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Determination of bupropion and its main metabolite in rat plasma by LC-MS and its application to pharmacokinetics

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A sensitive and selective liquid chromatograpy-mass spectrometry method for the determination of bupropion and its main metabolite, hydroxubupropion, in rat plasma was developed and validated. After addition of carbamazepine as internal standard (IS) and precipitation of protein with acetonitrile, the plasma samples were analyzed on an Agilent Zorbax SB-C_{18} (2.1 mm × 50 mm, 3.5 μm) column at 30 °C, with acetonitrile-0.1% formic acid as mobile phase at a flow rate of 0.4 mL min^{-1}. The detection was carried out in the selective ion monitoring mode with a positive electrospray ionization interface. The calibration curve was linear over the 10–2000 ng mL^{-1} for bupropion and 5–1000 ng mL^{-1} for hydroxybupropion in plasma. RSD of inter-day and intra-day precision was less than 7% for bupropion, 9% for hydroxybupropion. The developed method was successfully applied to pharmacokinetic studies after single intragastric administration of bupropion 15 mg kg^{-1} to rats.

1. Introduction

Bupropion ((±)-2-(*tert*-butylamino)-30-chloropropiophenone hydrochloride) is a second-generation antidepressant agent with neurochemical properties different from common tricyclic antidepressants (Yeniceli and Dogrukol-Ak 2009). It is also used as a non-nicotine drug for smoking cessation (Lau and Chang 2009). Bupropion (BUP) is metabolized to three metabolites: hydroxybupropion (HBUP), erythrohydrobupropion, and threohydroburpropion (Golden et al. 1988; Hesse et al. 2000; Schroeder 1983). HBUP is the major metabolite, since the plasma concentration of HBUP is nearly 4- to 6-fold greater and the AUC of HBUP is 10- to 16-fold greater compared to BUP (Faucette et al. 2001).

CYP2B6 is a member of the Cytochrome P450 (CYP450) group of enzymes and makes up approximately 2–10% of total hepatic CYP450 content (Wang and Tompkins 2008). Most of beta blockers and several antipsychotics and antidepressants are metabolized by CYP2B6 (Zanger et al. 2008). CYP2B6 is highly inducible by several drugs and other xenobiotics, furthermore a wide interindividual variability in the hepatic expression of CYP2B6 has been reported (Code et al. 1997; Wang and Negishi 2003). The previous investigations of the role of CYP2B6 in drug metabolism have been limited by the unavailability of selective metabolic probes for this enzyme (Faucette et al. 2000), therefore a phenotypic marker can be useful to assess CYP2B6 activity *in vitro* and *in vivo*.

In vitro studies have demonstrated that HBUP is generated from BUP almost exclusively by CYP2B6 (Faucette et al. 2000). Therefore, BUP was recognized as a probe drug of CYP2B6 *in vitro* and *in vivo* (Faucette et al. 2000; Turpeinen et al. 2004). Thus cocktail assays including BUP were developed to simultaneously measure the activities of CYP2B6 and other cytochrome P450 (CYP) enzymes to study whether other chemicals or drugs may induce or inhibit the cytochrome P450 (CYP) enzyme system and predict the potential drug-drug interactions (Dixit et al. 2007).

Numerous methods have been reported for the determination of BUP, included gas chromatography (Rohrig and Ray 1992), absorption spectrometry (Ramcharitar et al. 1992), fluorimetry (Pokrajac et al. 1991), and high-performance liquid chromatography (Loboz et al. 2005; Zhang et al. 2003). However, it seems reasonable to evaluate the activity of CYP2B6 by determining BUP and HBUP simultaneously. Reported HPLC methods for the determination of BUP and its metabolite using UV detection have poor sensitivity and are time-consuming (Cooper et al. 1984; Jennison et al. 1995; Zhang et al. 2003). The LC–MS method is time saving and has greater sensitivity than the published HPLC methods. The sample preparation methods described in these report include liquid–liquid extraction (Borges et al. 2004) and protein precipitation (Yeniceli et al. 2011).

The aim of this study was to develop a rapid and selective LC–ESI-MS (ion trap) method for the simultaneous determination of BUP and HBUP with a new sample preparation method, in order to obtain the pharmacokinetic profile of BUP and HBUP in Sprague Dawley rats.

2. Investigations and results

2.1. Method development

The feasibility of electrospray in positive and negative ion modes of detection was evaluated during the early stages of assay development. It was found that electro spray ionization with positive ion detection resulted in a better signal-to-noise ratio, the spectrum is shown in Fig. 1.

