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# Development and validation of a simple LC method for the determination of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone and their metabolites as markers of cytochromes 1A2, 2A6, 2C11, 2E1 and 3A2 in rat microsomal medium

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Cytochrome P450 enzymes are responsible for the oxidative metabolism of most pharmaceutical compounds. A "cocktail" approach which employs simultaneous administration of a mixture of substrates of CYP enzymes was often used to assess the metabolic activity of multiple P450 forms in one experiment. Phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone are commonly used as probe substrates to evaluate cytochrome P450 function. An analytical strategy to simultaneously extract and analyze the five probe substrates and their major metabolites by HPLC-DAD was developed. The incubation was done with all the substrates in one step. The ten analytes were extracted simultaneously by solid-phase extraction (SPE) from rat liver microsomes. A C18 analytical column and mobile phase composed of acetonitrile and 0.02% aqueous phosphoric acid were used for the chromatographic separation with DAD detection. Limits of quantification varied between 0.02378 and 0.2361 μg/mL which contributed to quantify all these drugs and metabolites with UV detection. The method is applicable for the modeling and description of pharmacological interactions on rat cytochromes P450 or can be used for *in vitro* evaluation of cytochromes 1A2, 2A6, 2C11, 2E1 and 3A2.

## 1. Introduction

A "cocktail" approach employing simultaneous administration of two or more of the probe substrates was often used to assess the metabolic activity of multiple P450 forms in one experiment (Fuhr et al. 2007; Zhou et al. 2004). A well-known advantage of this cocktail method is obtaining information of multiple P450 enzymes in a single experiment (Turpaule et al. 2009). Therefore, the cocktail approach was widely used and is now one of the basic analytical tools in initial drug evaluation (Tang et al. 2008). The probe substrates and their enzyme-specific metabolites can be assessed either simultaneously or separately (Jeong et al. 2009; Yuan et al. 2002; Zgheib et al. 2006; Wahlberg et al. 2003). Unfortunately, most of the studies have employed separate methods for extraction and analysis of these probe substrates and their metabolites (Zgheib et al. 2006; Wahlberg et al. 2003; Blakey et al. 2004; Sharma et al. 2004). These analytical strategies require a larger amount of the sample matrix and are labor-intensive due to multiple extraction and analysis steps. Therefore, a simpler assay procedure for analyzing multiple probes and their major metabolites from a single sample appears attractive.

Phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone are metabolized to acetaminophen, 7-hydroxycoumarin, 4-hydroxytolbutamide, 6-hydroxychlorzoxazone and 6β-hydroxytestosterone by CYP1A2, CYP2A6, CYP2C11, CYP2E1 and CYP3A2, respectively (Fig. 1). None of these five substrates are competitive inhibitors or inducers of CYP metabolism. Therefore, phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone are used as probe substrates to assess CYP1A2, CYP2A6, CYP2C11, CYP2E1 and CYP3A2 activity in both humans and animals (FDA 2006). Since most of currently marketed drugs are metabolized by these five isozymes (Yuan et al. 2002), administration of this probe-substrate cocktail can provide comprehensive information about CYP functionality in an individual.

For quantifying the mixture of markers and their metabolites in biological samples, researchers have been investigating different analytical strategies, such as high-performance liquid chromatography (HPLC) (Jurica et al. 2010; Wang et al. 2010; Elbarbry et al. 2009), LC-MS (Zhang et al. 2010; Dostalek et al. 2010; Zhang et al. 2008; Kumar et al. 2007), *etc*. Some research groups have developed LC-MS or LC–MS/MS methods for analysis of various groups of markers and their specific metabolites in incubation media (Lutz et al. 2010; Smith et al. 2007; Yao et al. 2007). However, some studies just focus on the determination of the probe substrates (Hamdy et al. 2010). Some studies which are quantitative analyses of probe substrates and their enzyme-specific metabolites, the probe substrates and the metabolites were assessed separately (Kumar et al. 2007). Moreover, to the best of our knowledge, the reports described the analysis of the probe substrates of multiple P450 enzymes pay little attention to CYP2A6 and CYP2E1 which are important enzymes for pre-carcinogen activation and oxidation of certain drugs (Kumar et al. 2007; Lutz et al. 2010; Smith et al. 2007; Hamdy et al. 2010).

