

Full-length article

Identification of some benproperine metabolites in humans and investigation of their antitussive effect¹

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Key words

benproperine; metabolism; antitussive agents; hydroxylation; glucuronide; high performance liquid chromatography; mass spectrometry

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Abstract

Aim: To identify 4 unknown metabolites of benproperine (BPP, 1) in human urine after a *po* dose, and to investigate the antitussive effect of monohydroxylate metabolites. **Methods:** The putative metabolite references were prepared using chemical synthesis. Their structures were identified using ¹H and ¹³C nuclear magnetic resonance, and mass spectrometry. The metabolites in human urine were separated and assayed using liquid chromatography-ion trap mass spectrometry (LC/MS/MS), and further confirmed by comparison of their mass spectra and chromatographic retention times with those of synthesized reference substances. The antitussive effects of metabolites were evaluated on coughs induced by 7.5% citric acid in conscious guinea pigs. **Results:** 1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]-ethyl]-4-piperidinol (2), 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3) and their glucuronides 4 and 5 were obtained from chemical synthesis. Four urinary metabolites in human urine showed peaks with the same chromatographic retention times and mass spectra in LC/MS/MS as synthetic substances 2, 3, 4 and 5. Phosphates of compounds 2 and 3 prolonged the latency of cough and reduced the number of coughs during the 3 min test using citric acid, but did not reduce the number of coughs during the 5 min immediately after the test in conscious guinea pigs. **Conclusion:** Compounds 2, 3, 4, and 5 were identified as the metabolites of BPP in human urine. Among them, compounds 2 and 3 are inactive in the antitussive effect.

Introduction

Benproperine, 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidine (BPP, 1, Figure 1) is widely used as a cough suppressant for non-productive coughs. It has a peripheral and central action and can be given to humans *po* in forms of embonate or phosphate^[1]. The antitussive activity is comparable to that of codeine, but is devoid of the undesirable codeine's side effects^[2].

In general, drugs are metabolized to more polar, hydrophilic entities, which can be excreted from the body more easily. At the same time, drugs can be inactivated, be activated or become a toxicant. There is a growing interest in identifying metabolites and in establishing their pharma-

cokinetic, pharmacological, and toxicological properties. Activated metabolites are sometimes drug candidates for the treatment of a variety of diseases.

In our previous research, 5 mono-hydroxylated metabolites of benproperine and their conjugates were detected in human urine and 2 of them that were hydroxylated in phenyl rings have been identified. Mass spectra indicated that the other 2 mono-hydroxylates were probably hydroxylated in the piperidyl ring^[3]. To identify metabolites, liquid chromatography-tandem mass spectrometry is an efficient approach, which is used widely^[4-6]. A comparison of the high performance liquid chromatography (HPLC) retention times, as well as MS/MS spectra of putative metabolite and authentic standard, might be sufficient to make a more definitive identification^[4].

The purposes of the present study was to synthesize 4 putative benproperine metabolites: 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (2), 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3), as well as their glucuronides 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-4-yl]- β -*D*-glucopyranosiduronic acid (4) and 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-3-yl]- β -*D*-glucopyranosiduronic acid (5) (Figure 1), to identify the chemical structures of the metabolites and to evaluate the antitussive effects of 2 and 3 phosphates.

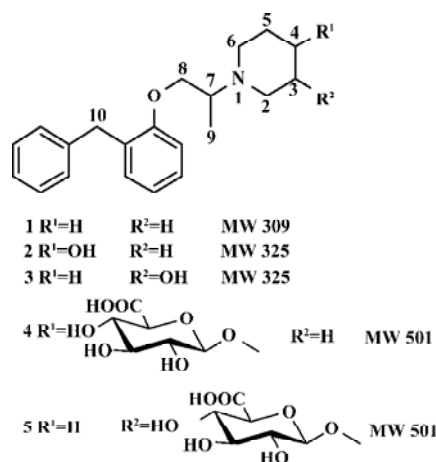


Figure 1. Benproperine and its possible metabolites

Materials and methods

Instruments and chemicals Melting points were determined in open capillary tubes and the thermometer was uncorrected. MS/MS spectra were recorded on a Finnigan LCQ ion trap mass spectrometer via an electrospray ionization (ESI) source in positive ion detection mode. ¹H and ¹³C

NMR spectra were recorded on a Bruker ARX-600 instrument with tetramethylsilane as an internal standard. Ultra-violet (UV) spectra were recorded on a Shimadzu UV-2201 instrument.

