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Identification and quantitative determination of benproperine metabolites in human plasma and urine by liquid chromatography–tandem mass spectrometry

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Abstract

Two novel metabolites of benproperine (BPP), 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3-OH-BPP) and 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (4-OH-BPP), were confirmed by comparison of retention times and mass spectra with those of synthetic standards using liquid chromatography–tandem mass spectrometry. Selective and sensitive procedures were developed for the simultaneous determination of BPP, 3-OH-BPP and 4-OH-BPP in human plasma and urine. The analytes were extracted from plasma sample and enzymatically hydrolyzed urine samples by liquid–liquid extraction, separated through a Diamonsil C_{18} column (150 mm × 4.6 mm i.d.) and determined by tandem mass spectrometry with an electrospray ionization interface in selected reaction monitoring mode. Dextromethorphan was used as internal standard. The mobile phase consisted of acetonitrile–water–formic acid (34:66:1, v/v/v), and flow-rate was 0.5 ml min^{-1}. This method has a lower limit of quantification (LLOQ) of 60, 4.0 and 4.0 nmol l^{-1} for BPP, 3-OH-BPP and 4-OH-BPP in plasma, 4.9, 4.7 and 2.4 nmol l^{-1} in urine, respectively. The intra- and inter-run precision were measured to be below 9.2%, and the accuracy was within ±4.3% for the analytes. The method was successfully used to determine BPP, 3-OH-BPP and 4-OH-BPP in plasma and urine for pharmacokinetic investigation. The results indicated residue of 3-OH-BPP in the body at least 192 h after an oral dose of BPP.

Keywords: Benproperine; 3-Hydroxybenproperine; 4-Hydroxybenproperine

1. Introduction

Benproperine (BPP, Fig. 1), 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidine, is widely used as a cough suppressant in non-productive cough. It is reported to have a peripheral and central action and has been given to humans via the mouth in usual doses of 25–50 mg two to four times daily as an embonate or phosphate [1]. Its antitussive activity is comparable to that of codeine while devoid of the undesirable side effect [2].

A few reports about urinary metabolites and the pharmacokinetics of BPP were published [3–5]. Five mono-hydroxylated metabolites were detected, which conjugated mostly with glucuronic acid in human urine. Two of them were identified as hydroxylated BPP in the phenyl ring. Mass spectra indicated that two of the others were probably hydroxylated in the piperidyl ring [3].

It is well known that there are some circumstances when the levels of metabolites would be helpful for further studies aimed at exploring the role of metabolites from a clinical point of view. It was indicated that drug accumulation perhaps occurred with the usual dosage of BPP in humans in a previous pharmacokinetic study within 24 h post-dose [4]. This result impelled us to investigate the pharmacokinetics and excretion of BPP and its metabolites and to further clarify it. The published methods were not sensitive enough for the determination of BPP in human urine [4,5]. An HPLC-UV method was used for quantification of BPP in human plasma with the lower limit of quantification (LLOQ) being 20 ng ml^{-1} (ca. 65 nmol l^{-1}). Two-step extraction had to be performed for sample preparation procedure since the selectivity of UV detector was limited [4]. Because of its high selectivity and sensitivity, liquid chromatography–tandem mass spectrometry (LC/MS/MS) has become the method of choice

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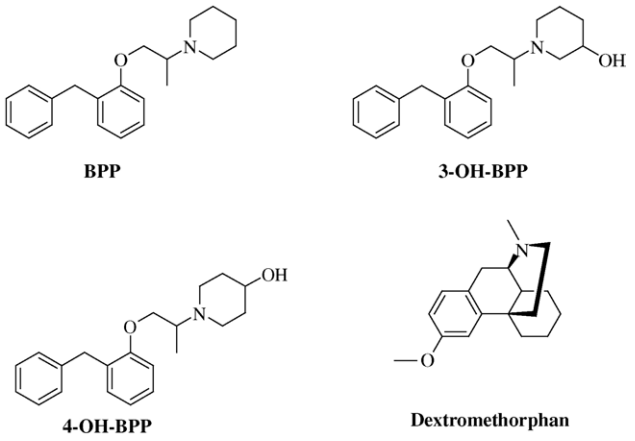

Fig. 1. Chemical structures of BPP, 3-OH-BPP, 4-OH-BPP and internal standard dextromethorphan.

for the quantitative determination of analytes in biological samples [6–8]. The unique selectivity of LC/MS/MS has allowed for simpler sample preparation procedures and shorter analysis time [9]. To our knowledge, the quantitative determination of BPP metabolites has not been reported up to now; and the LC/MS/MS method has not been used in the quantification of BPP and its metabolites.

The purpose of this study is to identify two novel metabolites of BPP in human plasma and urine and to develop a selective and sensitive LC/MS/MS method for the simultaneous determination of BPP and its two metabolites in human plasma and urine for pharmacokinetic investigation.

2. Experimental

2.1. Chemicals and reagents

Benproperine phosphate capsule was purchased from Shenyang Pharmaceutical Co. Ltd. (Liaoning, China). Benproperine phosphate was kindly supplied by Aosen Pharmaceutical Co. Ltd. (Liaoning, China) and was recrystallized by us; the purity was 98.5% by HPLC. Dextromethorphan hydrobromide (internal standard) was a generous gift of Tongyong Sanyang Pharmaceutical Co. Ltd. (Hainan, China). 1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (4-OH-BPP, Fig. 1) and 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3-OH-BPP, Fig. 1) were synthesized by us. Their phosphates were used in this study. β-Glucuronidase, GG-4259, was purchased from Sigma Chemical Co., St Louis, USA. Acetonitrile and methanol was of HPLC grade, and the other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study. Heparinized blank human plasma was purchased from Shenyang Blood Center (Liaoning, China).