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and short an analysis time) and appropriate ionization. Various combinations of acetonitrile and 0.1% formic acid in water with changed

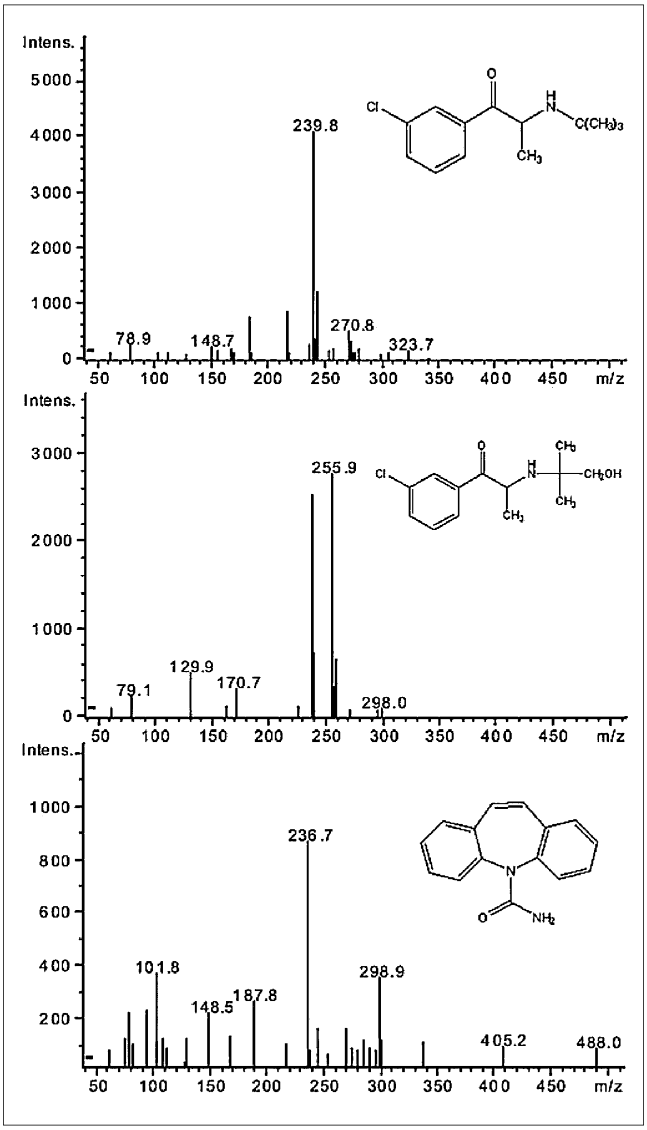

Fig. 1: MS spectrum of BUP, HBUP and carbamazepine (IS) with [M + H]+

content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic solvent because of its sharper peak shape, lower pressure and higher stability compared to methanol. Formic acid added into the mobile phase could not improve sensitivity, therefore acetonitrile was chosen as mobile phase. Gradient elution provided better peak symmetry, proper retention time, and avoided the matrix effects for the analytes and IS compared to isocratic elution. A flow rate of 0.4 mL min^{-1} produced good peak shapes and permitted a run time of 10 min.

Plasma protein precipitation was used for pretreatment of rat plasma samples as it is widely used and has a high recovery. Furthermore, it is more simple and rapid than the liquid-liquid extraction method. In this paper, three kinds of precipitation reagents, methanol, acetonitrile and 10% trichloro-aceticacid, were used to precipitate rat plasma protein. The supernatant was directly injected into the LC-MS system for analysis. The results showed that the acetonitrile proved to be the best reagent in terms of the peak shape obtained by LC-MS.

2.2. *Selectivity and matrix effect*

Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma spiked with BUP, HBUP and IS, and a plasma sample obtained at 0.15 h after a single oral adminstration of 15 mg kg^{-1} BUP. No interfering endogenous substances were observed at the retention times of the two analytes and IS.

The value of matrix effect for BUP and HBUP at concentrations of 20, 160, 1600 ng mL^{-1} and 10, 80, 800 ng mL^{-1} were 92, 91, 99% and 95, 97, 101% (n = 3), respectively. The ME of IS (50 ng mL^{-1}) was 91%. The result demonstrated that matrix effect from plasma was negligible in this method.

2.3. *Calibration curve and sensitivity*

The calibration curves of BUP and HBUP in rat plasma were linear in the range of 10–2000 ng mL^{-1} for BUP and 5–500 ng mL^{-1} for HBUP. The typical equation of the calibration curves were: $y = (0.0114 \pm 0.000954)$ x- (0.057033 ± 0.048133), $r^2 = 0.9985 \pm 0.001114$ for BUP and $y = (0.005 \pm 0.000283)$ x - (0.101467 ± 0.081919), $r^2 = 0.9913 \pm 0.006655$ for HBUP, where y represents the ratios of peak area to that of IS and x represents the plasma concentration. The LLOQ for the determination in plasma was 5 ng mL^{-1} for BUP, and 10 ng mL^{-1} for HBUP. The results show that the method was sensitive enough for pharmacokinetics studies.

