

Development of Four-Probe Drugs Cocktail Approach Evaluating Major Drug Metabolizing Enzymes Activity

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Abstract: A simple, fast and sensitive HPLC Method was developed for the simultaneous quantification of phenacetin, dapson, omeprazole and chlorzoxazone, reflecting the activities of CYP1A2, CYP3A4, CYP2C19 and CYP2E1 isozyme, respectively. HPLC analyses were performed on a reverse phase C₁₈ column (5 µm, 4.6×250 mm) operating at 30°C. The mobile phase consisted of a acetonitrile and phosphate buffer (pH 6.4-6.5, 34:66). The eluate was detected at 280 nm and the retention times for each compound were within 10 min. The method was validated over the concentration ranges 0.25-25 µg mL⁻¹ for dapson, omeprazole and chlorzoxazone, 0.5-25 µg mL⁻¹ for phenacetine. The intra and inter day precisions were 0.88-5.80 and 3.26-7.75%, respectively and the accuracy ranged from 94.17-103.74 and 93.45-107.08%. The Limit of Quantification (LOQ) was 0.25 µg mL⁻¹ for dapson, omeprazole and chlorzoxazone, 0.5 µg mL⁻¹ for phenacetin. The present method provided a robust, fast and sensitive analytical approach for the four cytochrome P450 probe drug cocktail.

Key words: HPLC, cytochrome P450, Cocktail Method, probe drug, dapson, sensitive

INTRODUCTION

Cytochrome P450 (CYP) enzymes constitute a large superfamily of haem-thiolate proteins involved in the metabolism of a wide variety of both exogenous and endogenous compounds. The CYP activities have been shown to be involved in numerous interactions among drugs (Sirisangtrakul and Sripanidkulchai, 2011). The majority of serious cases of drug interactions are as a result of the interference of the metabolic clearance of one drug by yet another co-administered drug, food or natural product (Saxena *et al.*, 2008). For example, CYP3A4 inhibitors can increase levels of cyclosporine or tacrolimus in body and lead to nephrotoxicity. Levels of the anticholinergic darifenacin can be increased so much by potent CYP3A4 inhibitors that this combination is contraindicated (Michel *et al.*, 2009).

Due to the significance of such drug interactions, probe drugs have been widely employed, alone or in combinations, to determine various individual CYP activities. Recently, the use of multiple probe drugs, i.e., a cocktail approach has become popular in pharmacogenetic studies as this provides a high-throughput method in evaluating CYP isozyme activities (Yin *et al.*, 2004). Several cocktail combinations have been described including the Pittsburgh cocktail (Zhang *et al.*,

2004), Sharma cocktail (Sharma, 2004), Karolinska cocktail (Zhu *et al.*, 2001) and others (Yin *et al.*, 2004). So, far most of the analytical methods for these cocktails are tedious and usually require a separate High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) or Liquid Chromatography/Mass Spectrometry (LC/MS) technique for each probe drug, almost not for all probe drugs in a single run (Yin *et al.*, 2004; Liu *et al.*, 2009).

To eliminate insufficiency in this study, researchers developed and optimized a simplified, rapid, selective HPLC method for the simultaneous extraction and analysis of the four probe drugs in rat serum and applied to the analyses of rat plasma samples. The four-drug cocktail was composited of phenacetin (Zhang *et al.*, 2008; Gao *et al.*, 2007; Testino and Patonay, 2003), Dapson (Tang *et al.*, 2008; qheib *et al.*, 2006; Liu *et al.*, 2005), Omeprazole (Gao *et al.*, 2007; Testino and Patonay, 2003; Tang *et al.*, 2008) and chlorzoxazone (Tang *et al.*, 2008; Zqheib *et al.*, 2006; Liu *et al.*, 2005; Xu *et al.*, 2008), reflecting the activities of CYP1A2, CYP3A4, CYP2C19 and CYP2E1 isozyme, respectively. This approach was an effective means of studying the effects of drugs on activity of CYP enzymes and the interactions among drugs (Brown *et al.*, 2006; Tomalik-Schart *et al.*, 2005).