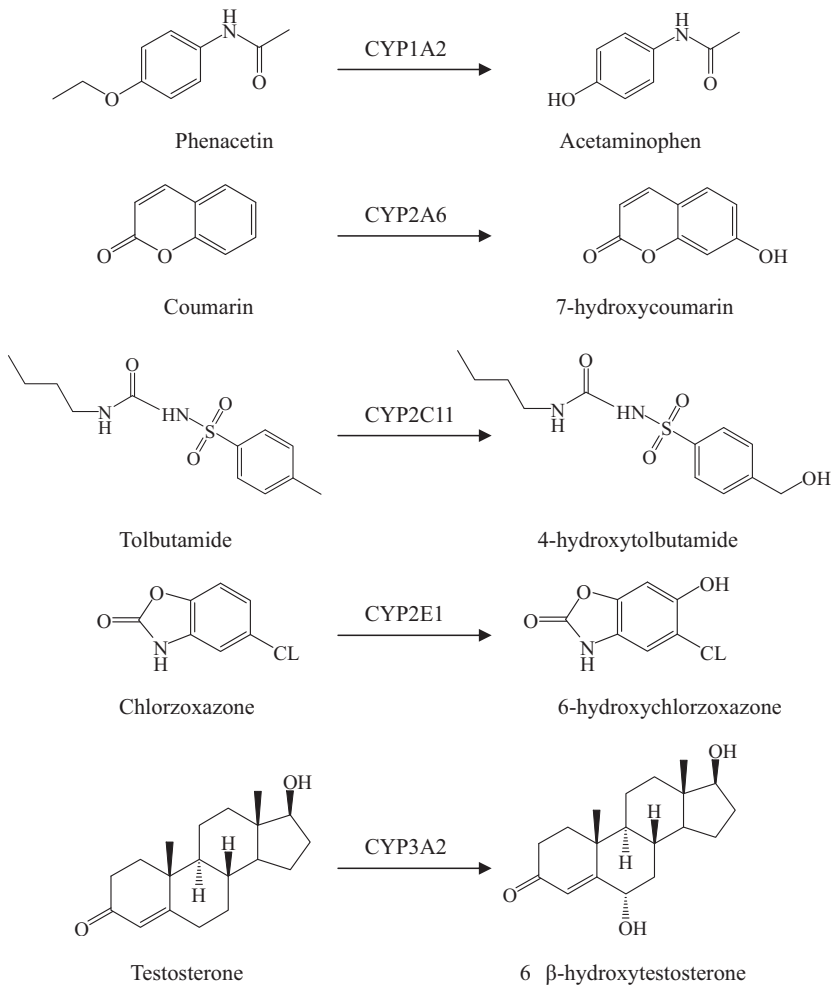


Fig. 1: Chemical structures of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone, and their metabolites

Here we describe a new, simple, rapid and repeatable HPLC method for the simultaneous determination of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone and their enzyme-specific metabolites from rat liver microsomes. The method is applicable for the modeling and description of pharmacological interactions on rat cytochromes P450.

## 2. Investigations, results and discussion

### *2.1. Optimization of HPLC conditions*

Hypersil ODS2 column (250 mm × 4.6 mm i.d. with 5.0 μm particle size), ZORBAX SB C18 column (250 mm × 4.6 mm i.d. with 5.0 μm particle size) and YMC PACK ODS column (250 mm × 4.6 mm i.d. with 5.0 μm particle size) were investigated and the result indicated that the Hypersil ODS2 column showed better separation in this study.

Acetonitrile-water and methanol-water systems were employed to separate the investigated components in sample solutions, and the acetonitrile-water system providing the better separation performance was used.

Formic acid, acetic acid and phosphoric acid are commonly used additives to depress the peak tailing of phenolic compounds. In this study, 0.5% formic acid, 1% acetic acid and 0.2% phosphoric acid (*v/v*) were tested. The result showed that the peak shapes were improved by the use of 0.2% phosphoric acid. Furthermore, effect of the concentration of phosphoric acid was also investigated, and 0.02% phosphoric acid giving the better separation was used.

Column temperatures of 20, 25, 30, 35, and 40 °C were examined. Considering the total resolution of the chromatographic separation, the running time and solvent/reagent consumption, 25 °C was chosen as the column temperature for the separation.

### *2.2. Extraction conditions*

The samples were prepared using acetonitrile precipitation extraction, liquid-liquid extraction and solid-phase extraction (SPE), respectively. The SPE was chosen due to its good stability and recovery. Several commercial SPE columns such as Oasis HLB, ODS, PS2, and Polyamide were examined in this study. As Oasis HLB is packed with polymer, it has both lipophilic and hydrophilic functions as sorbent. Therefore, Oasis HLB was the best among others to extract all of the analytes.

### *2.3. Method validation*

The method was validated for its selectivity, accuracy, precision, recovery, linearity and stability according to the principles of the relevant Food and Drug Administration guidance (FDA 2006). Biological samples were quantified using the peak area ratios of analytes to internal standard. To evaluate linearity, calibration standards were prepared in inactivated microsomes and assayed in sextuple. The precision was assessed by determining the analytes at three concentration levels which were analyzed in septuplicate in one day for intra-day precision and analyzed on six continuous days for inter-day precision. The concentrations of analytes for recovery test were at three levels and analyzed in

