaction and the reaction time were determined. A comparison of the areas obtained when 50, 60 or 70 μl 1 mg/ml PS were added, showed that the volume was preferably 70 μl, as the yield for some of the derivatized metabolites was significantly higher in this situation. For all metabolites, temperature and reaction time were correlated: higher temperature required shorter reaction time to get the same yield. The upper limit of the reaction temperature was 80 °C, as a higher temperature (90 °C) led to a high variability (RSD > 15%; data not shown). Furthermore, the samples needed to be cooled down on ice for 10 min in order to decrease variability.

The promotion of organic reactions by microwave energy has been suggested in many studies, as reviewed by Kappe and Dallinger [19]. Due to the direct delivery of the energy to the sample, there is a rapid rise in temperature and consequently a fast completion of the reaction [20]. As the purpose of the method under development was to be as short and easy as possible, the use of microwave-assisted heating was evaluated. A 20 s irradiation of the sample in an 850 W microwave oven, showed comparable yield compared to the heating block (60 °C, 10 min) procedure. As indicated before, a beaker with 60 ml water was placed on the microwave turning table, alongside the vials, to absorb excess energy [13,21]. The use of the microwave-assisted heating significantly decreased the total time of analysis, achieving equal overall performance and this method was finally adopted throughout.

3.3. Optimization of the LC–MS/MS analysis of PS-derivatized metabolites

The chromatographic separation was optimized through the use of gradient elution. The linear increase of eluent B (acetonitrile + 0.1% formic acid) from 20% to 90% during 9 min resulted in a chromatographic separation as depicted in Fig. 4. As internal standard for the analysis, levallorphan (LA) was selected. Not only can this molecule be derivatized by PS, but it can also be eluted in the middle of the analytical run.

Through multiple injections of the derivatized standard solutions, the MS parameters were tuned for each individual compound. For the source temperature, gas flows (both nitrogen and argon) and capillary voltage, a compromise was made to get optimal sensitivity for all the components (see Section 2). The MRM transitions, cone voltage and collision energy were optimized for each (derivatized) metabolite and are depicted in Table 1.

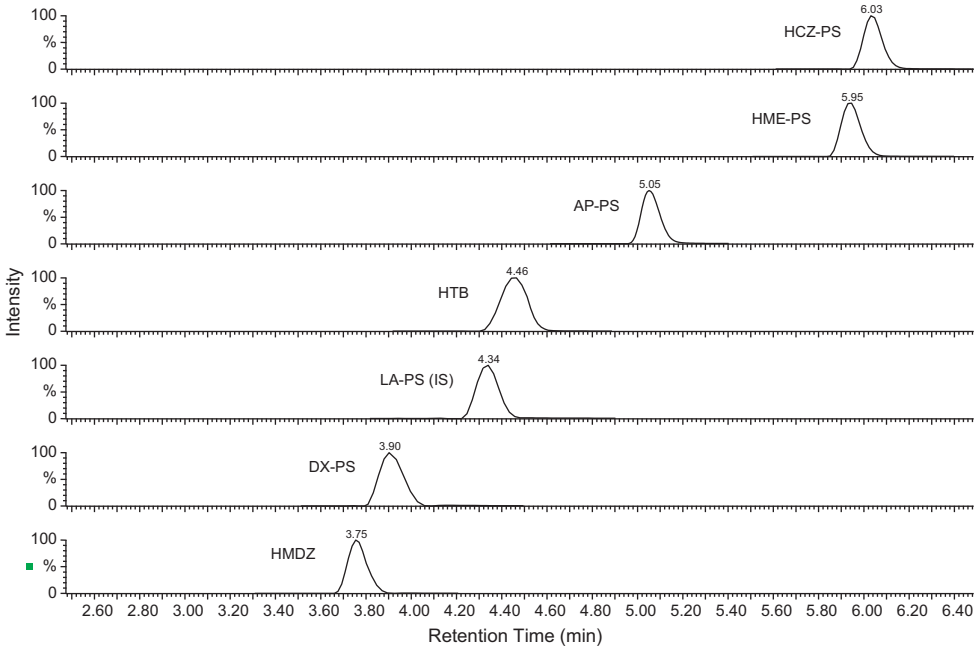

Fig. 4. Chromatogram of the (PS-derivatized) metabolites and the internal standard after gradient elution (compound abbreviation identification as in Table 1).

3.4. Method validation

The method was validated according to the FDA guidelines. The optimal calibration curves were statistically calculated and the relation between ratio and concentration showed a linear correlation for all compounds. A $1/x^2$ weighting factor was used to obtain the best residuals and consequently the best accuracy. Ranges of the calibration curves were partially deduced from the article by Walsky and Obach [22] (mainly enzyme kinetics driven) and were decided in relation to our particular application. The LLOQ was established at the lowest concentration of the calibration curve. Precision (<9.82% RSD) and accuracy (91.30–108.70%) at the LLOQ of all analytes were within the limits of the FDA guideline (<20% RSD and between 80 and 120%, respectively).

