Determination of Tolbutamide and Hydroxytolbutamide by LC–MS/MS in Rat and its Application to Assessment of CYP2C9 Activity

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SUMMARY. A sensitive and selective liquid chromatography–tandem mass spectrometry method (LC–MS/MS) for the determination of tolbutamide (TB) and its metabolite hydroxytolbutamide (HTB) in rat plasma was developed using carbamazepine as an internal standard. Chromatographic separation was performed by an Agilent Zorbax SB-C18 column (150 mmx2.1 mm, 3.5 μm), using the gradient elution of 0.1 % formic acid in water and acetonitrile. Calibration plots were linear over range of 5–1000 ng/mL for TB and 10–2000 ng/mL for HTB in rat plasma. The intra- and inter-day relative standard deviations of the assay were less than 10 % for both TB and HTB. The validated method is successfully used to analyze the influence of bupropion on cytochrome P450-mediated metabolism of TB. The biotransformation rates of TB administered either separately or both simultaneously were compared in this study. The results revealed that bupropion had no significant effect on TB hydroxylation.

INTRODUCTION

Specific probe drugs have been widely used for assessing various individual cytochrome P450 (CYP) enzymes activities. In addition to phenotyping, this approach can be used to evaluate the potential inhibitory or abductive effects of a New Chemical Entity (NCE) on the pharmacokinetics of representative probes of CYP enzymes and hence, in combination with the in vitro data, provide the basis for a rational and efficient approach to a drug-drug interaction strategy. Therefore, a number of drug metabolism cocktail methods have been described and developed 1-7.

Unfortunately, this approach also has certain limitations and disadvantages, such as mutual interactions between probe drugs, the frequent occurrence of side effects, and analytical complexities 8. For example, interactions between chlorozoxazone (CYP2E1) and midazolam (CYP3A) 9, dextromethorphan (CYP2D6) and chloroguanide (CYP2C19) 10, and caffeine (CYP1A2) and chlorozoxazone (CYP2E1) 11 have been reported. Thus, it is essential to ensure that probe drugs and metabolites in a serum or urine sample do not give rise to analytical interference.

Tolbutamide(TB) is an oral hypoglycaemic agent and has been extensively studied in humans, as it is a probe drug for CYP2C9 in drug oxidation 12. The pharmacological studies required the simultaneous measurement of the concentration of TB and its metabolite hydroxytolbutamide (HTB) in biological fluids. The method of gas chromatography (GC) 13 and liquid chromatography with ultraviolet detection (LC-UV) 14 have been reported, but lack of sensitivity and specificity and time-consuming. Thus, a fast and sensitive LC–MS/MS method for the determination of TB and HTB in rat plasma using one-step protein precipitation was developed and validated. Our current work was to develop a rapid and selective LC–MS/MS method for the simulta-
neous determination of TB and HTB in rat plasma as well as urine and evaluate the influence of bupropion on cytochrome P450-mediated metabolism of TB.

MATERIALS AND METHODS

Chemicals and Reagents

TB (purity > 98.0 %), HTB (purity > 98.0 %) and bupropion (purity > 98.0 %) were purchased from Sigma-Aldrich Company. Carbamazepine (purity > 98 %) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). Ultra-pure water (resistance > 18mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA) was used to make mobile phase.

Animals

Twelve male Sprague-Dawley rats, weighing between 200-250 g, were all obtained from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China). The rats were housed individually in standard plastic cages and maintained on normal rat purina chow and fresh water ad libitum in a room controlled for temperature (23-25 °C) and lighting (8:00-20:00 light, 20:00-8:00 dark). After the first week acclimatization period, rats were used for experiments and all efforts were made to minimize any animal suffering. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Instrumentation and Analytical Conditions

For the analysis of TB and its metabolite HTB, a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source were used. Chromatographic separation was performed on a Agilent Zorbax SB-C18 column (150 mm x 2.1 mm, 3.5 µm particle) at 30 °C by using the gradient elution of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B) as follows: 0-1.5 min (10-85 % B), 1.5–6.0 min (85-85 % B), 6.0-7.0 min (85-10 % B), 7.0-10.0 min (10-10 % B). The flow rate was 0.4 mL/min.