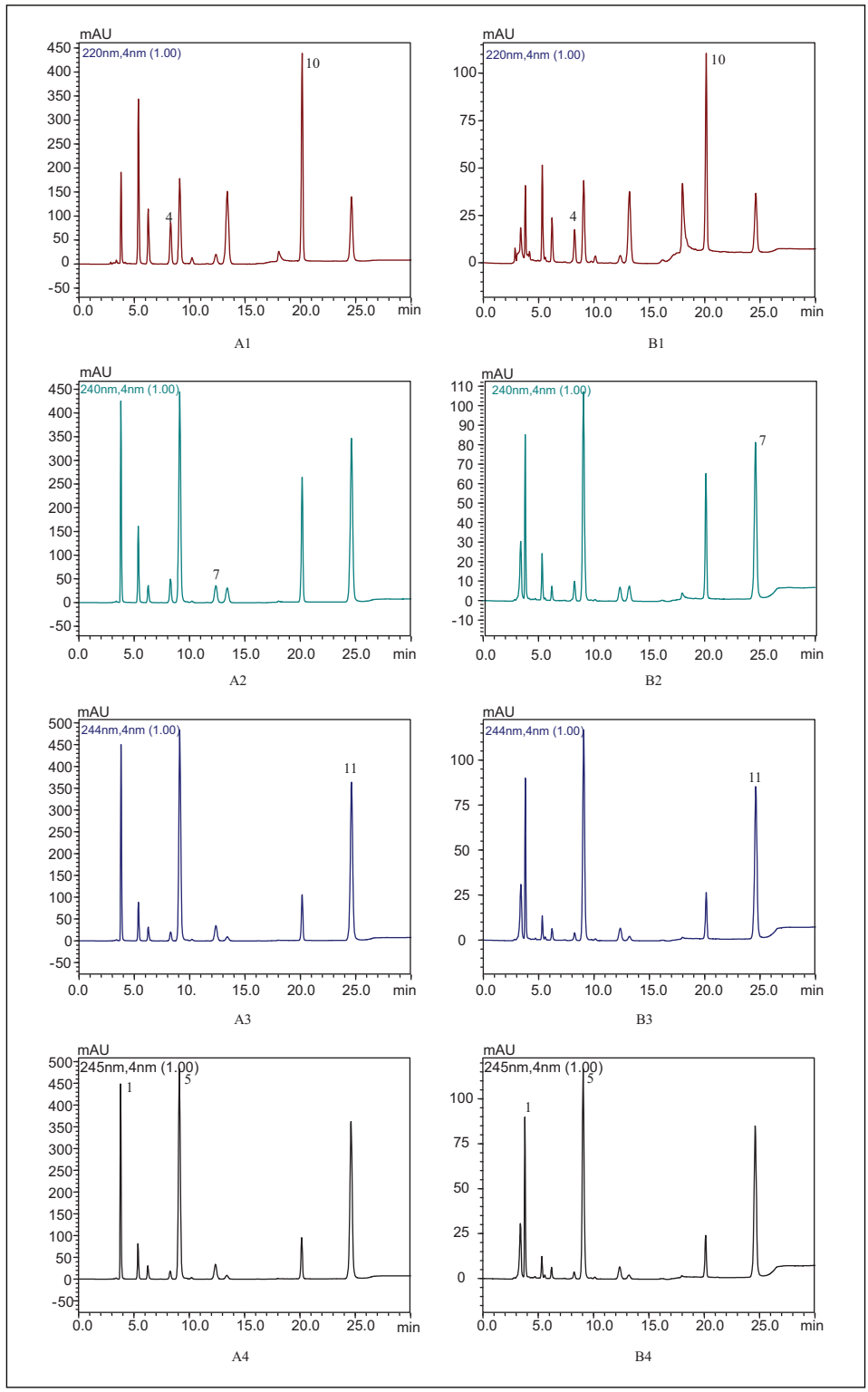


Fig. 2: The typical chromatograms of extracts of (A) medium spiked with standards (B) rat microsomal medium (30 min incubation of phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone) at different detection wavelengths. The peaks marked with 1–11 are acetaminophen, 6-hydroxychlorzoxazone, 7-hydroxycoumarin, 4-hydroxytolbutamide, phenacetin, coumarin, 6β-hydroxytestosterone, chlorzoxazone, dextromethorphan, tolbutamide and testosterone, respectively

septuplicate at each concentration. The stability of the sample extracts in the mobile phase was examined at room temperature for 12 h. The analytes in methanol stored at 4 °C was assessed in septuplicate.