3-Piperidinol hydrochloride and 4-piperidinol were obtained from the Aldrich Chemical Co. We prepared 1-[2-(phenylmethyl)phenoxy]-2-propanol *p*-toluenesulfonate (6) ourselves, according to published procedures^[7,8], from *o*-benzylphenol, which was kindly supplied by Aosen Pharmaceutical Co. Methyl (2,3,4-tri-*O*-acetyl-1-*O*-trichloroacetimidoyl)- α -*D*-glucopyranuronate (7) was prepared by us, according to Soliman *et al*^[9] from *D*(+)-glucuronolactone, which was obtained from Wako Chemical Co. The structures of 6 and 7 were identified using ¹H and ¹³C NMR. Column chromatography was carried out on silica gel BW-820MH, which was obtained from Fuji Silysia Chemical Co. Benproperine phosphate capsule was obtained from Shenyang Pharmaceutical Co. Benproperine phosphate was kindly supplied by Aosen Pharmaceutical Co and was recrystallized by us; the purity was 98.5% by HPLC. Citric acid was obtained from Shenyang Dongxing Reagent Factory. Test compounds and citric acid used to investigate antitussive were all dissolved in physiological saline. Methanol was of HPLC grade, and the other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used in LC/MS/MS.

The putative metabolites were synthesized with the procedure shown in Figure 2.

1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (2) A total of 3.64 g (36.0 mmol) of 4-piperidinol and 3.6 g (9.0 mmol) of 1-[2-(phenylmethyl)phenoxy]-2-propanol *p*-toluenesulfonate (6)^[7,8] were fused on an oil bath at 100°C for 3 h, and stirred. After cooling to an ambient temperature, 15 mL of water was introduced. The resultant was extracted with dichloromethane (20 mL×3). The combined dichloromethane extracts were washed with water and

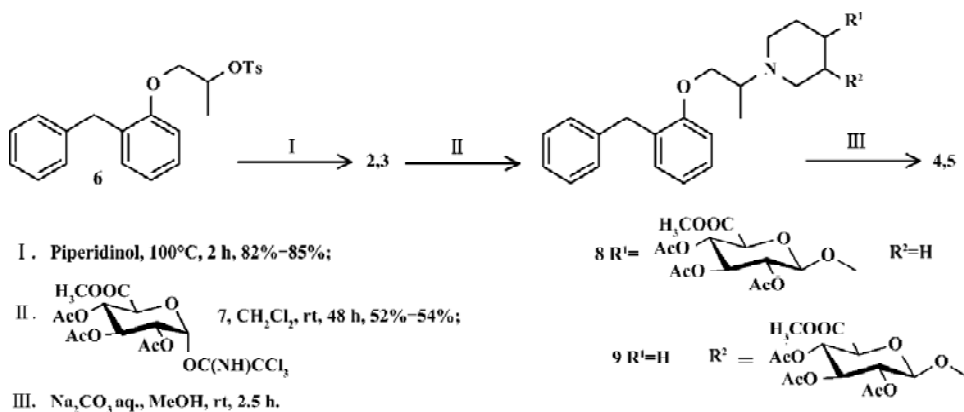


Figure 2. Synthesis of benproperine metabolites 2, 3, 4, and 5.

brine, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, leaving a brown oil. The residue was purified using silica gel column chromatography, eluting with $\text{CHCl}_3/\text{MeOH}$ to leave the titled compound 2 (2.49 g, 85.3%) as a pale-yellow oil. The oil 2 (2.49 g, 0.77 mmol) was dissolved in an aqueous solution of 0.3 mol/L H_3PO_4 (25 mL) and the water was removed under reduced pressure. The residue was recrystallized from ethanol, leaving phosphate of 2 (2.60 g, 80.2%) as a colorless needle.

1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3) 3-Piperidinol was treated as in the procedure described for 4-piperidinol, leaving 3 as a pale yellow oil (82.1%) and its phosphate of 3 as a white powder.