2.2. Synthesis of 3-OH-BPP, 4-OH-BPP and their phosphates

4-OH-BPP was prepared according to the followed procedure: 3.64 g of 4-piperidinol (36 mmol) and 3.6 g of 1-methyl-2[2-(phenylmethyl)phenoxy]ethyl *p*-toluenesulfonate [10] (10 mmol) were fused in an oil bath at 100 °C for 3 h. After cooling to ambient temperature, 15 ml of water was introduced. The resultant was extracted with dichloromethane (10 ml × 3). The combined dichloromethane extracts were washed with water and brine, and dried over anhydrous Na_2CO_3. The solvent was removed under reduced pressure leaving a brown oil. The residue was purified on silica gel column chromatography eluted with acetone to give 2.49 g (77%) of 4-OH-BPP as pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.11(d, 3H), 1.53 (m, 2H), 1.87 (m, 2H), 2.41(m, 2H), 2.81 (m, 2H), 3.05 (m, 1H), 3.62 (m, 1H), 3.88 (m, 1H), 3.98 (s, 2H), 4.02 (m, 1H), 6.88–7.27 (m, 9H). ^{13}C NMR ($CDCl_3$) δ 13.2, 34.7, 36.1, 46.8, 58.4, 67.9, 69.7, 111.1, 120.5, 125.7, 127.4, 128.2, 128.7, 129.4, 130.7, 141.0, 156.5. MS (*m/z*), MS^1 326, MS^2 142, 308. The phosphate was of 98.0% purity by HPLC, mp 167–170 °C. UV–vis (λ_{max}, H_2O), 269 nm, ε 1.9×10^3.

3-OH-BPP was prepared from 3-piperidinol, which was treated as described above for 4-piperidinol. 3-OH-BPP was obtained as pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.11(d, 3H), 1.50–1.75 (m, 4H), 2.40–2.64 (m, 4H), 3.01 (m, 1H), 3.78–3.98 (m, 5H), 6.88–7.27 (m, 9H). ^{13}C NMR ($CDCl_3$) δ 13.1, 21.9, 31.8, 36.1, 50.0, 56.4, 58.4, 65.9, 69.6, 111.1, 120.5, 125.7, 127.4, 128.2, 128.7, 129.4, 130.6, 141.0, 156.6. MS (*m/z*), MS^1 326, MS^2 308, 142. The phosphate was of 97.5% purity by HPLC, mp 146–149 °C. UV–vis (λ_{max}, H_2O), 269 nm, ε 1.9×10^3.

The phosphates of 3-OH-BPP and 4-OH-BPP were used in this study.

2.3. LC/MS/MS instrumentation and analytical conditions

A Shimadzu LC-10AD pump (Kyoto, Japan) was used in the LC/MS/MS system. Chromatography was performed on a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Dikma, Beijing, China), which was coupled to a Security Guard C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex, Torrance, CA, USA). The components were eluted with an isocratic mobile phase of acetonitrile–water–formic acid (34:66:1, v/v/v), and the flow-rate was 0.5 ml min^{-1}. The column temperature was maintained at 25 °C.

A Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) interfaced with liquid chromatography via an electrospray ionization (ESI) source was used for mass analysis and detection. The mass spectrometer was operated in positive ion detection mode with the spray voltage set at 4.5 kV. Nitrogen was used as the sheath gas (0.75 l min^{-1}) and auxiliary gas (0.15 l min^{-1}) for nebulization. The heated capillary temperature was set at 180 °C to assist in desolvation. A divert valve directed the HPLC-flow in the first 3.0 min of the chromatographic run to waste container and afterwards to the ion source. Helium was used as the collision gas at the flow rate of 0.2 ml min^{-1}.

Selected reaction monitoring (SRM) mode was chosen for quantitative analysis. The precursor ions were isolated and activated to produce fragment ions with the optimum relative collision energy of 36% for each analyte and the internal standard. The transitions were monitored at *m/z* 310 → 126 for BPP, *m/z*

326 → 308 + 142 for both 3-OH-BPP and 4-OH-BPP, and *m/z* 272 → 215 for the internal standard.

Data acquisition was performed with Xcalibur 1.1 software (Finnigan). Peak integration and calibration were performed using Finnigan LCquan software.

2.4. Preparation of standard solutions and quality control samples

Stock solutions of BPP·H_3PO_4, 3-OH-BPP·H_3PO_4 and 4-OH-BPP·H_3PO_4 were prepared in water to give final concentration of 1600 μmol l^{-1} for plasma study and 982.8, 945.6 and 472.8 μmol l^{-1} for urine study. The solutions were then serially diluted with water to obtain the desired concentrations. Internal standard working solution (60 nmol l^{-1}) was prepared by diluting the 3.0 mmol l^{-1} stock solution of dextromethorphan hydrobromide with methanol. The resulting working solutions were kept in the refrigerator (4 °C). The standard solutions (50 μl) were spiked in blank urine samples (500 μl) with 0.05 mol l^{-1} $NH_4H_2PO_4$ (pH 5.0, 500 μl) or blank plasma (500 μl), either for calibration curves of all analytes or for QC samples in the pre-study validation and during the pharmacokinetic investigation.