### *2.3.1. Selectivity, linearity and detection limits*

The retention times of the analytes were 3.791, 5.348, 6.244, 8.272, 9.094, 10.183, 12.382, 13.338, 20.171 and 24.640 min for acetaminophen, 6-hydroxychlorzoxazone, 7-hydroxycoumarin, 4-hydroxytolbutamide, phenacetin, coumarin, 6β-hydroxytestosterone, chlorzoxazone, tolbutamide and testosterone, respectively. Fig. 2A shows chromatograms of the rat liver microsomes spiked with standards. Typical chromatograms of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone and their metabolites in rat microsomal incubation are shown in Fig. 2B. No interfering peak was observed at the retention times of the analytes. In all of the

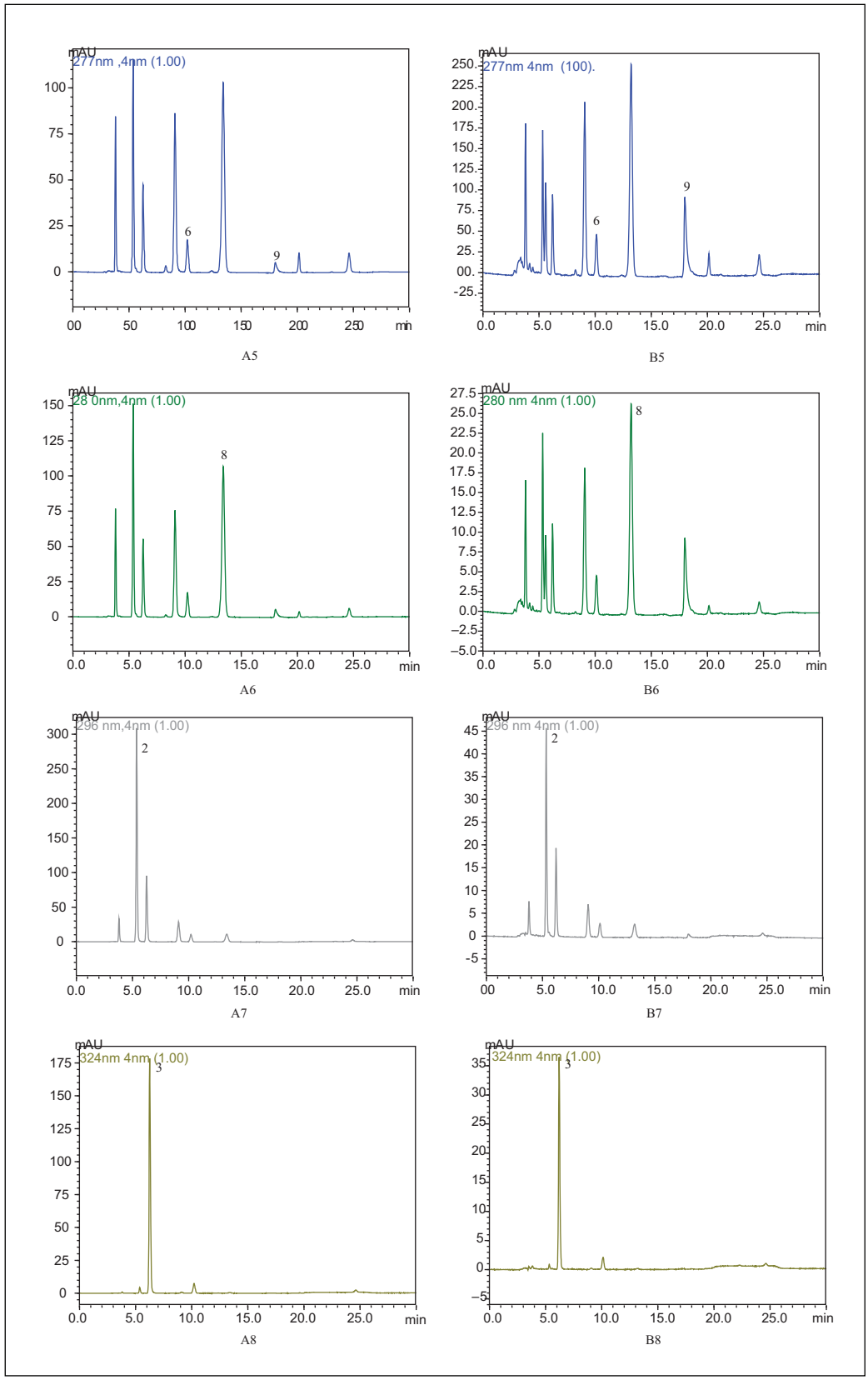


Fig. 2: (*Continued*)

chromatograms presented, the samples were extracted using the same procedure described in the microsomal incubation and sample extraction, employing dextromethorphan as an internal standard.

Because of the different UV characteristic of these ten compounds investigated, the detections at eight wavelengths (220 nm for tolbutamide and 4-hydroxytolbutamide, 240 nm for 6β-hydroxytestosterone, 244 nm for testosterone, 245 nm for phenacetin and acetaminophen, 277 nm for coumarin, 280 nm for chlorzoxazone, 296 nm for 6-hydroxychlorzoxazone and 324 nm for 7-hydroxycoumarin, respectively) were carried out to improve the sensitivity and selectivity for the quantitative