2.4. *Precision, accuracy and recovery*

The precision of the method was determined by analyzing three different concentration levels (20, 160, 1600 ng mL^{-1} for BUP and 10, 80, 800 ng mL^{-1} for HBUP) over three validation days. Intra-day precision was 3.81% to 5.96% for BUP and 5.2% to 6.8% for HBUP, and the inter-day precision was 4.93% to 6.90% for BUP and 3.6% to 8.7% for HBUP at each QC level. Accuracy ranged from 105.02% to 106.63% for BUP, 101.53% to 109.65% for HBUP. Assay performance data are showed in Table 1. It was demonstrated that the values were within the acceptable range and proved the good precise and accurate of the developed method.

Mean recoveries were 83.67, 81.75, and 85.78% (n = 6) for BUP, and 52.67, 52.56, 53.39% (n = 6) for HBUP at concentrations of 20, 160, 1600 ng mL^{-1} for BUP and 10, 80, 800 ng mL^{-1} for HBUP.

2.5. *Stability*

The stability results indicated that BUP, HBUP and IS spiked into the rat plasma were stable at room temperature for 2 h, in the sample solvent on autosampler for 24 h, during freeze/thaw cycles and at −20 °C for 30 days. The results of the stability experiments are shown in Table 2.

2.6. *Application of the method*

The method was applied to a pharmacokinetic study after a single oral administration to rats. The mean plasma concentration-time curves after administration of a single 15 mg/kg oral dose of BUP and its main metabolite, HBUP are presented in Fig. 3. The main pharmacokinetic parameters from two-compartment model analysis in six rats were calculated and summarized in Table 3.

3. Experimental

3.1. *Chemicals and reagents*

Bupropion (purity > 98.0%) and hydroxubupropion (purity > 98.0%) were purchased from Sigma-Aldrich Company. Carbamazepine (purity > 98%) were purchased from the Nation Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC-grade acetonitrile and

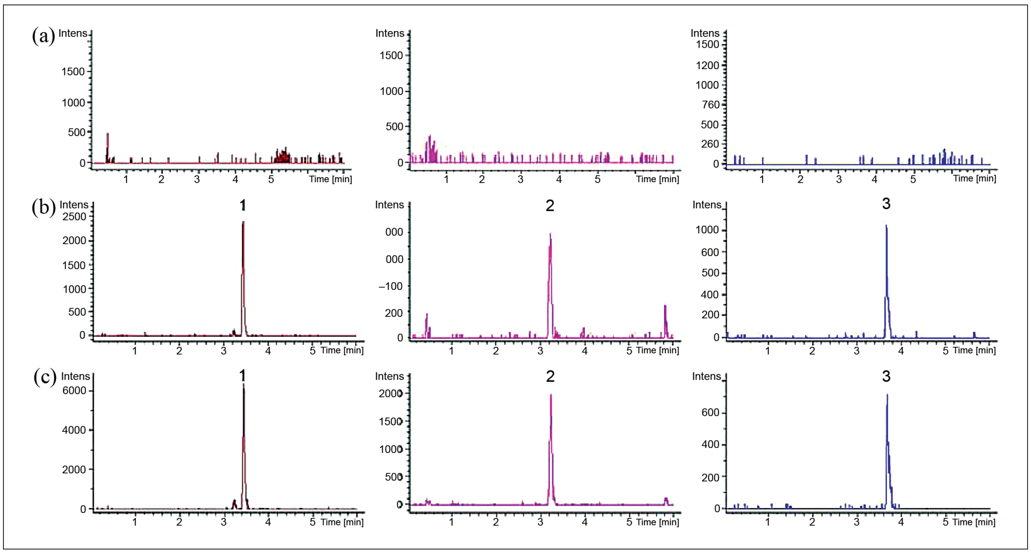

Fig. 2: Representative LC–MS chromatograms for BUP(1), HBUP(2) and carbamazepine (3;IS) in rat plasma samples: (a) blank plasma sample; (b) blank plasma sample spiked with BUP (500 ng mL^{-1}), HBUP (500 ng mL^{-1}) and IS (100 ng mL^{-1}); (c) rat plasma sample 0.25 h after oral administration of single dosage 15 mg kg^{-1} BUP

methanol were from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, USA). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

3.2. Sample preparation

The frozen plasma sample was thawed at room temperature. To 100 μL rat plasma sample, 10 μL of the internal standard working (1 μg mL^{-1}) and 200 μL of acetonitrile was added. After vortex mixing for 2 min and centrifugation at 13,000 rpm for 10 min, the supernatant (5 μL) was injected into LC-MS system for analysis.