MATERIALS AND METHODS

Chemicals and reagents: Phenacetin, chlorzoxazone, omeprazole were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dapsone, antipyrine (used as internal standard) were obtained from Fluka Chemical Co. (St. Louis, MO). All the above standard compounds possess purity of better than 98%. HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). HPLC-grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Distilled and deionized water was used throughout the study. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of analytical grade.

Instrument: A Waters LC System (Waters, USA) equipped with a quaternary pump, an autosampler, a thermostated column compartment and UV-detectors. An Waters Chemstation Software Package was used for instrument control, data acquisition and data handling. HPLC analyses were performed on a reverse phase C18 column (5 μm , 4.6 \times 250 mm) (Agilent, USA) coupled with a 4.6 \times 12.5 mm C18 guard column (Agilent, USA) operating at 30°C. Compounds were eluted by a degassed mobile phase consisting of a mixture of acetonitrile and phosphate buffer (pH: 6.4-6.5) which is a mixture water solution of 0.02 mol L⁻¹ potassium dihydrogen phosphate and 0.02 mol L⁻¹ triethylamine (34:66, v/v), at a flow rate of 1.0 mL min⁻¹. UV detection was 280 nm.

Standard solutions: The primary stock solutions of each probe drug were prepared at 1 mg mL⁻¹ in methanol, respectively. The stock solutions of the Internal Standards (IS), antipyrine were prepared at 1 mg mL⁻¹ in methanol. The working solution of each analyte was prepared by diluting the stock solution with methanol.

Serum standard and quality control samples: Standard calibration samples were prepared by spiking the blank rat plasma with the working solution of each analyte. The plasma concentrations for phenacetin were 0.5, 1, 5, 8, 10 and 25 $\mu\text{g mL}^{-1}$, 0.25, 0.5, 1, 5, 8, 10 and 25 $\mu\text{g mL}^{-1}$ for omeprazole and chlorzoxazone, 0.25, 0.5, 1, 5, 10 and 50 $\mu\text{g mL}^{-1}$ for dapsone. Quality Control (QC) samples were prepared in a similar manner at their different concentrations i.e., at the low, medium and high concentrations of 0.5, 5 and 25 $\mu\text{g mL}^{-1}$.

Sample preparation: Serum sample (0.1 mL) containing four probe drugs was extracted with 650 μL chloroform after the addition of 30 μL of internal standard (antipyrine,

100 $\mu\text{g mL}^{-1}$) in a Polyethylene (PE) tube. After mixing for 2 min and centrifuging at 5000 rpm for 5 min, the organic phase 500 μL was removed and evaporated under a gentle stream of nitrogen at 40°C. The residue obtained was dissolved in 100 μL of the initial mobile phase and 30 μL was injected into the HPLC system.

HPLC chromatographic conditions for probe drugs: A Waters e2695 system was used. Chromatography was performed on a C₁₈ column (5 μm , 4.6 \times 250 mm) coupled with a 4.6 \times 12.5 mm C₁₈ guard column operating at 30°C. Compounds were eluted by a degassed mobile phase consisting of a mixture of acetonitrile and phosphate buffer (pH: 6.4-6.5) (34:66, v/v), at a flow rate of 1.0 mL min⁻¹. UV detection was 280 nm. The injection volume was 30 μL for each time and the total run time is 10 min per sample.

Method validation: According to the Yin *et al.* (2004), Liu *et al.* (2009) and Tang *et al.* (2008) and guidelines of SFDA, China, validation of the assay was implemented in connection with the specificity, linearity, sensitivity, relative recovery, precision and accuracy. Applied the assay procedure described in the previous section, three QC samples (at low, medium and high concentration levels) were employed for these tests.

Specificity: To determine assay specificity, blank serum, serum spiked with probe drugs and serum after injection of probe drugs were analyzed to investigate the potential interferences at the LC peak region for each analyte and IS. The experiment was implemented in duplicate.