1-O-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-4-yl]- β -D-glucopyrano-siduronic acid (4) Phosphate of 2 (85 mg, 0.20 mmol) was suspended and stirred in CH_2Cl_2 (4.0 mL) at -15°C under N_2 atmosphere. $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (10 μL) was added to in one portion. After 10 min of stirring, methyl (2,3,4-tri-*O*-acetyl-1-*O*-trichloroacetimidoyl)- α -D-glucopyranuronate(7)^[9] (95 mg, 0.20 mmol) was added. Stirring was continued for 48 h at an ambient temperature. EtOAc (50 mL) was added, and the mixture was washed with saturated Na_2CO_3 aqueous solution (10 mL \times 2) and brine (10 mL), and dried over Na_2SO_4 . The solution was evaporated and purified on silica gel column chromatography, eluted with *n*- C_6H_{14} - EtOAc - MeOH in gradient. 1-*O*-[1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-4-yl]-2,3,4-tri-*O*-acetyl- β -D-glu-copyranosiduronic acid methyl ester (8, 65 mg, 50.4%) was obtained as a colorless syrup. Compound 8 was dissolved in MeOH (2 mL), and Na_2CO_3 aqueous solution (0.1 mol/L, 1 mL) was added at an ambient temperature and stirred. After 2.5 h of stirring, the mixture was desalted and concentrated. The titled compound 4 was obtained as a colorless syrup.

1-O-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-3-yl]- β -D-glucopyranosiduronic acid(3-OH-BPP glucuronide, 5) Phosphate of 3 was treated as in the procedure described above for phosphate of 2 to give the intermediate 9. Then, the titled compound 5 was obtained as a pale-yellow syrup after hydrolysis.

Urine sample Seven healthy male volunteers, whose mean (SD) age and weight were 22.7 (0.6) years and 62.2 (5.6) kg, gave written informed consent to take part in the study and local ethics approval was obtained. Each of the volunteers swallowed 3 benproperine phosphate capsules (60 mg BPP) with 200 mL of water. Urine samples were collected at 0 h–24 h. Blank urine was collected from the same volunteer directly before being given the capsules. The urine samples were stored at -20°C until analysis.

A 1.0 mL urine sample was filtered through 0.45 μm of precut membrane. The filter was applied to a 3.0-mL Bond-Elute C_{18} cartridge (0.5 g silica gel) preconditioned with 2 mL aliquots of methanol and water. After loading the sample, the column was washed with 1 mL of water. Metabolites were eluted with 1 mL of methanol. The eluate was evaporated to dryness at 40°C under a gentle stream of N_2 . 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.

A 1.0 mL portion of blank urine sample was treated as in the above procedure, in which the solutions of putative metabolites (2, 3, 4, or 5) in mobile phase were used instead of mobile phase to obtain the spiked urine sample for LC/MS/MS analysis.

LC/MS/MS analysis and identification of metabolites A Shimadzu LC-10AD pump was used in the LC/MS/MS system. Chromatography was performed on a Diamonsil C_{18} column (150 \times 4.6 mm inner diameter, 5 μm , Dikma), which was coupled with a Security Guard C_{18} guard column (4 \times 3.0 mm inner diameter, Phenomenex). The components were eluted with an isocratic mobile phase of methanol-water-formic acid (50:50:1, *v:v:v*), and the flow rate was 0.5 mL/min. The column temperature was maintained at 25°C . A Finnigan LCQ ion trap mass spectrometer interfaced with liquid chromatography via an electrospray ionization (ESI) source was used for mass analysis in positive ion detection mode. The capillary voltage was fixed at 16 V, and its temperature was maintained at 200°C . The spray voltage was set at 4.25 kV. The HPLC fluid was nebulized using N_2 as both the sheath gas at a flow rate of 0.75 L/min, and the auxiliary gas at a flow rate of 0.15 L/min. The MS/MS spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with He present in the mass analyzer, and the relative collision energy was set at 30%–40%. Data were collected and analyzed using the Navigator software (version 1.2).

Animals Dunkin Hartley guinea pigs of both sexes, weighing 200–300 g (Experimental Animal Center of Shenyang Pharmaceutical University, Shenyang, China) were used. All animal studies were in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988) and Implementing Regulations of the Administration on Medical Experiments on Animals (China, 1989). The guinea pigs were maintained in standard animal rooms, with food and water freely available, and on a natural light-dark cycle. They were allowed to adapt to the conditions for at least 1 week before being used in experiments.