3.3. Instrumentation and conditions

All analyses were performed with 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 ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 μm) column at 30 °C, with acetonitrile-0.1% formic acid as mobile phase. The flow rate was 0.4 mL min^{-1}. The HPLC gradient profile can be seen in Table 4.

Drying gas flow and nebuliser 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 239.9 for BUP, m/z 255.9 for HBUP and m/z 236.8 for carbamazepine (IS) in positive ion electrospray ionization interface.

3.4. Calibration standards and quality control samples

Primary stock solutions were prepared separately to give the concentration of 1.0 mg mL^{-1} for BUP and 0.34 mg mL^{-1} for HBUP in methanol and then stored at 4 °C. Working solutions for calibration (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20 μg mL^{-1} for BUP, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 μg mL^{-1} for HBUP) were prepared with methanol from the stock solution. The stock solution of IS at 1 mg mL^{-1} was prepared and diluted to obtain the IS working solution of 1 μg mL^{-1} with methanol. Controls (0.2, 1.6, 16 μg mL^{-1} for BUP and 0.1, 0.2, 8 μg mL^{-1} for HBUP) were prepared from the stock solutions by dilution in methanol.

Table 1: Precision and accuracy for BUP and HBUP of quality control sample in rat plasma (n = 6)

Drug	Concentration (ng mL^{-1})	RSD (%)		RE (%)		Recovery	
		Intra-day	Inter-day	Intra-day	Inter-day	Mean	RSD (%)
BUP	20	4.80	6.08	1.45	6.63	83.67	6.25
	160	5.96	4.82	3.99	−2.85	81.75	5.24
	1600	2.64	4.72	−1.62	−3.73	85.78	2.92
HBUP	10	6.83	5.97	9.65	5.46	52.67	2.19
	80	2.30	3.65	−3.55	−4.43	52.56	2.13
	800	2.85	8.71	−2.63	3.07	53.39	2.12

Table 2: Stability of BUP and HBUP in rat plasma under different conditions (n = 3)

Concentration (ng mL^{-1})		2h room temperature		24h room temperature		Freeze-thaw		−20 (30days)	
		Remained (%)	RSD (%)	Remained (%)	RSD (%)	Remained (%)	RSD (%)	Remained (%)	RSD (%)
BUP	20	100.55	3.20	98.60	4.06	100.94	6.57	99.41	7.22
	160	101.70	3.97	100.79	3.54	107.81	7.25	104.96	6.05
	1600	98.37	2.06	102.90	5.63	99.68	2.72	100.16	4.66
HBUP	10	101.18	4.33	94.60	7.44	96.44	5.33	97.41	8.79
	80	98.15	5.15	104.41	7.17	96.95	4.83	104.96	8.69
	800	100.98	4.43	105.40	5.00	101.99	5.56	98.91	9.77

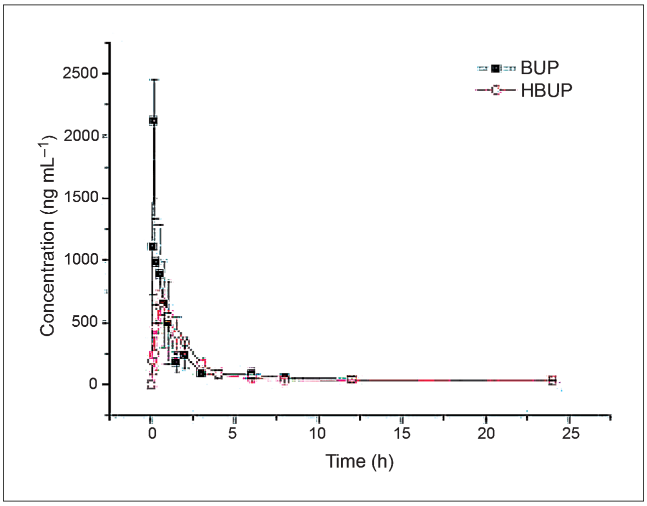

Fig. 3: The plasma concentration-time curves of BUP and HBUP after administration of a single 15 mg kg^{-1} oral dose of BUP

Calibration standards were prepared at the concentrations of 10, 25, 50, 100, 250, 500, 1000, 2000 ng mL^{-1} for BUP and 5, 10, 25, 50, 100, 250, 500, 1000 ng mL^{-1} for HBUP by spiking 90 μL blank rat plasma and 10 μL working solutions. The quality-control (QC) samples were prepared in the same way to give three different plasma concentrations of 20, 160, 1600 ng mL^{-1} for BUP and 10, 80, 800 ng mL^{-1} for HBUP, respectively.