Linearity and sensitivity: Calibration standards were prepared and analyzed in triplicate in five independent runs. Calibration curves were obtained by utilizing the analyte/IS peak area ratio versus the analyte concentration and were fitted by linear least-squares regression analysis with or without weighting (weighting factor of 1, 1/x, 1/x², x = Concentration). The model with lowest total bias and the most constant bias across the concentration range was identified to be the best fit. To assess linearity, deviations of the mean calculated concentrations over three runs were set at $\pm 15\%$ of nominal concentration, except for the lower limit of quantification where a deviation of $\pm 20\%$ was permitted. The Limit of Quantification (LOQ) was determined based on the criteria that the analyte response at LOQ is 10 times the baseline noise and the analyte response at LOQ can be determined with precision of $\leq 20\%$ and accuracy of 80-120%.

Recovery: The relative recoveries for the serum were analyzed drug-spiked plasma and comparing to the added amount. For each analyte, the recovery experiment was implemented with three QC concentrations (low, medium and high) with five copies of determinations at each concentration.

Precision and accuracy: The intraday precision and accuracy are determined by analyzing the QC samples in five replicates on the same day while inter-day precision and accuracy are determined on each of three separate days. Precision was calculated according to the Relative Standard Deviation (RSD) in which accuracy was assessed as the percentage bias from the nominal concentration (% bias). The acceptable intra and inter day precision and bias were set at $\pm 15\%$ except at the LOQ where they were set at $\pm 20\%$. After analyzing drug-spiked plasma and comparing to the added amount, accuracy was expressed as percent recovery.

Application to non-clinical study: The assay method was applied to a non-clinical study in 6 Wistar rats, demonstrating its feasibility and reliability. Fasted for 12 h with free access to water, 0.5 mL/100 g water solution containing phenacetin (10 mg kg^{-1}), dapsone (5 mg kg^{-1}), omeprazole (10 mg kg^{-1}) and chlorzoxazone (10 mg kg^{-1}) was injected through the caudal vein to the six rats. All animal procedures were performed in accordance with current China legislation.

Venous blood samples were collected in tubes using an indwelling cannula at designated time intervals after administration within 12 h. Each blood sample was immediately centrifuged and stored frozen at -80°C until analysis. Exactly 100 μL of serum was removed to a PE tube with 30 μL Internal Standard (IS, antipyrine). Extracted with 650 μL of chloroform, mixed for 2 min and centrifuged for 5 min under 5000 rpm, exactly 500 μL of the organic layer was transferred to another tube and then evaporated to dryness under a gentle stream of nitrogen, the residue was dissolved in 100 μL of the mobile phase and 30 μL was injected into HPLC.

RESULTS AND DISCUSSION

Method validation

Specificity: For all of serum samples, analyte and IS peaks were free from interference. Well separated from the IS antipyrine, an earlier eluting endogenous peak was observed in the serum sample. Figure 1 for the chromatograms of extracted blank serum, serum spiked with probe drugs and serum after injection of probe drugs.

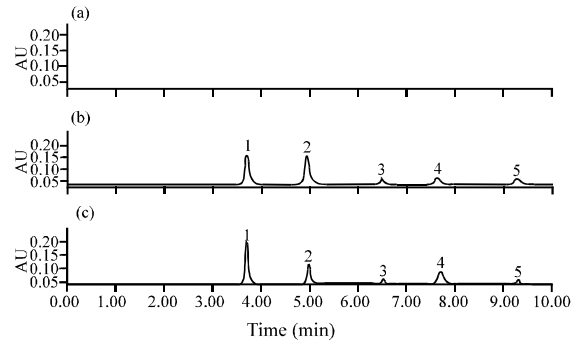


Fig. 1: Chromatograms of four probe drugs and the internal standard in rat serum; a) Blank serum sample; b) drugs added the drug-free serum and c) drug and internal standard extracted from a rat serum after injection of the cocktail, Peaks: 1 = Antipyrine (internal standard), 2 = Dapson, 3 = Phenacetin, 4 = Omeprazole and 5 = Chlorzoxazone

Linearity and sensitivity: The assay was linear over the concentration ranging from 0.25-25 $\mu\text{g mL}^{-1}$ for dapson, omeprazole and chlorzoxazone, 0.5-25 $\mu\text{g mL}^{-1}$ for phenacetin. Utilizing a weighting factor of $1/x$ except for dapson using a weighting factor of $1/x^2$, the best-fit line of the calibration curve and excellent coefficient for each analyte was obtained. Applying the present method, LOQ was 0.5 $\mu\text{g mL}^{-1}$ for phenacetin, 0.25 $\mu\text{g mL}^{-1}$ for dapson, omeprazole and chlorzoxazone (Table 1).