Investigation of antitussive effect The antitussive effect of BPP, 2 and 3 phosphates was evaluated in conscious guinea-pigs against citric acid-induced coughs^[10,11]. Guinea pigs were placed individually in a transparent plexiglass cylinder chamber (10 cm×10 cm×21 cm) and exposed to a nebulized solution of 7.5% citric acid for 3 min. An ultrasonic nebulizer (402 AI, Shanghai Yuyue Medical Facilities) was used to produce an aerosol with particles with an aerodynamic mass median diameter of 1 mm; the volume of solution aerosolized was approximately 0.6 mL/min. Animals were selected for the study according to the number of coughs observed 24 h before the test, and animals with more than 20 or fewer than 6 coughs during the 3 min test were not used. The compounds and the vehicle (physiological) saline were given ip (2 mL/kg) for 1.5 h (for BPP×H₃PO₄) or for 40 min (for 2×H₃PO₄ and 3×H₃PO₄) before the test. The number of coughs during the 3 min test and during the 5 min immediately after the test was determined. The animals with different doses (3 mg/kg, 9 mg/kg, 27 mg/kg for 2×H₃PO₄ and 3×H₃PO₄, 27 mg/kg for BPP×H₃PO₄) of drugs and the vehicle were grouped according to a random table. Animals were used only once because of tachyphylaxis of the cough response. Coughing sounds were recorded and amplified using a microphone and loudspeaker. During the experiment, the animals were continuously watched by a trained observer who was unaware of the treatment. Sneezes and coughs were differentiated by visual observation of the animals.

Statistical analysis Pharmacological results are represented as mean±SEM. Data were analyzed using one-way analysis of variance (ANOVA). Whenever ANOVA was significant, further comparisons between vehicle- and drug-treatment groups were performed using Dunnett's *t*-test. The above analysis was performed using the software SPSS V11.0 for Windows.

Results

Chemical synthesis Compound 6, which was synthesized from 2-benzylphenol^[8], was fused with 4-piperidinol to give 2 in 85% of yield. 3-Piperidinol was treated according to the procedure for 4-piperidinol to give 3 in 82% of yield. Glucuronides were synthesized from mono-hydroxylates (2 and 3) treated with trichloroacetimidate donor (7) followed by basic hydrolysis (Figure 2). The structures of products were identified using ESI-MS, ¹H NMR, and ¹³C NMR (Table 1).

Metabolites of benproperine Compared with corresponding blank samples, the urine after being given of BPP showed 5 peaks corresponding to hydroxylated metabolites and 5 peaks corresponding to their glucuronides in LC/MS/MS analysis (Figure 3).

Metabolites M1 and M2 M1 and M2 gave the same pseudo-molecular ions [M+H]⁺ at *m/z* 326, similar MS/MS spectra (Figure 4), but different retention times (Figure 3),