2.5. Sample preparation

2.5.1. Plasma sample

To 500 μl of the plasma sample, 50 μl of water, 50 μl of the internal standard solution and 100 μl of Na_2CO_3 saturated aqueous solution were added. The mixture (ca. pH 11) was extracted with 3 ml of diethyl ether by vortex for 1 min and shaken for 15 min. After centrifugation at 2900 × *g* for 10 min, the upper organic layer was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of air. The residue was dissolved in 100 μl of the mobile phase. A 20 μl aliquot of the resulting solution was injected onto the LC/MS/MS system for analysis.

2.5.2. Urine sample

To 500 μl of the urine sample at each time interval from each subject, β-glucuronidase (1000 units) in 0.05 mol l^{-1} $NH_4H_2PO_4$ (500 μl, pH 5.0) was added. The samples were incubated for 14 h at 37 °C in a water bath. Hydrolyzed urine sample was treated according to the procedure described in Section 2.5.1 for the plasma sample.

2.6. Method validation

Calibration curves were established by spiking blank samples with appropriate amounts of BPP, 3-OH-BPP and 4-OH-BPP using six different concentrations in the range of 60–2.400 × 10^4, 4.0–1600 and 4.0–1600 nmol l^{-1} for plasma samples, and the range of 4.9–2457, 4.7–2364 and 2.4–1182 nmol l^{-1} for urine samples. The much lower concentration of BPP in urine resulted in different calibration ranges in urine and plasma. The peak area ratios of BPP, 3-OH-BPP and 4-OH-BPP over internal standard were plotted against the nominal concentrations, using least-square regression analysis with $1/C^2$ weighting. The concentrations of quality control (QC) samples and unknown samples were calculated from the linear regression equation of the peak area ratio against concentrations.

Spiked samples of analytes in plasma/urine were prepared at three different concentrations as QC samples (Table 1). Six

Table 1
Summary of precision, accuracy and extraction recovery from QCs in human plasma and urine extracts (n = 3 days, six replicates per day for precision and accuracy)

Analytes	Nominal concentration (nmol l^{-1})	Found concentration (nmol l^{-1})	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)	Extraction recovery (%)
In plasma						
BPP	120.0	121.1	4.6	5.4	0.9	67.8
	1200	1210	5.6	8.6	0.9	61.3
	1.920 × 10^4	1.934 × 10^4	6.1	2.4	0.8	62.4
3-OH-BPP	8.0	7.7	8.4	6.4	−3.3	75.2
	80.0	79.1	6.7	5.1	−0.1	72.2
	1280	1259	5.4	6.7	−1.7	72.2
4-OH-BPP	8.0	8.1	7.4	5.9	1.2	75.5
	80.0	80.8	5.6	9.0	0.9	76.1
	1280	1285	5.5	4.1	0.4	73.4
In urine						
BPP	9.8	9.5	7.8	7.2	−3.1	82.0
	393.1	408.7	5.9	5.7	4.0	78.6
	1965	1900	5.2	6.3	−3.3	79.6
3-OH-BPP	9.5	9.7	7.8	6.3	2.1	87.9
	378.2	382.5	5.6	3.4	1.1	85.3
	1891	1899	7.5	7.0	0.4	86.6
4-OH-BPP	4.7	4.9	9.2	7.4	4.3	86.8
	189.1	196.5	3.0	3.8	3.9	87.2
	945.6	961.8	7.3	3.9	1.7	84.5

replicates of QC samples were included in each run to determine the intra- and inter-day precisions. The relative standard deviation (R.S.D.) was used to report the precision. Accuracy was calculated from (measured − nominal)/nominal × 100%.

The matrix effect of analytical methods in plasma/urine was examined by comparing the MS/MS response (peak areas, B) of each analyte (at three concentration levels of QCs) or IS spiked into extracts originating from six different sources of plasma/urine, to the MS/MS response (A) of the same analyte or IS present in the neat mobile phase. The value ($B/A \times 100\%$) was considered as an absolute matrix effect. The assessment of the presence of a relative matrix effect was made based on the direct comparison of B values. The variability in these responses, expressed as CV%, was considered as a measure of the relative matrix effect for each analyte or IS.

The extraction recoveries of BPP, 3-OH-BPP, and 4-OH-BPP were determined in triplicate at three different concentrations by comparing peak areas obtained from samples spiked before extraction which were pretreated according to the described extraction procedure (Section 2.4), with that obtained from blank plasma/urine samples that were extracted and spiked post-extraction with standards.

The "cross talk" between MS/MS channels was assessed as follows: (a) injecting each analyte separately at the highest concentration on calibration curves (without IS added) and monitoring the response in the IS and all other channels, and (b) by injecting plasma/urine extract containing only the IS and monitoring the response at the MS/MS channels used for monitoring BPP and its metabolites.

The freeze–thaw stability of the analytes was determined by measuring the assay precision and accuracy for the samples that underwent three freeze–thaw cycles. In each freeze–thaw cycle, the frozen samples were thawed at ambient temperature for 3–4 h, and refrozen for about 20 h. After completion of each cycle the samples were analyzed and results were compared with that from the zero cycle.

The short-term stability at ambient temperature and the long-term stability at −20 °C were evaluated by measuring the assay precision and accuracy for the samples that were kept in ambient temperature for 24 h and in −20 °C for 30 days.