The between-run and within-run precision were <14.06% and <9.58% (RSD), respectively, and accuracy ranged from 85.48% to 114.94%, for all metabolites, derivatized and underivatized ones. No interference was observed at the retention times of the analytes and the IS when analyzing blank microsomes from six independent sources. Thus, the method is considered selective.

Derivatized samples were proven to be stable for at least three freeze–thaw cycles (paired *t*-test, $p > 0.05$; accuracy 92.96–109.62%), except for DX-PS at low concentrations ($p < 0.05$). However, the mean of the calculated concentration is still within the 85–115% limit (104.09%) of the calculated concentration of the freshly prepared sample, and therefore acceptable. Except for AP-PS, stability at 4 °C was acceptable (paired *t*-test, $p > 0.05$) for 10 days after derivatization. Nevertheless, for AP-PS the calculated concentration was still within the aforementioned limits, and therefore again acceptable. Poor autosampler stability (24 h, room temperature around 28 °C) was observed for almost all the metabolites. Cooling down the derivatized samples in the sampler will be mandatory.

The IS-normalized matrix factors (MFs), determined by the analysis of microsomes prepared from five different patient samples (matrices A–E), are summarized in Table 2. All samples were ana-

Table 2
Internal standard (IS)-normalized matrix factors (MFs) were determined in microsomes prepared from five different patient samples (patients A–E): the maximum permitted CV (%) = 15%. Conc.: concentration; CV: coefficient of variation (compound abbreviation identification as in Table 1).

IS-normalized MF ($n = 2$)	HMDZ			DX-PS			HTB		
	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3
Matrix A	0.99	1.06	0.98	1.00	0.93	1.01		1.26	1.24
Matrix B	1.33	1.01	0.88	1.14	0.94	1.00		1.14	1.16
Matrix C	0.96	0.81	0.67	0.92	0.89	1.05		0.87	0.85
Matrix D	1.06	0.79	0.78	1.01	1.09	1.06		1.03	1.09
Matrix E	0.98	1.04	0.92	0.96	1.03	1.00		1.20	1.25
CV (%)	14.48	13.88	14.47	8.32	8.30	2.90		14.03	14.61
IS-normalized MF ($n = 2$)	HME-PS			AP-PS			HCZ-PS		
	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3	Conc. 1	Conc. 2	Conc. 3
Matrix A	0.81	0.98	0.58	0.95	0.89	0.73		1.02	2.89
Matrix B	1.03	0.97	0.55	1.05	0.84	0.69		1.48	2.65
Matrix C	0.82	1.18	0.70	1.02	1.00	0.79		1.16	2.75
Matrix D	1.05	1.08	0.77	1.11	1.01	0.84		1.11	2.85
Matrix E	1.03	1.14	0.63	1.11	0.99	0.69		1.27	3.15
CV (%)	12.65	8.78	13.36	6.53	7.75	9.04		14.65	6.53

Table 3
Relative ion intensities of qualifiers (% of base peak) (compound abbreviation identification as in Table 1).

	n	Relative intensity (% of base peak)	Maximum permitted tolerance (relative)	% of samples exceeding tolerance
HMDZ	80	35.86	±25%	2.50
DX-PS	54	6.74	±50%	0
HTB	50	25.57	±25%	0
HME-PS	78	18.12	±30%	0
AP-PS	76	45.36	±25%	1.32
HCZ-PS	71	58.54	±20%	1.41

Table 4
Comparison of the LOQ's of the detection of the metabolites prior and after derivatization (compound abbreviation identification as in Fig. 1).

LOQ (ng/ml)					
Prior to derivatization			After derivatization		
HMDZ	2.90	(ESI+)	HMDZ	2.30	(ESI+)
DX	2.10	(ESI+)	DX-PS	1.10	(ESI+)
HTB	25.00	(ESI−)	HTB	25.00	(ESI+)
AP	63.00	(ESI+)	AP-PS	5.60	(ESI+)
HME	971.40	(ESI−)	HME-PS	6.90	(ESI+)
HCZ	256.20	(ESI−)	HCZ-PS	128.10	(ESI+)

Table 5
Results of the incubations of rat microsomes ($n = 2$) with the six probe substrates. Incubation conditions: 0.25 mg microsomal protein/ml; reaction time: 15 min; reaction temperature: 37 °C. The amount of DX-PS exceeded the ULOQ: the samples were diluted and re-analyzed. SD: standard deviation (compound abbreviation identification as in Table 1).

		Metabolite concentration Mean (±SD) in ng/ml	Enzyme activity Mean (±SD) in pmol/mg/min
HMDZ	3A4	148.55 (±11.49)	509.99 (±39.45)
DX-PS	2D6	283.00 (±12.80)	1290.18 (±58.36)
HTB	2C9	93.10 (±6.94)	381.48 (±28.45)
HME-PS	2C19	10.15 (±0.50)	50.84 (±2.48)
AP-PS	1A2	100.00 (±13.50)	776.22 (±104.81)
HCZ-PS	2E1	114.75 (±19.03)	725.59 (±120.36)

lyzed in duplicate. For some compounds, the ionization is enhanced in the presence of the matrix (IS-normalized MF > 1). For other compounds, ionization is suppressed (IS-normalized MF < 1). The CV at the different concentration levels is <15% for all the compounds. The maximum permitted tolerances in deviation of the ion intensities are depicted in Table 3. Based on a statistically significant number of randomly chosen analyses, it can be seen that for all compounds less than 2.5% of the samples would need to be excluded based on a deviation of the ion ratio.