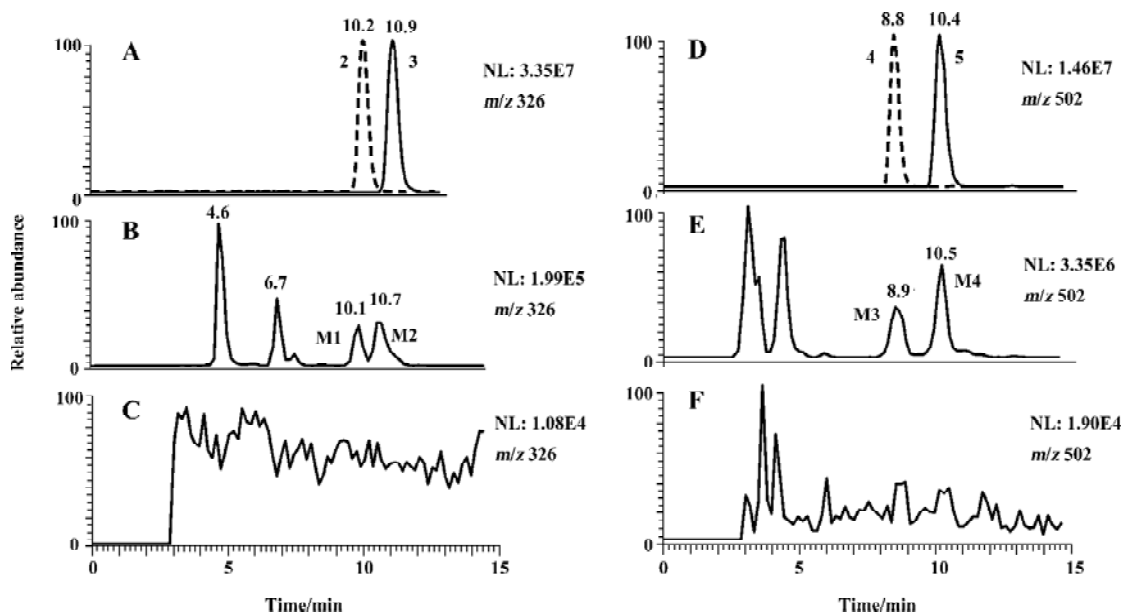


Figure 3. Liquid chromatography-ion trap mass spectrometry SIM chromatogram of blank urine spiked with synthetic references 2, 3, 4, and 5 (A and D), urine sample after benproperine administration (B and E), and blank urine sample (C and F). (SIM: Selected ion monitoring; NL: Normalized level).

Table 1. Data of benproperine metabolites. Ultraviolet (UV) and melting point (Mp) data are of corresponding phosphates. MS: Mass spectrum.

Compound	¹ H δ (ppm)	¹³ C δ (ppm)	MS	UV-vis	Mp (°C)
2	CDCl ₃ , 1.11(3H, d, J 6.8 Hz, H-9), 1.54 (2H, m, H-3a and H-5a), 1.67 (2H, m, H-3e and H-5e), 2.41(2H, m, H-2a and H-6a), 2.81 (2H, m, H-2e and H-6e), 3.05 (1H, m, H-7), 3.62 (1H, m, H-4), 3.88 (1H, m, H-8), 3.98 (2H, s, H-10), 4.02 (1H, m, H-8), 6.88 (2H, m, ArH), 7.08 (1H, m, ArH), 7.25 (6H, m, ArH)	CDCl ₃ , 13.2 (C-9), 34.7 (C-3 and C-5), 36.1 (C-10), 46.8 (C-2 and C-6), 58.4 (C-7), 67.9 (C-4), 69.7 (C-8), 111.1 (C-16), 120.5 (C-14), 125.7 (C-20), 127.4 (C-15), 128.2 (C-19 and C-21), 128.7 (C-18 and C-22), 129.4 (C-12), 130.7 (C-13), 141.0 (C-17), 156.5 (C-11)	MS ¹ 326, MS ² 142, 308	269 nm, ε 1.9×10 ³ (λ _{max} , H ₂ O)	168–170
3	CDCl ₃ , 1.11(3H, d, J 6.8 Hz, H-9), 1.50-1.75 (4H, m, H-4 and H-5), 2.40-2.64 (4H, m, H-2 and H-6), 3.01 (1H, m, H-7), 3.75 (1H, m, H-3), 3.82 (1H, m, H-8), 3.98 (2H, s, H-10), 4.02 (1H, m, H-8), 6.88 (2H, m, ArH), 7.08 (1H, m, ArH), 7.25 (6H, m, ArH)	CDCl ₃ , 13.1 (C-9), 21.9 (C-5), 31.8 (C-4), 36.1 (C-10), 50.0 (C-6), 56.4 (C-2), 58.4 (C-7), 65.9 (C-3), 69.6 (C-8), 111.1 (C-16), 120.5 (C-14), 125.7, 127.4 (C-15), 128.2 (C-19 and C-21), 128.7 (C-18 and C-22), 129.4 (C-12), 130.6 (C-13), 141.0 (C-17), 156.6 (C-11)	MS ¹ 326, MS ² 308, 142	269 nm, ε 1.9×10 ³ (λ _{max} , H ₂ O)	147–149
4	CD ₃ OD, 1.00 (3H, d, J 7.2Hz, H-9), 1.50 (2H, m, H-3a and H-5a), 1.84 (2H, m, H-3e and H-5e), 2.25-2.35 (2H, m, H-2a and H-6a), 2.72 (2H, m, H-2e and H-6e), 2.87 (1H, m, H-7), 3.10 (1H, m, H-3'), 3.31 (2H, m, H-2' and H-4'), 3.45 (1H, d, J 9.6 Hz, H-5'), 3.67 (1H, m, H-4), 3.81 (1H, m, H-8), 3.87 (2H, s, H-10), 3.93 (1H, m, H-8), 4.27 (1H, d, J 7.8Hz, H-1'), 6.78 (1H, m, ArH), 6.83 (1H, m, ArH), 7.03 (4H, m, ArH), 7.10 (3H, m, ArH)		MS ¹ 502, MS ² 326, MS ³ 142, 308		
5	CD ₃ OD, 1.00 (3H, d, J 7.2Hz, H-9), 1.50 (2H, m, H-4a and H-5a), 1.68 (2H, m, H-4e and H-5e), 2.42 (2H, m, H-2a and H-6a), 2.52 (2H, m, H-2e and H-6e), 2.81 (1H, m, H-7), 3.13 (1H, m, H-3'), 3.30 (2H, m, H-2' and H-4'), 3.45 (1H, m, H-5), 3.70-3.93 (3H, m, H-8 and H-3), 3.87 (2H, s, H-10), 4.46 (1H, d, J 7.8Hz, H-1'), 6.97 (1H, m, ArH), 7.03 (1H, m, ArH), 7.09 (4H, m, ArH), 7.10 (3H, m, ArH)		MS ¹ 502, MS ² 326, MS ³ 308, 142		