3.5. Method validation

The selectivity of the method was evaluated by analyzing blank rat plasma and blank plasma spiked BUP, HBUP, IS, and a rat plasma sample to show that no interfering endogenous substances observed at the retention of BUP, HBUP and IS.

Calibration curves were obtained by analyzing spiked calibration samples for 3 days. Peak area ratios of BUP and HBUP to IS were plotted against analyte concentration, and standard curves were well fitted to the equations by weighted ($1/\chi 2$) least squares linear regression. The LLOQ, defined as the lowest concentration for which precision (RSD) was better than 20%, was estimated in the process of calibration curve construction.

Table 3: Main pharmacokinetic parameters of BUP and HBUP after oral administration of single dosage 15 mg kg^{-1} BUP to rats (n = 6)

Pharmacokinetic	BUP	HBUP
parameters	Mean (±SD)	Mean (±SD)
T1/2 (h)	11.33 ± 6.98	18.68 ± 8.82
MRT(0-t) (h)	5.53 ± 1.23	5.74 ± 0.08
MRT(0-∞) (h)	12.02 ± 6.88	17.85 ± 8.93
CL (L/h)	5.31 ± 0.06	5.44 ± 1.23
Cmax (μg/L)	2,121.84 ± 333.57	667.21 ± 85.59
AUC (0-t) (μg h/L)	2,362.86 ± 402.09	2,104.18 ± 71.95
AUC (0-∞) (μg h/L)	2,826.41 ± 32.45	2,847.27 ± 592.18

Table 4: HPLC gradient for detection of BUP and HBUP in rat plasma

Load time (min)	Pump flow (μL/min)	Formic acid%	Acetonitrile %
0.0	400	90	10
1.5	400	15	85
6.0	400	15	85
7.0	400	90	10
10.0	400	90	10

To study the matrix effect, three different blank rat plasma were investigated for BUP and HBUP at the concentration of 20, 160, 1600 ng mL^{-1} and 10, 80, 800 ng mL^{-1}, respectively. After the protein was precipitated then two analytes were spiked into the blank rat plasma at the concentration above. The corresponding peak areas of the two analytes in spiked plasma after-protein precipitation were compared with those of standard solutions at equivalent concentrations. The evaluation of IS at 1 μg mL^{-1} was performed in the same manner.

Accuracy and precision of the method were assessed by analyzing QC samples at three concentration levels (20, 160, 1600 ng mL^{-1} for BUP, 10, 80, 800 ng mL^{-1} for HBUP) over three validation days (n = 6). The accuracy was expressed by relative error (RE) and the precision was expressed by calculating the relative standard deviation (RSD). The accuracy must be within ± 15%, the intra-day and inter-day precisions should be below 15%.

The recoveries of BUP and HBUP at three QC levels (n = 6) were determined by comparing the peak area ratios of the analytes to IS in QC samples to which the analytes were added after protein precipitation at equivalent concentrations. The recovery of the IS was determined in the similar way using the QC samples at the concentration of 160 ng mL^{-1} for BUP and 80 ng mL^{-1} for HBUP.

The stabilities of BUP and HBUP in rat plasma were evaluated by analyzing replicates (n = 3) of plasma samples at the concentrations of 20, 160, 1600 ng mL^{-1} for BUP and 10, 80, 800 ng mL^{-1} for HBUP in different conditions. The short-term stability was determined after keeping the samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) at room temperature for 24 h in the HPLC autosampler. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 30 days. The results were compared with those obtained for freshly prepared plasma samples. The stability was evaluated for IS (50 ng mL^{-1}) in a similar way.

3.6. Pharmacokinetic study

Six Male Sprague-Dawley rats (200–250 g) from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China) were used to study the pharmacokinetics of BUP and HBUP. Before the study, diet was prohibited for 12 h but water was freely available. Blood samples (0.3 mL) from the tail vein were collected into heparinized ploythene tubes before and 0.083, 0.16, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after oral administration of BUP (15 mg kg^{-1}). After the samples were centrifuged at 13,000 rpm for 10 min, the plasma obtained (100 μL) was transfered into 1.5 mL heparinized ploythene tubes and stored at −20 °C until analysis. The pharmacokinetic parameters of BUP and HBUP were calculated by DAS software (Version 2.0, Medical College of Wenzhou, China).

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