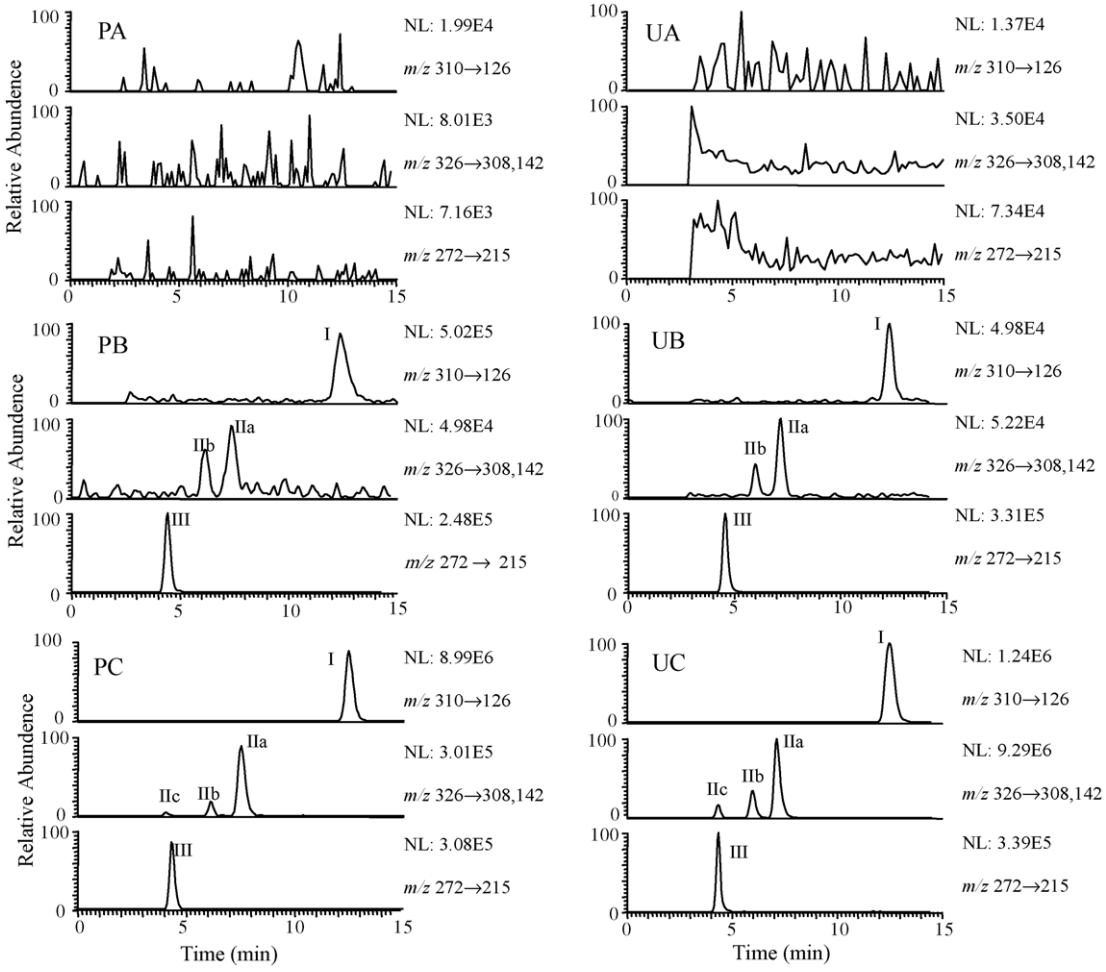

Fig. 2. Representative SRM chromatograms (PA) a blank plasma sample; (PB) a plasma sample spiked with BPP (I), 3-OH-BPP (IIa) and 4-OH-BPP (IIb) at the lower limit of quantification (60, 4.0 and 4.0 nmol l^{-1}) and the internal standard dextromethorphan (III, IS); (PC) a plasma sample from a volunteer 1 h after administration of 60 mg benproperine. (UA) a blank urine sample; (UB) a urine sample spiked with BPP (I), 3-OH-BPP (IIa) and 4-OH-BPP (IIb) at the lower limit of quantification (4.9, 4.7 and 2.4 nmol l^{-1}) and the internal standard dextromethorphan (III, IS); (UC) a urine sample from a volunteer 8–12 h after administration of 60 mg benproperine.

2.7. Collection of samples

Seven healthy male volunteers, whose mean (S.D.) age and weight were 22.7 (0.6) years and 62.2 (5.3) kg, gave written informed consent to take part in the study and a permission of the local ethics review board was obtained.

Each of them swallowed three benproperine phosphate capsules (ca. 60 mg BPP, 194 μmol) with 200 ml of water. Blood samples were collected in heparinized 10 ml tube at pre-dose and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0 and 96.0 h post-dose and centrifuged to separate plasma fraction. Urine samples were collected at time intervals of 0–4, 4–8, 8–12 h, and every 12 h intervals from 12 to 192 h post-dose. Blank urine was collected from the same volunteer directly before administration. The volume of urine was measured directly after collection. The plasma samples and urine samples were stored at −20 °C till analysis.

3. Results and discussion

3.1. Identification of metabolites

To identify the chemical structures of metabolites, a comparison of the HPLC retention time, as well as MS and MS/MS spectra of putative metabolite and authentic standard, may be sufficient to make the assignment more definitive [11]. Compared with corresponding blank samples, the plasma or enzyme hydrolyzed urine after administration of BPP showed two peaks in LC/MS/MS analysis corresponding to hydroxylated metabolites. The retention times and mass spectra of two metabolites were identical to that of synthetic compounds 3-OH-BPP and 4-OH-BPP (Fig. 2IIa and IIb), respectively. It confirmed that these compounds were metabolites of BPP. 3-OH-BPP and 4-OH-BPP were recovered from urine mainly as glucuronides, and in very low concentrations as free forms. In plasma samples, they were present mainly as free forms; their glucuronides were found only in traces.

3.2. Mass spectrometry

BPP, 3-OH-BPP, 4-OH-BPP and the internal standard dextromethorphan were at first characterized by MS and MS/MS by direct injection from syringe pump to ascertain their precursor ions and to select product ions for use in SRM, respectively.