indicating that they were isomers. Their pseudo-molecular ions were 16 u higher than that of the parent drug, indicating the addition of a hydroxyl group to the parent drug.

The MS² spectra of M1 and M2 displayed the same fragment ions at *m/z* 308 and *m/z* 142 (Figure 4). The presence of the prominent ion at *m/z* 308 was 18 u lower than precursor ions, indicating the loss of a water molecule from pseudo-

molecular ions and further proving the presence of hydroxyl group. The presence of the ion at *m/z* 142 indicates that the hydroxyl group was in the piperidylpropanyl moiety. However, the MS/MS data did not provide useful information for assigning the exact site of hydroxylation. Therefore, M1 and M2 were definitely confirmed by comparing their retention time and mass spectra with synthesized 2 and 3

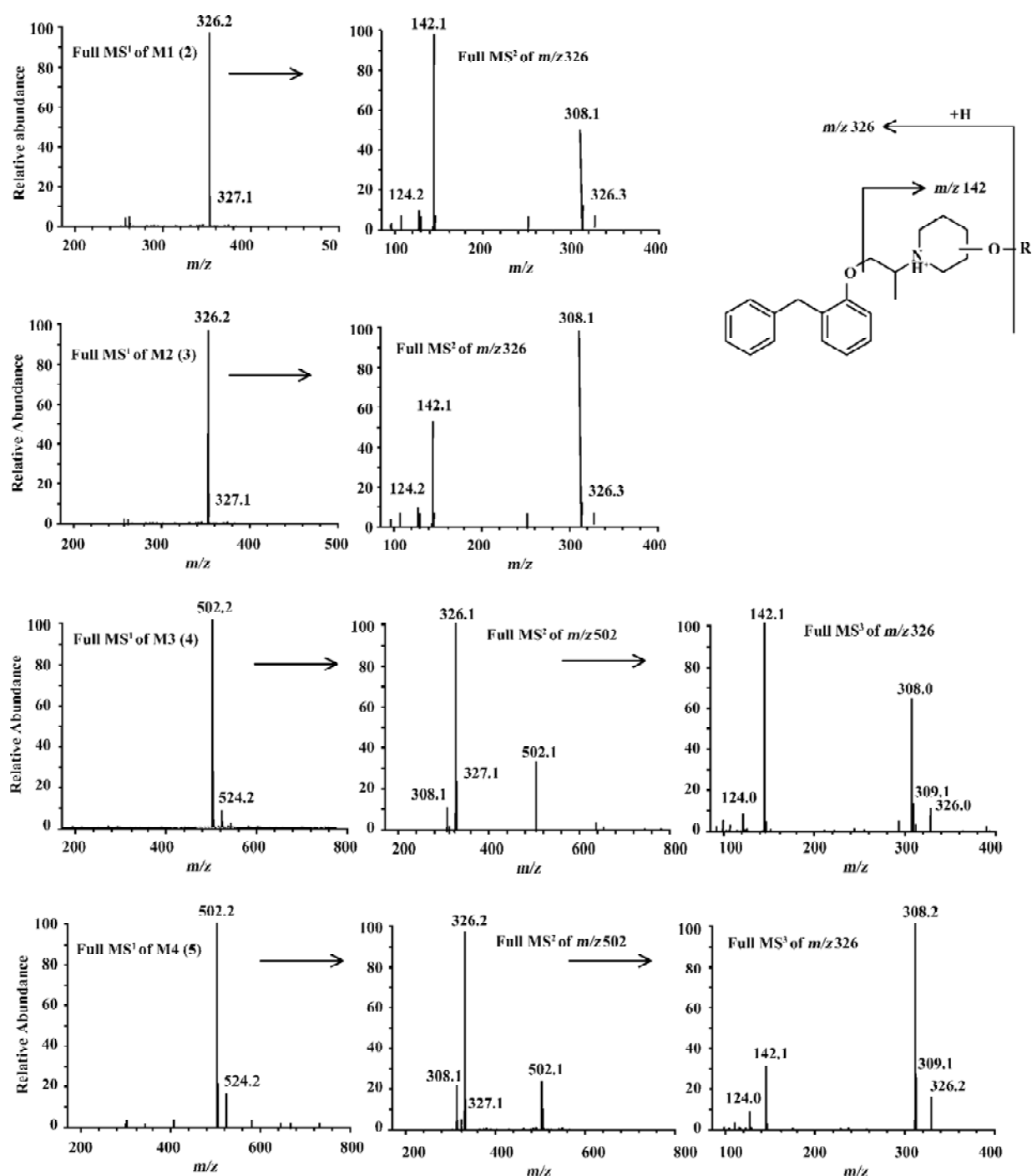


Figure 4. MS/MS spectra of benproperine metabolites (synthetic references 2, 3, 4, and 5).

(Figures 3 and 4). M1 possessed the same retention time and mass spectra with 2, and was confirmed as 1-[1-methyl-2-[2-(phenylmethyl)-phenoxy]ethyl]-4-piperidinol and, in the same way, M2 was identified as 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]-ethyl]-3-piperidinol.

Metabolites M3 and M4 M3 and M4 gave the same

pseudo-molecular ions $[M + H]^+$ at m/z 502, which were 176 u higher than monohydroxylate metabolites, indicating conjugation of a glucuronic acid. The MS^2 spectra of M3 and M4 displayed same fragment ions at m/z 326, which possessed the same mass to charge ratio as the pseudo-molecular ions of M1 and M2. The MS^3 spectra of M3 and M4 displayed

the fragment ions at m/z 308 and m/z 142, which were consistent with MS² spectra of M1 and M2, respectively (Figure 4). It indicated that M3 and M4 were the glucuronides of M1 and M2, respectively. The further confirmation was obtained by comparing their retention times and MS/MS spectra with the synthesized glucuronides 4 and 5 (Figure 3 and Figure 4). M3 was identified as 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]-ethyl]-piperidin-4-yl]-β-*D*-glucopyranosiduronic acid. In the same way, M4 was identified as 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-3-yl]-β-*D*-glucopyranosiduronic acid.