3.5. Comparison with non-derivatized method

A comparison of the detection method for non-derivatized molecules and the newly developed method is summarized in Table 4. After derivatization, the detection of HCZ was made possible in positive ESI, with a lower LOQ. Furthermore, the sensitivity of the detection of AP and HME was increased significantly. The sensitivity of the detection of the metabolites that are not derivatized (HMDZ and HTB) was not affected by the procedure.

3.6. Application of the method

After incubation of rat microsomes with the probe substrates, the metabolites in the incubation medium were analyzed with the described method. Table 5 depicts the results of the analysis. These results show that derivatization was advantageous, as the amount of HME was far below the LOQ of the method without derivatization. Also, all 6 components can be quantified using the same electrospray ionization mode (ESI+).

4. Conclusion

A chemical derivatization method was developed for the quantification of the probe metabolites formed during *in vitro* cytochrome P450 activity determination. The derivatization with pyridine-3-sulfonyl chloride of the metabolites containing a phenolic OH-function in their structure improved the ionization efficiency in positive ES and enabled the detection of all six metabolites in the ES+ mode. The method is easy and fast, as derivatization proceeds directly in the aqueous incubation medium, without additional evaporation, extraction or reconstitution steps. The method met all requirements of sensitivity, selectivity, precision, accuracy and stability following the FDA guidelines for bioanalytical methods. Proof-of-concept samples from the intended (pharmacokinetics) application were successfully analyzed using the newly developed procedure. To our knowledge, this is the first report on using aqueous derivatization in the LC–MS analysis of CYP activity probe metabolites. Moreover, we believe that the simplicity of the approach put forward here can remove some of the disinclination towards the use of derivatization in LC–MS for hard to ionize molecules in other application fields.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] A. Lahoz, L. Gombau, M.T. Donato, J.V. Castell, M.J. Gomez-Lechon, Mini-Rev. Med. Chem. 6 (2006) 1053.
[2] Z.E. Wilson, A. Rostami-Hodjegan, J.L. Burn, A. Tooley, J. Boyle, S.W. Ellis, G.T. Tucker, Br. J. Clin. Pharmacol. 56 (2003) 433.
[3] G.T. Tucker, J.B. Houston, S.M. Huang, Br. J. Clin. Pharmacol. 52 (2001) 107.
[4] J.M.E. Quirke, C.L. Adams, G.J. Vanberkel, Anal. Chem. 66 (1994) 1302.
[5] C. Gros, B. Labouess, Eur. J. Biochem. 7 (1969) 463–470.
[6] H. Chang, Y. Wan, J. Naile, X.W. Zhang, S. Wiseman, M. Hecker, M.H.W. Lam, J.P. Giesy, P.D. Jones, J. Chromatogr. A 1217 (2010) 506.
[7] G.W. Lien, C.Y. Chen, G.S. Wang, J. Chromatogr. A 1216 (2009) 956.
[8] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.F. Li, Anal. Chim. Acta 627 (2008) 91.
[9] M.R. Anari, R. Bakhtiar, B. Zhu, S. Huskey, R.B. Franklin, D.C. Evans, Anal. Chem. 74 (2002) 4136.
[10] W.Z. Shou, X.Y. Jiang, N.D. Weng, Biomed. Chromatogr. 18 (2004) 414.
[11] L. Xu, D.C. Spink, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 855 (2007) 159.
[12] L. Xu, D.C. Spink, Anal. Biochem. 375 (2008) 105.
[13] W. Sun, S.J. Gao, L.J. Wang, Y. Chen, S.Z. Wu, X.R. Wang, D.X. Zheng, Y.H. Gao, Mol. Cell. Proteomics 5 (2006) 769.
[14] FDA-CDER and CVM Guidance for Industry, Bioanalytical Method Validation, May 2002.
[15] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J. 9 (2007) E30–E42.
[16] Off. J. Eur. Commun. (2002).
[17] R. Yuan, S. Madani, X.X. Wei, K. Reynolds, S.M. Huang, Drug Metab. Dispos. 30 (2002) 1311.
[18] F. Beaudry, S.A. Guénette, A. Winterborn, J.F. Marier, P. Vachon, J. Pharm. Biomed. Anal. 39 (2005) 411.
[19] C.O. Kappe, D. Dallinger, Mol. Diversity 13 (2009) 71.
[20] T.Y. Chu, C.H. Chang, Y.C. Liao, Y.C. Chen, Talanta 54 (2001) 1163.
[21] A.K. Bose, B.K. Banik, N. Lavlinskaia, M. Jayaraman, M.S. Manhas, ChemTech 27 (1997) 18.
[22] R.L. Walsky, R.S. Obach, Drug Metab. Dispos. 32 (2004) 647.