In positive-ion mode, BPP, 3-OH-BPP and 4-OH-BPP formed protonated molecules $[M+H]^+$ at *m/z* 310, *m/z* 326 and *m/z* 326 as major ion peaks, which were chosen as the precursor ions for the analytes.

The product ion spectra of the analytes and internal standard were presented in Fig. 3. The base peak was observed at *m/z* 126 in the product spectrum of BPP. Structurally BPP consists of an *o*-benzylphenyl group linked a 2-piperidylpropyl group by an ether bond (see Fig. 1). The fragment ion at *m/z* 126 formed by cleavage of the ether bond was chosen in the SRM acquisition for BPP. 3-OH-BPP and 4-OH-BPP are two isomers; each of them

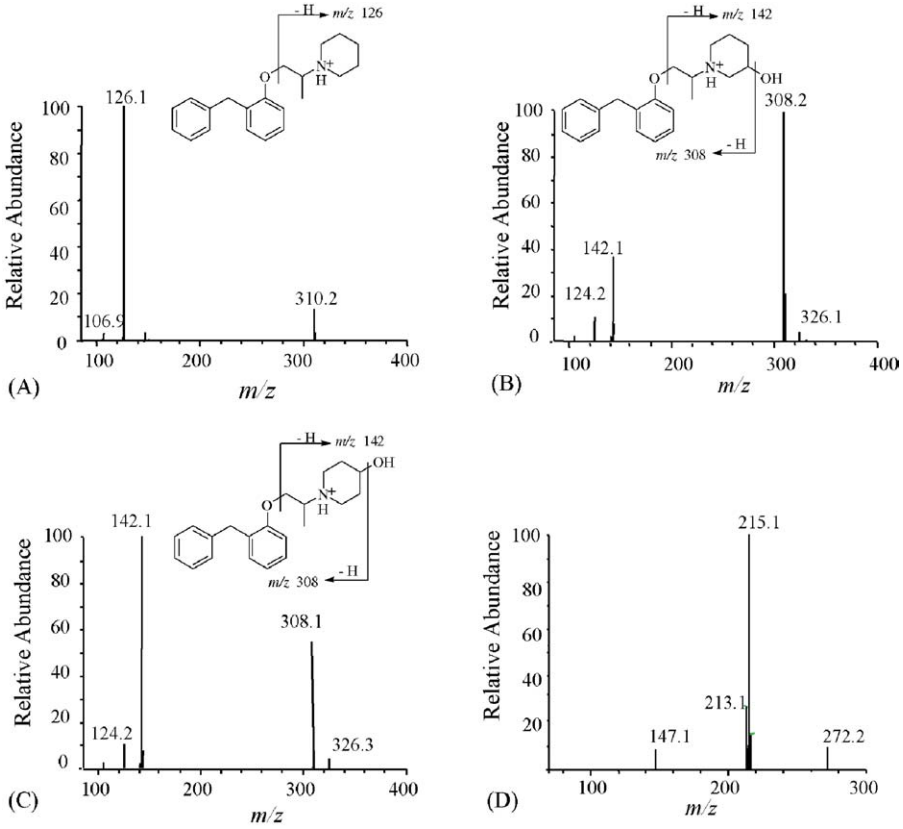

Fig. 3. Full-scan product ion spectra of $[M+H]^+$ of BPP (A), 3-OH-BPP (B), 4-OH-BPP (C) and dextromethorphan (D).

containing a hydroxyl group in piperidyl ring and an ether bond as BPP. The same product ions were observed in the full scan of MS/MS spectra of both 3-OH-BPP and 4-OH-BPP, *m/z* 308 and 142, which was formed by the dehydration and cleavage of the ether bond, respectively. Dehydration was easier in 3-OH-BPP than in 4-OH-BPP because p–π conjugated system was formed in the former compound (see Fig. 3). Therefore, *m/z* 308 was the base peak for 3-OH-BPP and *m/z* 142 was the base peak for 4-OH-BPP. The two product ions at *m/z* 308 and 142 were chosen as a sum in the SRM acquisition for 3-OH-BPP and 4-OH-BPP.

Using similar procedures the precursor ion of the internal standard was determined at *m/z* 272 as the base peak. The SRM transition of *m/z* 272 → 215 was selected.

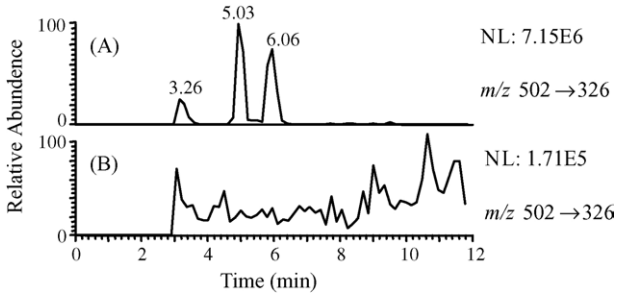

Fig. 4. SRM chromatograms of glucuronic acid conjugated compounds from the volunteer urine samples without (A) and with (B) enzymatic hydrolysis.

3.3. Chromatography

The contents of mobile phase were adjusted by flow injection analysis with the mobile phase containing varying percentages of organic solvents. It was found that the low organic solvent content in HPLC system decreased the background noise, and provided stable MS signal throughout an analytical run, allowing enhancement of sensitivity.