The peaks at 4.6 min and 6.7 min (Figure 3) corresponded to the two identified monohydroxylate metabolites^[3]. Two peaks gave the same pseudo-molecular ions [M + H]⁺ at m/z 326. The MS/MS spectra showed the fragment ion at m/z 163 and 126 for the peak at 4.6 min and m/z 126 for the peak at 6.7 min, respectively.

Pharmacology Exposure to a nebulized solution of 7.5% citric acid aerosol caused coughing in control animals within 88.1±5.9 s ($n=10$), and both test compounds prolonged the latency of cough without dose-dependency as shown in Table 2. Immediately after exposure to citric acid, there is a hypersensitive period and the animals tend to cough continuously. For the control group, the number of coughs during the 3 min test was 8.2±1.2; all three compounds significantly decreased the number of coughs during the 3 min test, but 2 and 3 failed to decrease the number of coughs during the 5 min immediately after the test (Table 2).

Discussion

In synthesis of putative metabolites 4 and 5, the form of basic acceptor, reaction promoter, and the order of addition are all important variables in glucuronidation. The strong electron-withdrawing character of the 6β-methoxycarbonyl

group in any activated glucuronate makes such species notoriously poor donors^[12], so that glucuronidation is difficult. The phosphate of 2 was treated with a trichloroacetimidate donor in the presence of BF₃×Et₂O to give protected glucuronide 4 in 52% of yield. However, in free form of 2, no desired product was obtained. The salt form of the basic acceptor was better than its free form for glucuronidation, with Lewis acid used as the promoter in the present study. When trimethylsilyl trifluoromethane sulfonate (TMSOTf)^[9] was used as a promoter instead of BF₃×Et₂O, more of the by-product, 2 acetylate, was obtained and the yield of the desired product was increased. An inverse addition, which means trichloroacetimidate 7 was added into the mixed solution of 2 phosphate and BF₃×Et₂O, improved the yield to 52% from 21%. This result is consistent with the prior report for glucuronidation of other compounds^[13].