The quantification was performed by the peak-area method. Drying gas flow and nebulizer pressure was set at 6 L min⁻¹ and 20 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3,500 V, respectively. LC-MS was performed with SIM mode using target ions at m/z 271.2 for TB, m/z 286.9 for HTB and m/z 236.8 for carbamazepine (IS) in positive ion electrospray ionization interface.

Preparation of Calibration Standards and Quality-Control Samples

Individual stock solutions of TB (1.0 mg/mL), HTB (1.0 mg/mL) and carbamazepine (internal standard, IS, 100.0 µg/mL) were prepared in acetonitrile. Working solutions for calibration and controls were prepared from the stock solution by dilution using acetonitrile. All of the solutions were stored at 4 °C and were brought to room temperature before use.

Calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the stock solutions. Calibration plots were constructed in the range of 5-1000 ng/mL for TB (5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL) and 10-2000 ng/mL for HTB (10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards (10, 80, and 800 ng/mL for TB and 20, 160, and 1600 ng/mL for HTB). The analytical standards and QC samples were stored at -20 °C.

Sample Preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (1.0 µg/mL) was added to 0.2 mL of collected plasma sample followed by the addition of 0.4 mL of acetonitrile. The tubes were vortex mixed for 1 min. After centrifugation at 13,000 rpm for 10 min, the supernatant (10 µL) was injected into the LC-MS/MS system for analysis.

Method Validation

The selectivity of the method was evaluated by analyzing blank rat plasma and blank plasma spiked TB, HTB and IS. Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of TB and HTB to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the con-
centration squared \((1/x^2)\) in the concentration range of 5-1000 ng/mL for TB and 10-2000 ng/mL for HTB.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in five replicates (10, 80, and 800 ng/mL for TB and 20, 160, and 1600 ng/mL for HTB) in three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ± 15%.

The recoveries of both TB and HTB at three QC levels \((n = 5)\) were determined by comparing the peak area obtained from the extracted plasma samples with the peak area obtained by the direct injection of the corresponding concentration spiked standard solution in the extracted blank plasma. The IS solution was added to both sets of samples post-protein precipitation.

The stabilities of TB and HTB in rat plasma were evaluated by analyzing replicates \((n = 3)\) of plasma samples at three QC levels in different conditions. The short-term stability was determined after the spiked samples kept at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) kept at room temperature for 24 h in the HPLC autosampler. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 °C to room temperature) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at -20 °C for 14 days. The results were compared with those obtained for freshly prepared plasma samples.

**HTB/TB urinary metabolic ratio study**

This was an open, randomized crossover study. Twelve male Sprague-Dawley rats were randomly divided into two groups \((n = 6)\). Before the study, diet was prohibited for 12 h until 2 h after drug administration, but water was freely available. In the first cycle, one group took TB (3 mg/kg) alone and another group received TB (3 mg/kg) and bupropion (15 mg/kg) in combination after an overnight fast. Drugs were administered by gastric irrigation. Urine was collected during time intervals of 0 to 8 hours, 8 to 12 hours, and 12 to 24 hours after drug intake. Before determination, each urine sample was diluted appropriate concentration.

**Pharmacokinetic study**

The above 12 rats were raised for a 2-weeks recovery stage for the secondary administration. In the secondary cycle, oral administration in the first cycle were repeated. Blood samples (0.3 mL) from the tail vein were collected immediately into heparinized ploythene tubes before drug administration and 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 36, and 48 h thereafter. After the samples centrifuged at 13,000 rpm for 10 min, the plasma obtained (100 µL) was transferred into 1.5 mL heparinized ploythene tubes and stored at -20 °C until analysis.

**Statistical analysis**

Results were expressed as means ± SD. All analyses for comparing the datas determined from TB alone and in combination were performed with the SPSS software system version 16.0(SPSS Inc., Chicago) by use of Student’s t-test. The level of significance was set at \(P < 0.05\). Plasma concentration versus time data for each rat was analyzed by DAS software (Version 2.0, Medical College of Wenzhou, China).