Though it was not necessary for mass spectrometry to separate all the analytes, 3-OH-BPP and 4-OH-BPP are isomers with the same precursor ions and product ions. The chromatographic separation of the two isomers has to be implemented. Therefore, we investigated different organic modifiers and their percentages to improve the separation of the two isomers. Acetonitrile was found to be more favorable for the chromatographic separation of these two isomers than methanol, while the analysis speed was the same. Generally, the addition of acidic modifiers can improve peak shape by promoting the ionization of the basic analytes in reversed-phase HPLC system. It was also demonstrated that good peak symmetry and high sensitivity of these two analytes were obtained after the addition of formic acid in the mobile phase. As shown in Fig. 2, the two isomers were fully separated at retention times of 7.5 min (IIa) and 6.3 min (IIb) corresponding to 3-OH-BPP and 4-OH-BPP, respectively.

There are two couples of diastereomers of 3-OH-BPP because of two chiral centers in the molecule. These diastereomers can be separated partly using the above-described column but with a mobile phase of methanol–water–formic acid (50:50:1, v/v/v) at 14.6 and 15.3 min, respectively. In that chromatographic condition, the retention time of BPP is 22.1 min. To simplify the experiment and shorten the analysis time, the mobile phase of acetonitrile–water–formic acid (34:66:1, v/v/v) was chosen. In this case, the diastereomers of 3-OH-BPP were detected as the overall amount without separation.

3.4. Sample preparation

Because 3-OH-BPP and 4-OH-BPP are mainly excreted as glucuronides, each urine sample had to be hydrolyzed by β-glucuronidase in order to determine the sum of free and conjugated analytes. The results from the urine samples, with and without enzymatic hydrolysis are shown in Fig. 4. The peaks at 5.03 and 6.06 min are of the glucuronides of 4-OH-BPP and 3-OH-BPP, respectively. It was indicated that deconjugation proceeded almost completely under the experimental condition.

The influence of the enzymatic hydrolysis on free BPP, 3-OH-BPP and 4-OH-BPP in the urine sample were investigated as follows. The standard solution (50 μl) at the medium level (see Table 1) was spiked in blank urine sample (500 μl), treated according to the procedure mentioned in "Section 2.5.2", and the spiked sample with enzymatic hydrolysis was obtained. The same standard solution (50 μl) was spiked in blank urine sample (500 μl) with 0.05 mol l^{-1} $NH_4H_2PO_4$ (pH 5.0, 500 μl), treated according to the procedure mentioned in "Section 2.5.1", and the spiked sample without enzymatic hydrolysis was obtained. On comparing the peak areas obtained from the spiked samples with and without enzymatic hydrolysis, no significant difference was found.

It was reported that two-step extraction was performed when the UV detector was used to determine BPP in plasma [4]. This operation is complicated but necessary since the detection wavelength was so short (220 nm) that many endogenous substances could interfere with the determination. However, simple extraction method could be used in LC/MS/MS analysis without interference from endogenous substances because of its high selectivity. Ethyl acetate, ethyl acetate–isopropanol, diethyl ether–hexane, diethyl ether, and so on, were tested to one-step extraction for the analytes in plasma and urine. The extraction recoveries of hexane–isopropanol and diethyl ether were both higher than the others. Diethyl ether did not produce emulsions and therefore was selected as the extraction solvent.

3.5. Method validation

3.5.1. Selectivity

The selectivity of the method was demonstrated by comparing the chromatograms of blank samples and spiked samples. Fig. 2 demonstrated that there was no interference from endogenous substances with the analytes and internal standard. The peak at 4.5 min of *m/z* 326 (Fig. 2, PC and UC) represented another possible hyhroxylated metabolite whose chemical structure has not been identified. The retention time of two known metabolites [3] were 4.8 and 5.3 min, whose product ions (*m/z* 126 and 163) were different from that of IIa and IIb, and they did not present in the detection channel (*m/z* 326 → 142 + 308)

Table 2
Matrix effect data for analytes and IS in six different lots of human plasma and urine

Analytes	Nominal concentration ($nmol\,l^{-1}$)	Mean peak area[a]		Absolute matrix effect ($B/A \times 100\%$)	Relative matrix effect (CV% of B)
		A[b]	B[c]		
In plasma					
BPP	120	90.1	87.1	97	8.4
	1200	673.0	638.0	95	6.9
	1.920×10^4	1.266×10^4	1.189×10^4	94	6.7
3-OH-BPP	8.0	14.1	14.2	101	9.8
	80.0	121.7	111.8	92	8.9
	1280	1820	1717.	94	5.4
4-OH-BPP	8.0	9.0	9.2	102	9.9
	80.0	61.4	64.7	105	8.3
	1280	1049	1060.	101	5.9
IS	60.0	160.8	151.2	94	7.6
In urine					
BPP	9.8	10.7	11.6	109	9.4
	393.1	372.5	410.6	110	9.3
	1965	1961	2002.	102	6.1
3-OH-BPP	9.5	16.1	17.7	109	8.1
	378.2	489.3	470.7	96	6.9
	1891	2558	2330.	91	4.8
4-OH-BPP	4.7	8.2	7.7	94	7.5
	189.1	293.7	283.3	96	6.4
	945.6	748.2	692.6	93	5.4
IS	60.0	145.5	138.4	95	5.2

[a] In arbitrary units, $\times 10^5$, $n = 6$.
[b] Peak areas of each analyte or IS spiked into extracts originating from five different lots (sources) of plasma/urine.
[c] Peak areas of the same analyte or IS present in the neat mobile phase.

for IIa and IIb. The glucuronide metabolites could not be extracted in this condition, so that they did not interfere with the analysis.