Recovery: The recoveries of probe drugs from drug-free in rat serum were 94.17-103.74% and RSDs (Relative Standard Deviation) were 0.88-5.80% (Table 2).

Precision and accuracy: Table 2 for data of precision and accuracy for each analyte. The intraday RSDs ranges from 0.88-5.80% while interday RSDs ranges from 3.26-7.75%. The interday accuracy is in the scope of 94.17-103.74% while interday accuracy is in the scope of 93.45-107.08%, according to regulatory requirements.

Result of non-clinical study: Novel cocktail was injected through the caudal vein to the six rats, the mean serum concentration-time of four probe drugs in rats were shown in Fig. 2. Analyte and IS peaks were free of interference of endogenous peak and separated. Therefore, the method of simultaneously determining four probe drugs by HPLC is suitable for application to evaluate the activity of CYP1A2, 3A4, 2C19 and 2E1 isozyme, respectively. An important general limitation in the use of multiple probe drug cocktail for the evaluation of CYP isozyme

Table 1: Regression equation, correlation coefficients of linear calibration graphs, concentration range, limits of quantitation for probe drugs

Analyte	Regression equation	Correlation coefficient	Concentration range ($\mu\text{L mL}^{-1}$)	Limit of quantitation ($\mu\text{g mL}^{-1}$)
Phenacetin	$Y = 65.717X + 0.066$	0.999	0.5-25	0.5
Dapson	$Y = 7.754X - 0.007$	0.993	0.25-25	0.25
Omeprazole	$Y = 26.449X - 0.037$	0.998	0.25-25	0.25
Chlorzoxazone	$Y = 30.560X - 0.016$	0.997	0.25-25	0.25

Table 2: Intra and inter day precisions and accuracy of all probe drugs in rat serum

Analyte	Spiked concentration ($\mu\text{g mL}^{-1}$)	Intra day (n = 5)		Inter day (3 days, n = 5)	
		Precision (RSD %)	Accuracy (%)	Precision (RSD %)	Accuracy (%)
Phenacetin	0.5	5.80	100.00±5.80	5.57	103.31±5.76
	5.0	1.02	96.57±0.98	3.77	101.31±3.82
	25.0	3.08	101.71±3.13	3.34	102.21±3.41
Dapson	0.5	0.87	95.6±0.830	6.29	94.09±5.92
	5.0	4.52	98.8±4.470	5.45	101.31±5.52
	25.0	4.14	95.2±3.940	4.68	99.49±4.66
Omeprazole	0.5	3.39	94.17±3.19	7.75	96.97±7.52
	5.0	4.37	101.49±4.43	4.69	104.50±4.90
	25.0	3.13	97.44±3.05	3.48	99.27±3.45
Chlorzoxazone	0.5	3.04	99.56±3.02	3.26	98.64±3.22
	5.0	1.76	99.09±1.74	4.59	104.54±4.80
	25.0	4.14	103.74±4.29	4.98	107.08±5.34

Intraday accuracy was expressed as percent recovery after analyzing drug-spiked plasma and comparing to the added amount