**Table 1: Regression analysis on calibration curves and detection limits (n = 3)**

| Components | Regression equation ($y = ax + b$)[a] | $R^2$ | Linear range (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|
| Phenacetin | $y = (0.2014 \pm 0.012)\ x + (0.1025 \pm 0.010)$ | 0.9987 | 0.1301 − 16.65 | 0.1301 ± 0.0002 |
| Coumarin | $y = (0.0038 \pm 0.001)\ x + (0.0192 \pm 0.002)$ | 0.9974 | 0.2071 − 26.51 | 0.2071 ± 0.0003 |
| Tolbutamide | $y = (0.1233 \pm 0.042)\ x + (0.1219 \pm 0.044)$ | 0.9986 | 0.2361 − 30.23 | 0.2361 ± 0.0001 |
| Chlorzoxazone | $y = (0.0747 \pm 0.003)\ x + (0.0448 \pm 0.001)$ | 0.9988 | 0.1065 − 13.63 | 0.1065 ± 0.0002 |
| Testosterone | $y = (0.2724 \pm 0.017)\ x + (0.0367 \pm 0.001)$ | 0.9985 | 0.1471 − 18.83 | 0.1471 ± 0.0012 |
| Acetaminophen | $y = (0.1762 \pm 0.062)\ x - (0.0524 \pm 0.006)$ | 0.9995 | 0.05883 − 7.531 | 0.05883 ± 0.0057 |
| 7-Hydroxycoumarin | $y = (0.2367 \pm 0.042)\ x - (0.0185 \pm 0.007)$ | 0.9997 | 0.02536 − 3.246 | 0.02536 ± 0.0004 |
| 4-Hydroxytolbutamide | $y = (0.1843 \pm 0.038)\ x - (0.008 \pm 0.004)$ | 0.9996 | 0.03725 − 4.768 | 0.03725 ± 0.0023 |
| 6-Hydroxychlorzoxazone | $y = (0.1191 \pm 0.044)\ x - (0.0736 \pm 0.006)$ | 0.9991 | 0.08697 − 11.13 | 0.08697 ± 0.0082 |
| 6β-Hydroxytestosterone | $y = (0.1871 \pm 0.034)\ x - (0.0097 \pm 0.002)$ | 0.9997 | 0.02378 − 3.044 | 0.02378 ± 0.0077 |

a) $y$ is the peak area ratio of the analytes to dextromethorphan (0.0991 mg/mL), $x$ is the concentration of the analytes in the reaction mixture (500 μL) (μg/mL), $a$ is the slope and $b$ is the intercept of the regression line, respectively. Both values of $a$ and $b$ are given as the form of mean ± SD.

analysis. The target components in the chromatographic profile of the real sample solution were identified by comparing the retention times and the characteristic of the UV spectra of these peaks with those presented in the chromatogram of the mixture standard solution. The peak purity was confirmed by studying the photodiode array detector (DAD) data with peaks of ten analytes in which no indication for impurities could be found.
The calibration curves investigated under the optimum separation conditions exhibit good linear relationships over the tested concentration range for all the compounds. The limits of quantification (LOQ) were determined at S/N (the ratio of signal to noise) of 10, respectively. The linearity, linear range, as well as the LOQ for each compound are presented in Table 1.

### *2.3.2. Precision and stability*

The precision were defined by examining both intra-day precision and inter-day precision. The precision were expressed as relative standard deviation (RSD). The values of intra-day and inter-day precision were less than 9.96% for all the compounds (Table 2).

