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Liquid chromatography/tandem mass spectrometry method for simultaneous evaluation of activities of five cytochrome P450s using a five-drug cocktail and application to cytochrome P450 phenotyping studies in rats

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ABSTRACT

A reliable liquid chromatography/tandem mass spectrometry has been developed for simultaneous evaluation of the activities of five cytochrome P450s (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) in rat plasma and urine. The five-specific probe substrates/metabolites include phenacetin/paracetamol (CYP1A2), tolbutamide/4-hydroxytolbutamide and carboxytolbutamide (CYP2C9), mephenytoin/4′-hydroxymephenytoin (CYP2C19), dextromethorphan/dextrorphan (CYP2D6), and midazolam/1′-hydroxymidazolam (CYP3A). Internal standards were brodimoprim (for phenacetin, paracetamol, midazolam and 1′-hydroxymidazolam), ofloxacin (for 4′-hydroxymephenytoin, dextromethorphan and dextrorphan) and meloxicam (for tolbutamide, 4-hydroxytolbutamide and carboxytolbutamide). Sample preparation was conducted with solid-phase extraction using Oasis® HLB cartridges. The chromatography was performed using a C_{18} column with mobile phase consisting of methanol/0.1% formic acid in 20 mM ammonium formate (75:25). The triple–quadrupole mass spectrometric detection was operated in both positive mode (for phenacetin, paracetamol, midazolam, 1′-hydroxymidazolam, brodimoprim, 4′-hydroxymephenytoin, dextromethorphan, dextrorphan and ofloxacin) and negative mode (for tolbutamide, 4-hydroxytolbutamide, carboxytolbutamide and meloxicam). Multiple reaction monitoring mode was used for data acquisition. Calibration ranges in plasma were 2.5–2500 ng/mL for phenacetin, 2.5–2500 ng/mL for paracetamol, 5–500 ng/mL for midazolam, and 0.5–500 ng/mL for 1′-hydroxymidazolam. In urine calibration ranges were 5–1000 ng/mL for dextromethorphan, 0.05–10 μg/mL for dextrorphan and 4′-hydroxymephenytoin, 5–2000 ng/mL for tolbutamide, 0.05–20 μg/mL for 4-hydroxytolbutamide and 0.025–10 μg/mL for carboxytolbutamide. The intra- and inter-day precision were 4.3–12.4% and 1.5–14.8%, respectively for all of the above analytes. The intra- and inter-day accuracy ranged from −9.1 to 8.3% and −10 to 9.2%, respectively for all of the above analytes. The lower limits of quantification were 2.5 ng/mL for phenacetin and paracetamol, 5 ng/mL for midazolam, 0.5 ng/mL for 1′-hydroxymidazolam, 5 ng/mL for dextromethorphan, 50 ng/mL for dextrorphan and 4′-hydroxymephenytoin, 5 ng/mL for tolbutamide, 50 ng/mL for 4-hydroxytolbutamide and 25 ng/mL for carboxytolbutamide. All the analytes were evaluated for short-term (24 h, room temperature), long-term (3 months, −20 °C), three freeze–thaw cycles and autosampler (24 h, 4 °C) stability. The stability of urine samples was also prepared with and without β-glucuronidase incubation (37 °C) and measured comparatively. No significant loss of the analytes was observed at any of the investigated conditions. The current method provides a robust and reliable analytical tool for the above five-probe drug cocktail, and has been successfully verified with known CYP inducers.

1. Introduction

Cytochrome P450 (CYP) system represents drug-metabolizing enzymes involved in phase I (oxidative) metabolism. Among the various CYP isozymes, CYP1A2, 2C9, 2C19, 2D6 and 3A are the major isoforms responsible for the metabolism of more than 90% of market drugs [1,2]. Administration of specific probe drugs followed by measurement of metabolism of those particular substrates can

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be used to determine the real-time activities of CYP450 [3–8]. Compared to the individual administration of specific probes in multiple studies, the "cocktail" approach can minimize the confounding influence of inter-subject and intra-subject variability over the time [9]. Therefore, a number of drug metabolism cocktails (particularly five- or six-drug cocktails) have been proposed and developed [3–5,10–13]. Recently, Jerdi et al. and Yin et al., respectively reported analytical methods for the simultaneous determination of CYP1A2, 2C9, 2C19, 2D6 and 3A substrates [14,15]. However, their cocktails still included some undesirable probe drugs (i.e. debrisoquine and flurbiprofen), so the practical application of both cocktails was limited. First of all, studies suggested that debrisoquine might not correlate well with the CYP2D6 probe sparteine *in vivo* [16,17]. Additionally, debrisoquine had the potential of causing significant hypotension [18]. Secondly, the use of flurbiprofen as a CYP2C9 marker is undesirable due to the weak correlation observed between the formation clearance of flurbiprofen to its CYP2C9-mediated metabolites and genotype [19]. Thirdly, omeprazole has been used as a probe drug for CYP2C19, but besides the CYP2C19-mediated 5-hydroxylation of omeprazole, CYP3A4-mediated sulfoxidation of both omeprazole and hydroxyomeprazole also occurred [20]. Thus the activity of CYP2C19 reflected by omeprazole and 5-hydroxylation is probably not objective.