In positive-ion mode, compounds 2 and 3 formed pseudo-molecular ions [M+H]⁺ at m/z 326. They are two isomers; each of them contains a hydroxyl group in the piperidyl ring and an ether bond. The same product ions were observed in the full scan of MS/MS spectra at m/z 308 and 142, with different relative abundance for 2 compounds. The fragment ion at m/z 142 was formed by cleavage of the ether bond (Figure 4). The fragment ion at m/z 308 was formed by dehydration, which was easier going in 3 than in 2 because *p*-π conjugated system was formed after dehydration in the former. Therefore, m/z 308 was the base peak for 3 and m/z 142 was the base peak for 2.

Hydroxylation and glucuronidation are 2 general pathways of drug metabolism, by which drug can be metabolized to more polar, hydrophilic entities, which can be excreted from the body more easily. The presence of M1 (2), M2 (3) and their glucuronides M3 (4) and M4 (5) is consistent with the general rule of metabolism. In human urine, some

Table 2. The antitussive effect of benproperine and its metabolites 2 and 3 phosphates using 7.5% citric acid aerosol in guinea pigs. $n=10$. Mean±SD. ^b $P<0.05$, ^c $P<0.01$ vs vehicle.

Drug	Dose/mg·kg ⁻¹	Time to onset of first cough/s	Coughs during 3 min challenge	Coughs during 5 min after the challenge
Vehicle	—	88.1±5.9	8.2±1.2	16.5±2.5
BPP×H ₃ PO ₄	27	129.9±14.0 ^b	2.7±1.0 ^c	4.7±1.2 ^c
2×H ₃ PO ₄	27	127.6±14.0 ^b	2.9±0.8 ^c	9.6±2.6
	9	125.2±9.6 ^c	3.6±0.9 ^c	16.2±2.5
	3	132.3±10.3 ^c	3.9±0.9 ^b	16.4±3.3
3×H ₃ PO ₄	27	131.2±13.8 ^b	4.0±0.9 ^b	14.3±3.3
	9	131.3±11.0 ^c	5.0±1.2	16.9±1.9
	3	126.8±10.0 ^b	3.9±0.8 ^c	15.6±1.5

dihydroxylate metabolites besides monohydroxylate were detected; no dealkylation metabolite was detected. In the present study, 4 monohydroxylate metabolites and 2 glucuronide metabolites were identified. The chromatogram (Figure 3) indicates that the hydroxylated metabolites of benproperine were recovered in urine mainly as glucuronides, and in very low concentrations as free forms. M1 and M2 were also found in human plasma.

The free forms of compound 2 (M1) and 3 (M2) are oils, which are difficult to be weighed, and the water-solubility of the free forms are poor. They are not suitable for use in the study of antitussive activity. Benproperine is generally used in clinical treatments as dihydrogenphosphate. Therefore, we converted the free form of compounds 2 and 3 to their phosphates for pharmacological study.

The experimental model to induce coughing using citric acid is the model most frequently adopted and extensively studied in animals and in humans^[14]. Citric acid can induce coughing in guinea pigs by increasing the volume of bronchial secretion^[10], by acting on capsaicin sensitive sensory neurons^[15,16] or by disturbing the pH of the airway surface liquid^[17]. However, it induces the airway hyper-reactivity in guinea pigs^[18]. Although phosphates of 2 and 3 produced an increase in the latency of the first cough and decreased the number of coughs during the 3 min test using citric acid, they did not decrease the number of coughs during the 5 min immediately after the test. The results showed that phosphates 2 and 3 did not inhibit the coughing induced by citric acid in guinea pigs.

In conclusion, 8 novel compounds, 2–5, 8, 9, and phosphates of 2 and 3 were synthesized successfully for the first time. 1-[1-Methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-4-piperidinol (2), 1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-3-piperidinol (3), 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)-phenoxy]ethyl]-piperidin-4-yl]- β -*D*-glucopyranosiduronic acid (4) and 1-*O*-[1-[1-methyl-2-[2-(phenylmethyl)phenoxy]ethyl]-piperidin-3-yl]- β -*D*-glucopyranosiduronic acid (5) were identified to be metabolites of benproperine in human urine. Compounds 2 and 3 are inactive metabolites of benproperine.

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