**RESULTS AND DISCUSSION**

**Optimization of HPLC and MS conditions**

Under the conditions described in the experimental part, the assay was highly specific, and no endogenous plasma materials interfered with the peak of TB, HTB and IS. TB, HTB and IS were eluted with retention times of 3.9, 3.5, and 3.7 min, respectively (Fig. 1). Figure 1 shows the typical chromatograms of a blank plasma sample, a blank plasma spiked with TB, HTB and IS, and a plasma sample obtained at 24.0 h after single oral adminstration of 3 mg/kg TB.

Different mobile phase compositions were assessed to increase the sensitivity and obtain better separation. Ammonium acetate–acetonitrile, ammonium acetate–methanol and water containing formic acid–acetonitrile were optimized as the mobile phase. On the basis of the total resolution, running time and reagent consumption in chromatographic separation, acetonitrile was chosen as the mobile phase because of its stronger elutive power than methanol. In addition, acetonitrile also reduced chromatographic separation time and resulted in sharper peak shape. We also found that addition of formic acid improved the separation and made peak shape sharper. Compared to isocratic elution, gradient elution provided better peak

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symmetry, proper retention time, and avoided the matrix effects for the analytes and IS. A flow rate of 0.4 mL/min produced good peak shapes and permitted a run time of 10 min.

An efficient clean-up for bio-samples to remove protein and potential interferences prior to LC-MS/MS analysis was an important point in the studies. The simple and effective protein precipitation was employed in our work, which could provide the acceptable recovery and avoid the matrix effects. Acetonitrile was chosen as the solvent for protein precipitation, because it exhibited better effect than methanol.

**Method Development**

The calibration curves of TB and HTB in rat plasma were linear in the range of 5-1000 ng/mL for TB and 10-2000 ng/mL for HTB. The typical equation of the calibration curves were: 
\[ y = 0.0011x - 0.0027, \quad r = 0.996 \text{ for TB} \]  
\[ y = 0.0034x - 0.032, \quad r = 0.992 \text{ for HTB} \]

where \( y \) represents the peak area ratio of TB or HTB to IS and \( x \) represents the plasma concentration of TB or HTB. The LLOQ for the determination in plasma was 5 ng/mL for TB and 10 ng/mL for HTB. The results show that the method was sensitive enough for the assessment of CYP2C9 activity.

The precision of the method was determined by analyzing three different concentration levels (10, 80, and 800 ng/mL for TB and 20, 160, and 1600 ng/mL for HTB) over three validation days. Intra-day and inter-day precision were both 10 % or less at each QC level for TB and HTB. Accuracy ranged from 104.67 to 106.27 % for TB, and from 101.32 to 106.22 % for HTB. As shown in Table 1, this method allowed good precision and accuracy. The recovery of TB and HTB from rat plasma was more than 80 %.

All the stability studies of TB and HTB in plasma were conducted at three QC level with three determinations for each under different storage conditions. The RSD of the mean test responses was within 10 % in all stability tests of TB and HTB in plasma. No effect on the quantitation was observed for plasma samples kept at room temperature for 2 h and 24 h in an autosampler. There was also no significant degradation when samples of TB and HTB in plasma were taken through three freeze (-20 °C)-thaw (room temperature) cycles. And TB and HTB were also stable at -20 °C for 14 days.
Application of the Method

The developed and validated method was applied to the study of the assessment of CYP2C9 activity administered TB alone or both combined with bupropion. Table 2 shows that TB partial metabolic clearance to HTB and renal clearance of TB and HTB did not change significantly whether TB was administered alone or in the presence of bupropion. Moreover, the HTB/TB urinary metabolic ratio was not affected over a 24-hour collection after simultaneous bupropion oral administration. Likewise, the individual and mean TB and HTB plasma concentration-time profiles (Fig. 2) were not significantly different whether TB was administered alone or with bupropion. Accordingly, there was no difference in the pharmacokinetic parameters for TB and HTB and for the plasma HTB/TB metabolic ratio in the presence or absence of bupropion. The results suggest that bupropion addition to TB had no significant effect on CYP2C9-mediated TB hydroxylation.

CONCLUSION

A sensitive, rapid and specific LC–MS/MS method for the determination of TB and HTB in rat was developed. The method was validated to meet the requirements for the determination of TB and HTB in rat plasma and urine, and also could be suitable for assessing the CYP2C9 activity. From the presented results, it may be suggested that bupropion had no significant effect on TB hydroxylation.

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