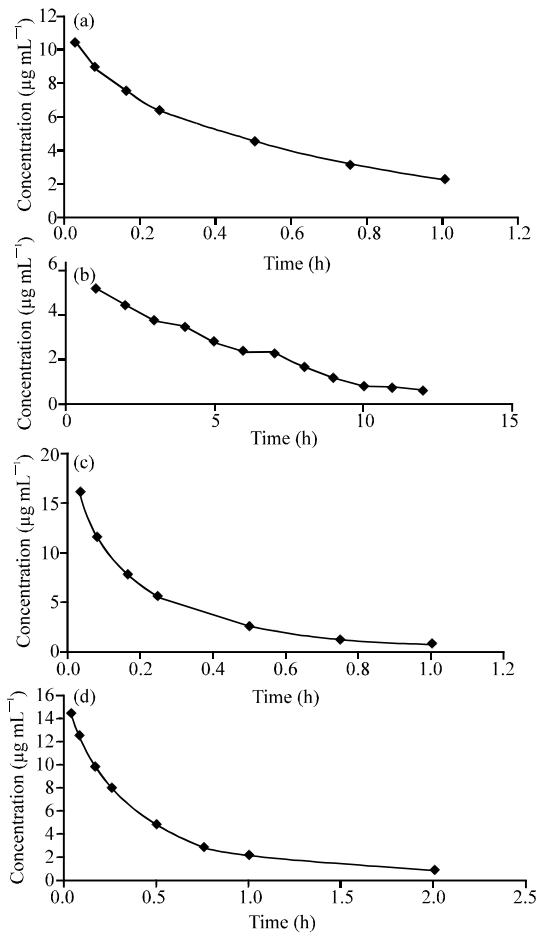


Fig. 2: Serum concentration-time profiles of four probe Drugs after Single Intravenous in Rats (n = 6) Peaks: a) phenacetin 10 mg kg^{-1} ; b) dapson 5 mg kg^{-1} ; c) omeprazole 10 mg kg^{-1} and d) chlorzoxazone 10 mg kg^{-1}

activities is the complicated and tedious assay. Thus, simultaneous determination of all the probe drugs using a simple, rapid, selective analytical method in a single run is most desirable (Yin *et al.*, 2004; Liu *et al.*, 2009).

HPLC with UV detection has been commonly employed in the analysis of probe drugs in blood samples. HPLC/MS/MS and HPLC/MS have also been used in the analysis of probe drugs and the sensitivity can be improved through optimization of analytical parameters. Method development in the present study was based on HPLC coupled with UV detector. For establishing the present analytical method, researchers investigated the different chromatographic conditions to improve the separation of the analytes and IS in a suitable time and good peak separation.

Different types of columns and mobile phase compositions were carefully tested in order to determine the optimal chromatographic conditions. It was found that better separation and peak shapes were achieved with an Agilent C18 column ($250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$) than with Agilent Zorbax SB-C18 column ($4.6 \times 150 \text{ mm}$, $5 \mu\text{m}$) or a C18 column ($250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$) (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China). For the mobile phase, acetonitrile as an organic modifier performed better than methanol and was thus chosen to constitute the mobile phase with phosphate buffer. To develop a suitable method, the influence of the mobile phase composition on the retention time of these compounds was evaluated. When the acetonitrile content of the mobile phase was 26%, the compounds did not separate well. When the acetonitrile content of the mobile phase was $>26\%$, the retention time of four drugs and IS is lengthened. So, researchers set a mobile phase containing 34% acetonitrile as an optimal condition and the total run time was 10 min per sample.

Researchers also investigated the influence of the pH value of the mobile phase, column temperature and the UV absorption wavelengths on retention time, peak width and the shape of the peak. Finally, the pH value was set at 6.4-6.5, the column temperature was at 30°C and chromatograms for their quantitative analyses were recorded at 280 nm.

In this study we used a liquid-liquid extraction procedure that enables to extract simultaneously all 5 analytes (including IS) from serum, followed by chromatography with UV detection. Chloroform and ethyl acetate were respectively selected to extract 5 analytes from serum. It was found that better extraction efficient were achieved with Chloroform than with ethyl acetate.

According to the current method, good sensitivity, precision and accuracy were established to extract 5 analytes from serum by chloroform. Moreover, the retention time is as short as 10 min which promoted the detection efficiency greatly. Compared to other validated cocktails reported in the literature, the present HPLC method is more popular and is much simpler and faster as it requires only a single extraction for a serum sample and the total run time for each sample is only 10 min.

CONCLUSION

A simple, fast, robust and sensitive HPLC-UV assay was developed for the simultaneous determination of a novel four probe-drug cocktail (phenacetine, dapson, omeprazole and chlorzoxazone) for evaluating CYP1A2, CYP3A4, CYP2C19 and CYP2E1 isozyme activity. The cocktail Method is suitable for non-clinical pharmacogenetic studies and also potentially applicable to clinical CYP enzyme research.

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