**Table 2: Intra-day precision and inter-day precision of the developed method (n = 6)**

| Components | Intra-day precision[a)] | | Inter-day precision[b)] | |
|---|---|---|---|---|
| | Contents (± SD, μg/mL) | R.S.D.(%) | Contents (± SD, μg/mL) | R.S.D.(%) |
| Phenacetin | 0.2212 ± 0.01891 | 8.55 | 0.2123 ± 0.01836 | 8.65 |
| | 0.4712 ± 0.02667 | 5.66 | 0.4627 ± 0.03077 | 6.65 |
| | 7.565 ± 0.3609 | 4.77 | 7.465 ± 0.3710 | 4.97 |
| Coumarin | 0.3614 ± 0.02949 | 8.16 | 0.3572 ± 0.02986 | 8.36 |
| | 0.7241 ± 0.06416 | 8.86 | 0.7146 ± 0.06188 | 8.66 |
| | 12.03 ± 0.9038 | 7.51 | 11.03 ± 0.8364 | 7.58 |
| Tolbutamide | 0.3822 ± 0.03233 | 8.46 | 0.3913 ± 0.03350 | 8.56 |
| | 0.8724 ± 0.04240 | 4.86 | 0.8646 ± 0.04288 | 4.96 |
| | 14.07 ± 0.5319 | 3.78 | 13.07 ± 0.5072 | 3.88 |
| Chlorzoxazone | 0.1835 ± 0.01356 | 7.39 | 0.1628 ± 0.01200 | 7.37 |
| | 0.3525 ± 0.02002 | 5.68 | 0.3446 ± 0.01988 | 5.77 |
| | 5.746 ± 0.2034 | 3.54 | 5.645 ± 0.2004 | 3.55 |
| Testosterone | 0.2204 ± 0.01622 | 6.34 | 0.2530 ± 0.01888 | 7.46 |
| | 0.5253 ± 0.03215 | 6.13 | 0.5307 ± 0.03264 | 6.15 |
| | 8.182 ± 0.1833 | 2.24 | 8.282 ± 0.2683 | 3.24 |
| Acetaminophen | 0.1076 ± 0.004455 | 4.14 | 0.09761 ± 0.005134 | 5.26 |
| | 0.2256 ± 0.007625 | 3.38 | 0.2115 ± 0.009644 | 4.56 |
| | 3.541 ± 0.08358 | 2.36 | 3.441 ± 0.08087 | 2.35 |
| 7-Hydroxycoumarin | 0.0441 ± 0.003166 | 7.18 | 0.04412 ± 0.002815 | 6.38 |
| | 0.0957 ± 0.005149 | 5.38 | 0.08543 ± 0.003913 | 4.58 |
| | 1.497 ± 0.07353 | 4.91 | 1.511 ± 0.04912 | 3.25 |
| 4-Hydroxytolbutamide | 0.06861 ± 0.006834 | 9.96 | 0.06432 ± 0.005699 | 8.86 |
| | 0.1289 ± 0.005826 | 4.52 | 0.1314 ± 0.008594 | 6.54 |
| | 2.126 ± 0.002870 | 1.35 | 2.131 ± 0.04944 | 2.32 |
| 6-Hydroxychlorzoxazone | 0.1547 ± 0.01021 | 6.6 | 0.1424 ± 0.01089 | 7.65 |
| | 0.3078 ± 0.01428 | 4.64 | 0.3138 ± 0.02052 | 6.54 |
| | 5.098 ± 0.16924 | 3.32 | 5.194 ± 0.1849 | 3.56 |
| 6β-Hydroxytestosterone | 0.04571 ± 0.004246 | 9.29 | 0.04667 ± 0.004182 | 8.96 |
| | 0.08413 ± 0.005426 | 6.45 | 0.08512 ± 0.006452 | 7.58 |
| | 1.374 ± 0.04643 | 3.38 | 1.397 ± 0.06078 | 4.35 |

a) The sample was analyzed 6 times during one day. b) The sample was analyzed once a day over six consecutive days.

**Table 3: Extraction recoveries of the analytes from rat microsomal medium (n = 6)**

| Components | Nominal concentration (μg/mL) | Estimated concentration[a)] (μg/mL) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| Phenacetin | 0.2601 | 0.2212 ± 0.01891 | 85.03 | 8.55 |
| | 0.5203 | 0.4712 ± 0.02667 | 90.56 | 5.66 |
| | 8.325 | 7.565 ± 0.3609 | 90.88 | 4.77 |
| Coumarin | 0.4142 | 0.3614 ± 0.02949 | 87.26 | 8.16 |
| | 0.8284 | 0.7241 ± 0.06416 | 87.41 | 8.86 |
| | 13.25 | 12.03 ± 0.9038 | 90.79 | 7.52 |
| Tolbutamide | 0.4723 | 0.3822 ± 0.03233 | 80.93 | 8.46 |
| | 0.9445 | 0.8724 ± 0.04240 | 92.36 | 4.86 |
| | 15.11 | 14.07 ± 0.5319 | 93.13 | 3.78 |
| Chlorzoxazone | 0.2130 | 0.1835 ± 0.01356 | 86.14 | 7.39 |
| | 0.4260 | 0.3525 ± 0.02002 | 82.74 | 5.68 |
| | 6.817 | 5.746 ± 0.2034 | 84.31 | 3.54 |
| Testosterone | 0. 2526 | 0.2204 ± 0.01622 | 87.25 | 6.34 |
| | 0.5886 | 0.5253 ± 0.03215 | 89.25 | 6.13 |
| | 9.417 | 8.182 ± 0.1833 | 86.89 | 2.24 |
| Acetaminophen | 0.1177 | 0.1076 ± 0.004455 | 91.45 | 4.15 |
| | 0.2353 | 0.2256 ± 0.007625 | 95.86 | 3.38 |
| | 3.765 | 3.541 ± 0.08358 | 94.06 | 2.36 |
| 7-Hydroxycoumarin | 0.0507 | 0.0441 ± 0.003166 | 86.96 | 7.18 |
| | 0.1014 | 0.0957 ± 0.005149 | 94.34 | 5.38 |
| | 1.623 | 1.497 ± 0.07353 | 92.27 | 4.92 |
| 4-Hydroxytolbutamide | 0.07450 | 0.06861 ± 0.006834 | 92.10 | 9.96 |
| | 0.1490 | 0.1289 ± 0.005826 | 86.52 | 4.52 |
| | 2.384 | 2.126 ± 0.002870 | 89.17 | 1.35 |
| 6-Hydroxychlorzoxazone | 0.1739 | 0.1547 ± 0.01021 | 88.95 | 6.62 |
| | 0.3479 | 0.3078 ± 0.01428 | 88.48 | 4.66 |
| | 5.566 | 5.098 ± 0.16924 | 91.59 | 3.32 |
| 6β-Hydroxytestosterone | 0.04757 | 0.04571 ± 0.004246 | 96.11 | 9.29 |
| | 0.09513 | 0.08413 ± 0.005426 | 88.44 | 6.46 |
| | 1.522 | 1.374 ± 0.04643 | 90.25 | 3.38 |

Recovery = (peak area after extraction)/(peak area of direct injection) × 100 (%). a) Express as mean ± SD (n = 6).