The absolute matrix effect for analytes and IS, calculated according to ($B/A \times 100\%$), was in the range of 91–110% (Table 2). No co-eluting endogenous substances interfered with the ionization of the analytes and internal standard. The absence of any significant relative matrix effect for each analyte or IS is also shown in Table 2, as the precision of the determination of B for every analytes and IS are all <9.8%. The major reason for the absence of the matrix effect should be that the analytes are relatively unpolar. At the described LC/MS/MS conditions, no "cross-talk" among the analytes was observed.

3.5.2. Linearity of calibration curves and lower limit of quantification

The typical equations of calibration curves are listed in Table 3. Linear calibration curves were obtained over the concentration range of 60–2.4×10^4, 4.0–1600 and 4.0–1600 $nmol\,l^{-1}$ for BPP, 3-OH-BPP, 4-OH-BPP, respectively in human plasma, and the range of 4.9–2457, 4.7–2364 and 2.4–1182 $nmol\,l^{-1}$, respectively in human urine. The lower limit of quantification was defined as the lowest concentration on the calibration curve for which an acceptable R.E. of 15% and R.S.D. below 15% were obtained.

The LLOQs were 60, 4.0 and 4.0 $nmol\,l^{-1}$ for BPP, 3-OH-BPP, 4-OH-BPP, respectively in plasma, and 4.9, 4.7 and 2.4 $nmol\,l^{-1}$, respectively in urine. Under present LLOQs, benproperine and its two metabolites can be determined till 96 h post-dose in plasma and till 192 h post-dose in urine, which is sensitive enough for investigating the pharmacokinetic behavior of benproperine in humans.

3.5.3. Precision and accuracy

Table 1 summarizes the intra- and inter-run precisions and accuracy for BPP, 3-OH-BPP and 4-OH-BPP from the QC samples. The intra-run precision was less than 8.4% for each QC level of all analytes in plasma; it was less than 9.2% in urine.

Table 3
Summary of calibration curves

Analytes	Intercept	Slope	r
In plasma			
BPP	-2.936×10^{-2}	2.710×10^{-2}	0.9974
3-OH-BPP	1.342×10^{-2}	6.224×10^{-2}	0.9968
4-OH-BPP	1.474×10^{-2}	3.856×10^{-2}	0.9968
In urine			
BPP	8.548×10^{-2}	8.221×10^{-2}	0.9990
3-OH-BPP	4.811×10^{-2}	9.875×10^{-2}	0.9988
4-OH-BPP	2.819×10^{-2}	5.516×10^{-2}	0.9986

Table 4
Stability of analytes in plasma samples ($n = 6$)

Analytes	Nominal concentration ($nmol\,l^{-1}$)	Found concentration ($nmol\,l^{-1}$)	Relative error (%)	Coefficient of variability (%)
Freeze and thaw stability				
BPP	120	122	1.7	4.6
	1.920×10^4	1.934×10^4	−3.2	2.3
3-OH-BPP	8.0	8.3	5.0	4.3
	1280	1311	2.4	2.1
4-OH-BPP	8.0	8.2	2.5	3.9
	1280	1276	−0.3	1.9
Short-term stability (at ambient temperature for 24 h)				
BPP	120	116	3.3	3.7
	1.920×10^4	1.900×10^4	−1.0	1.7
3-OH-BPP	8.0	7.8	−2.5	3.2
	1280	1341	4.8	1.4
4-OH-BPP	8.0	7.8	−2.5	4.3
	1280	1271	−0.7	2.5
Long-term stability (at −20 °C for 30 days)				
BPP	120	114	−5.0	3.1
	1.920×10^4	1.897×10^4	−1.2	1.3
3-OH-BPP	8.0	7.8	−2.5	4.1
	1280	1322	3.2	2.5
4-OH-BPP	8.0	8.3	3.8	3.6
	1280	1295	1.2	1.7

Table 5
Stability of analytes in urine samples ($n = 6$)

Analytes	Nominal concentration ($nmol\,l^{-1}$)	Found concentration ($nmol\,l^{-1}$)	Relative error (%)	Coefficient of variability (%)
Freeze and thaw stability				
BPP	9.8	9.6	−2.1	5.2
	1965	1893	−3.7	1.3
3-OH-BPP	9.5	9.8	3.2	3.1
	1891	1877	−0.7	1.6
4-OH-BPP	4.7	4.9	4.3	2.0
	945.6	901.1	−4.7	2.8
Short-term stability (at ambient temperature for 24 h)				
BPP	9.8	9.4	−4.1	2.1
	1965	1900	−3.3	6.9
3-OH-BPP	9.5	9.2	−3.2	5.4
	1891	1886	−4.8	4.2
4-OH-BPP	4.7	4.6	−2.1	3.8
	945.6	950.4	0.5	7.6
Long-term stability (at −20 °C for 30 days)				
BPP	9.8	9.9	1.0	4.0
	1965	1913	2.6	3.6
3-OH-BPP	9.5	9.8	3.2	5.9
	1891	1904	−3.9	6.2
4-OH-BPP	4.7	4.5	−4.2	2.9
	945.6	933.3	−1.3	4.0