The current study was proposed to (1) develop a modified phenotyping cocktail with superior probes; (2) choose the most appropriate biological matrix for accurate metabolic analysis of probes; (3) investigate a specific analytical method for the simultaneous evaluation of *in vivo* activities of five major CYP isozymes for drug biotransformation (1A2, 2C9, 2C19, 2D6 and 3A) in one test system. The probe drugs selected for the present study were phenacetin, tolbutamide, mephenytoin, dextromethorphan and midazolam. The chosen substrates have demonstrated as superior (in terms of safety, sensitivity and specificity of enzyme metabolism) to the probes included in previous studies of phenotyping cocktails. To the best of our knowledge, this is the first work combining these five probe drugs for these five CYPs. In this paper we describe the development and validation of a sensitive and selective liquid chromatography/mass spectrometry (LC–MS/MS) method for the simultaneous analysis of the probe drugs (phenacetin, tolbutamide, dextromethorphan and midazolam) and metabolites (paracetamol, 4-hydroxytolbutamide, carboxytolbutamide, 4′-hydroxymephenytoin, dextrorphan and 1′-hydroxymidazolam) in rat plasma and urine (Fig. 1). This method has also been successfully verified with known CYP inducers.

2. Experimental

2.1. Chemicals and animals

Midazolam, 1′-hydroxymidazolam, 4′-hydroxymephenytoin, dextrorphan, carboxytolbutamide, 4-hydroxytolbutamide and lyophilized β-glucuronidase (type VII–A) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenacetin was obtained from Tianjin Li Sheng Pharmaceutical Co. Ltd. (Tianjin, China). Paracetamol was kindly provided by Shen Yang Pharmaceutical University (Shenyang, China). Dextromethorphan, tolbutamide, brodimoprim, ofloxacin and meloxicam were supplied by Tianjin Institute of Pharmaceutical Research (Tianjin, China). All the above standard compounds possess purity of better than 99%. *S*-Mephenytoin was kindly provided by Tianjin Medical University (Tianjin, China). Phenobarbital was purchased from Shanghai Jin Shan Pharmaceutical Co. Ltd. (Shanghai, China). Rifampicin was obtained from Shenyang Hong Qi Pharmaceutical Co. Ltd. (Shenyang, China). HPLC-grade methanol was obtained from Tianjin Concord Tech Reagent Co. Ltd. (Tianjin, China). All other reagents were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China) and were of analytical grade. Deionized water was prepared using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., China) and used throughout the study. Oasis® HLB solid-phase extraction (SPE) cartridge (1 ml, 30 mg) were purchased from Waters Corp. (Milford, MA, USA).

Male Wistar rats weighting 180–220 g were purchased from Center of Experiment Animals, Tianjin Institute of Pharmaceutical Research (Certificate No. 2007-0001), Tianjin, China. Animals were fasted overnight before dose.

2.2. Instrumentation

Samples were analyzed by LC–MS/MS using a Thermo Electron (San Jose, CA, USA) LC–MS/MS system consisting of a Surveyor quaternary narrowbore LC pump, a Surveyor autosampler, fitted with a tempered tray and a column oven, coupled to a TSQ Quantum triple quadrupole tandem mass spectrometer which was equipped with an electrospray ionization (ESI) source. Instrument control and data acquisition was performed with the Xcalibur 1.1 software (Thermo Finnigan). Peak integration and calibration curves were made with LCQuan software (Thermo Finnigan). MS/MS conditions for analytes were optimized by infusing pure solutions (concentrations were approximately 100 ng/mL of each analyte) using the Quantum Tune Master® software (Thermo Electron).

2.3. LC–MS/MS conditions

The chromatographic separation was performed on a Shiseido C18 column (150 mm × 4.6 mm i.d., 5 μm; Shiseido Fine Chemicals, Japan). The column temperature was maintained at 35 °C. The tray temperature in the autosampler was kept at 4 °C. The mobile phase consisted of methanol and 20 mM ammonium formate containing 0.1% formic acid (75:25) with a flow rate of 0.4 mL/min. The total run time for each injection was 16 min (9.0 min for positive mode followed by 7.0 min for negative mode).

The electrospray ionization source was operated in either positive mode (4200 V) or negative mode (−4000 V). The capillary temperature was maintained at 280 °C. High purity nitrogen served both as sheath and auxiliary gas and set to 30 and 10 (arbitrary units), respectively. Argon (1.6 mTorr) was used as the collision-induced dissociation (CID) gas. Detection was carried out in the multiple reaction monitoring (MRM) mode. The MS/MS transitions and fragmentation conditions selected for individual analytes are shown in Table 1. The peak full width at half maximum (FWHM) was set at 0.7 Th for both Q1 and Q3. The scan time for each analyte was 0.2 s.

2.4. Calibration standards and quality control samples

The primary stock solutions of each probe drug and its metabolite were prepared at 1 mg/mL in methanol, except for 1′-hydroxymidazolam, 4′-hydroxymephenytoin and carboxytolbutamide which were 0.5 mg/mL in methanol. The stock solutions of internal standards, brodimoprim, ofloxacin and meloxicam, were prepared at 1 mg/mL in methanol, respectively. All the stock solutions were stored at −20 °C, and were stable for at least 6 months. The working solution of each analyte was prepared by diluting the stock solution with methanol/water (50:50).

Calibration standards (CSs) and quality control (QC) samples were prepared by spiking working solution of each analyte into blank plasma or urine. Calibration samples ($n = 3$) were made to achieved the final plasma concentrations of 2.5, 5, 25, 100,