The analytes were stable in methanol for at least 30 days when stored at 4 °C. The RSD of sample extracts between the initial concentrations (immediately after extraction) and the concentrations stored at room temperature (20 °C) for 12 h ranged from 0.03 to 7.89% for all the compounds, which indicated that the sample extracts were stable for at least 12 h at room temperature in the mobile phase.

#### *2.3.3. Recovery*

The extraction recoveries for all analytes were measured with three levels of concentration in spiked rat microsomal medium (Table 3). Eighteen replicates of these samples were extracted and analyzed according to the method described above. The recoveries of all analytes from rat microsomal medium were within an acceptable range of 80.9–96.1%.

### *2.4. Biological application*

Phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone were used as probe substrates to assess rat liver cytochrome CYP1A2, CYP2A6, CYP2C11, CYP2E1 and CYP3A2 activity. Incubation experiments were performed using rat liver microsomes. This HPLC method was used to determine the concentrations of phenacetin, coumarin, tolbutamide, chlorzoxazone, testosterone and their metabolites in rat microsomal medium (Table 4). The results suggested quite a high metabolic turnover of phenacetin, tolbutamide, chlorzoxazone, testosterone by rat CYP1A2, CYP2C11, CYP2E1 and CYP3A2 at the tested concentration level.

### *2.5. Conclusions*

Methods using reactions with more than one marker and subsequent chromatographic analysis are recommended by the FDA in initial drug evaluations for investigating pharmacokinetic interactions with P450 enzymes (FDA 2006). Some studies have used separate methods to extract and analyze various groups of analytes which requires multiple aliquots of sample matrix and was highly labor-intensive.

The method described in this study, in contrast, allows the simultaneous extraction of the five most commonly used probe substrates and their major metabolites from a single 500 μl sample. The chromatographic conditions were well optimized for the simultaneous separation of the target components, and the method validation proved the precision, accuracy and repeatability of this method. The sample was condensed in the process of solid-phase extraction. Therefore, the LLOQ of the analytes ranged from 23.78–236.1 ng/mL which allowed to quantify all these drugs and metabolites with UV detection.

This HPLC–DAD method showed optimal separation and good sensitivity for the determination of phenacetin, coumarin, tolbutamide, chlorzoxazone and testosterone and their enzyme-specific metabolites. The method could be utilized in testing the influence of drugs on the activity of CYP1A2, CYP2A6, CYP2C11, CYP2E1 and CYP3A2 in rat liver microsomes simultaneously, which can save time and material needed for the determination of each P450 enzyme activity separately. The method proved to be useful for this kind of experiment, due to its simplicity, precision, adequate sensitivity, and low sample volume.

**Table 4: Results of rat microsomal incubation experiments (n = 3)**

| Probe substrates | Amount before incubation (μg/mL) | Amount after incubation (μg/mL) | Metabolites | Amount after incubation (μg/mL) |
|---|---|---|---|---|
| Phenacetin | 8.947 | 1.319 ± 0.1039 | acetaminophen | 1.989 ± 0.1315 |
| Coumarin | 14.60 | 7.358 ± 0.09352 | 7-hydroxycoumarin | 3.288 ± 0.1653 |
| Tolbutamide | 16.22 | 4.782 ± 0.1946 | 4-hydroxytolbutamide | 3.542 ± 0.1432 |
| Chlorzoxazone | 8.397 | 1.009 ± 0.04239 | 6-hydroxychlorzoxazone | 2.015 ± 0.06123 |
| Testosterone | 12.40 | 1.615 ± 0.1125 | 6β-hydroxytestosterone | 5.817 ± 0.1860 |

## 3. Experimental

### *3.1. Materials*

Phenacetin, coumarin, tolbutamide and D-glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis. Mo., USA). 6-hydroxychlorzoxazone and 4-hydroxytolbutamide were purchased from Toronto Research Chemicals (North York, Ontario, Canada). 7-Hydroxycoumarin was purchased from Chem Service (West Chester, PA, USA). 6β-Hydroxytestosterone was purchased from Cerilliant Corporation (Round Rock, TX, USA). Chlorzoxazone and acetaminophen were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Testosterone was purchased from Dr. Ehrenstorfer (Augsburg, Germany). NADP and glucose-6-phosphate dehydrogenase were purchased from Roche Diagnostics (Laval, PQ, Canada). Rat liver microsomes were purchased from Research Institute for Liver Diseases Co LTD (Shanghai, China).
HPLC-grade methanol and ethanol were obtained from Kermel (Tianjin, China). Other reagents were analytical-grade or better. Water for HPLC analysis was purified using an ultrapure water purification system (Shanghai Ulupure Engineering Ltd., Shanghai, China).