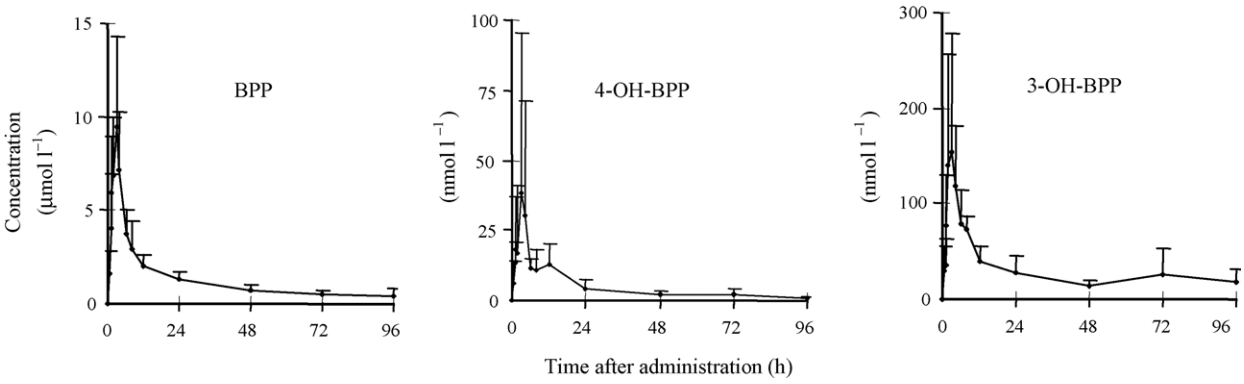

Fig. 5. Mean plasma concentration–time profiles of BPP, 3-OH-BPP and 4-OH-BPP after an oral dose of 60 mg (194 μmol) benproperine ($n = 7$).

The inter-run precision was less than 9.0% for each QC level of all analytes in plasma; it was less than 7.4% in urine. The accuracy derived from QC samples was within ±3.3% in plasma and within ±4.3% in urine for each QC level of BPP, 3-OH-BPP and 4-OH-BPP. The results were within the acceptable range for precision and accuracy.

3.5.4. Extraction recovery and stability

The extraction recoveries for BPP, 3-OH-BPP and 4-OH-BPP from the QC samples are summarized in Table 1. The spiked urine sample with the solution of β-glucuronidase in 0.05 mol l^{-1} $NH_4H_2PO_4$ (1000 units in 1 ml) was tested for extraction recovery. No significant differences were observed on comparison to using 0.05 mol l^{-1} $NH_4H_2PO_4$ alone. This indicated that the inclusion of gluronidase has no impact on extraction efficiencies. Therefore, the enzyme was economized in the samples for calibration curves and QC.

The stability of BPP, 3-OH-BPP and 4-OH-BPP in human plasma and urine was investigated under a variety of storage and process conditions. The analytes were found to be stable (>95%) in plasma and urine samples after three cycles of freeze (−20 °C)–thaw (room temperature) and for at least 24 h at room temperature. The analytes in the samples also showed to be stable for at least a month under −20 °C (Tables 4 and 5).

3.6. Application of the method to a pharmacokinetic investigation

After a single oral dosage of 60 mg (194 μmol) BPP to healthy volunteers, plasma and enzymatic hydrolyzed urine concentrations of BPP, 3-OH-BPP and 4-OH-BPP were determined simultaneously by the described LC/MS/MS method. Fig. 5 shows the mean plasma concentration–time profiles of BPP, 3-OH-BPP and 4-OH-BPP. This indicated that a significant individual variation in plasma concentration of metabolites. Fig. 6 shows mean urine cumulative excretion curves of BPP, 3-OH-BPP and 4-OH-BPP. The excreted amount of 3-OH-BPP was still increasing in the interval of 180–192 h post-dose, and the concentration in this interval was 34 nmol l^{-1} on average. This indicated residue of 3-OH-BPP in the body till at least 192 h post-dose. The overall excretion of these three compounds is up

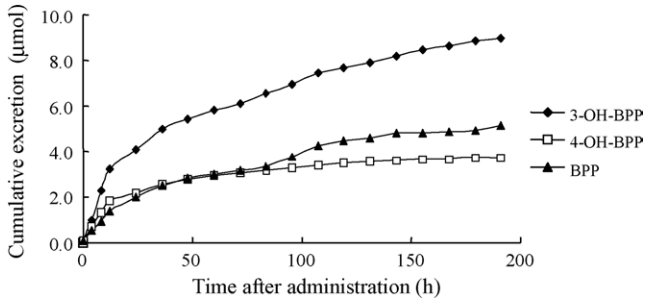

Fig. 6. Mean urine cumulative excretion of BPP, 3-OH-BPP and 4-OH-BPP after an oral dose of 60 mg (194 μmol) benproperine ($n = 3$).

to 10% dosage in human urine, which suggested other possible excretion routes. In general, compounds with molecular weights of less than 300 are almost exclusively excreted via kidney into urine. Drugs that are mainly excreted in bile have molecular weights in excess of 500. In addition to a high molecular weight, drugs excreted in bile usually required a strong polar group [12]. The formation of glucuronide increases the molecular weight of the compound by nearly 200, and increases the polarity as well. If most of 3-OH-BPP and 4-OH-BPP were conjugated with glucuronic acid in vivo, bile would be the major excretion route of these metabolites.

4. Conclusions

1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3-OH-BPP) and 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (4-OH-BPP) are identified as metabolites of benproperine in human plasma and urine. They present mainly as glucuronides in the urine. An LC/MS/MS method was developed and validated for the simultaneous determination of BPP, 3-OH-BPP and 4-OH-BPP in human plasma and urine. The method is selective, sensitive and reliable with the LLOQs of 60, 4.0, 4.0 nmol l^{-1} for BPP, 3-OH-BPP, 4-OH-BPP and 4.9, 4.7, 2.4 nmol l^{-1} in urine, respectively. The method was used successfully to evaluate the plasma pharmacokinetics and the urinary cumulative excretion of BPP, 3-OH-BPP and 4-OH-BPP in humans after an oral dose.

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