### *3.2. Analysis*

Analyses were performed using a Shimadzu LC-20A HPLC system (Kyoto, Japan) which consisted of a binary gradient pump (model LC-20A), a SPD-M20A diode array detector, a SIL-20A auto sampler and a DGU-20A3 degasser. The HPLC system was interfaced to a HP PC compatible computer using LC solution software.
All separations were carried out on a Hypersil ODS2 column (250 mm × 4.6 mm i.d. with 5.0 μm particle size). A linear gradient elution of mobile phase A (0.02% aqueous phosphoric acid) and mobile phase B (acetonitrile) was used to run the separation. The elution program was well optimized and conducted as follows: an isocratic elution of 25% mobile phase B with the first 11 min and then 40% mobile phase B with the range of 11–28 min. After a washing step of 8 min with 90% mobile phase B, the column was re-equibrated for 10 min with 25% mobile phase B. The solvent flow rate was 1.0 mL/min, the injection volume was 20 μl, and the column temperature was maintained at 25 °C. Eight detection wavelengths were used for the quantitative analysis (UV wavelength 220 nm for tolbutamide and 4-hydroxytolbutamide, 240 nm for 6β-hydroxytestosterone, 244 nm for testosterone, 245 nm for phenacetin and acetaminophen, 277 nm for coumarin, 280 nm for chlorzoxazone, 296 nm for 6-hydroxychlorzoxazone and 324 nm for 7-hydroxycoumarin).

### *3.3. Standard stock solutions, calibration standards and quality control*

The standard stock solutions of phenacetin (13.32 mg/mL), coumarin (25.34 mg/mL), tolbutamide (24.19 mg/mL), chlorzoxazone (13.64 mg/mL), testosterone (3.645 mg/mL), acetaminophen (0.8862 mg/mL), 7-hydroxycoumarin (1.008 mg/mL), 4-hydroxytolbutamide (1.195 mg/mL), 6-hydroxychlorzoxazone (0.9238 mg/mL) and 6β-hydroxytestosterone (0.6088 mg/mL) dextromethorphan (9.912 mg/mL) were prepared in methanol and stored protected from light at 4 °C. Working solutions of the lower concentration were prepared by appropriate dilution of the stock solution.
Calibration standards and quality control samples were prepared by spiking inactivated rat microsomal medium with the working standard solutions. Quality control samples were prepared at three different concentrations of each analyte (low, average and high) for the method validation studies. Each sample was prepared, extracted and diluted as described for microsomal incubation and sample extraction. A working internal standard solution was prepared at a final concentration of 0.0991 mg/mL.

### *3.4. Microsomal incubation and sample extraction*

The reaction mixture containing the following compounds with final concentrations described below was prepared on ice: 0.8 mg protein/mL rat liver microsomes, 0.1 mol/L potassium dihydrogeno/hydrogenophosphate buffer (pH 7.4), 49.92 μmol/L phenacetin, 99.88 μmol/L coumarin, 60.00 μmol/L tolbutamide, 49.52 μmol/L chlorzoxazone and 20.13 μmol/L testosterone (the concentrations were around Km). The incubation was done with all the substrates in one incubation with higher substrate concentrations in their linear ranges. After the reaction mixture was pre-incubated at 37 °C for 5 min, 50 μL NADPH generating system (final concentration: 3.3 mmol/L magnesium chloride, 3.3 mmol/L glucose-6-phosphate, 1 mmol/L NADP, and 0.4 IU/mL of glucose-6-phosphate dehydrogenase) was added to the mixture to initiate the reaction (final volume of the reaction mixture: 500 μL). The final concentration of the methanol added to the reaction mixture was set as < 1%. 30 min later, the reaction mixture was put on ice for 30 min to terminate reaction and extracted at once.
The reaction mixture (500 μL), 500 μL water, 20 μL strong phosphoric acid and 20 μL internal standard solution (0.0991 mg/mL) were mixed well with a vortex mixer. Oasis HLB SPE cartridges (30 mg, 1 mL) were conditioned with 1.0 mL methanol and 1.0 mL deionized water. The samples were added to the cartridges and vacuum was applied. The cartridges were washed with 1.0 mL 5% methanol-water (*v/v*). The sample was eluted using 1.0 mL methanol and evaporated to dryness in a water bath at 37 °C under the protection of nitrogen. The residue was reconstituted with 100 μL initial mobile phase. The solution was then centrifuged at 1600 rcf for 10 min and the supernatant obtained was analyzed by HPLC.

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