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Isolation and Identification of Urinary Metabolites of Berberine in Rats and Humans

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d) Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EtOH, ethanol; MeOH, methanol; ODS, octadecyl silane; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; ESI-MS, electrospray ionization-mass spectrometry; LC/MS^n, liquid chromatography-mass spectrometry at stage n.

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Abstract

The urinary metabolites of berberine, an isoquinoline alkaloid isolated from several Chinese herbal medicines, were investigated in rats and humans. Using macroporous adsorption resin chromatography, open ODS column chromatography, and preparative high performance liquid chromatography, seven metabolites (**HM1**-**HM7**) were isolated from human urine and five metabolites (**RM1**-**RM5**) from rat urine following oral administration. Their structures were elucidated by enzymatic deconjugation and analyses of MS, ^{1}H-NMR and NOESY spectra. Besides the three known metabolites demethyleneberberine-2-O-sulfate (**HM1, RM3**), jatrorrhizine-3-O-sulfate (**HM5**) and thalifendine (**RM5**), six new metabolites were identified, namely, jatrorrhizine-3-O-β-D-glucuronide (**HM2**), thalifendine-10-O-β-D-glucuronide (**HM3**), berberrubine-9-O-β-D-glucuronide (**HM4, RM2**), 3,10-demethylpalmatine-10-O-sulfate (**HM6, RM4**), columbamin-2-O-β-D-glucuronide (**HM7**), and demethyleneberberine-2,3-di-O-β-D-glucuronide (**RM1**). These findings suggest that berberine undergoes a similar biotransformation in rats and humans. Possible metabolic pathways of berberine in rats and humans are proposed.

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Introduction

Berberine is an isoquinoline alkaloid isolated from several Chinese herbal medicines such as Rhizoma Coptidis, Cortex Phellodendri and Caulis Mahoniae. Its chemical structure is 5,8-dihydro-9,10- dimethoxy-6H-Benzo(g)-1,3-benzodioxolo(5,6-a)quinolizine. Berberine exhibits a wide variety of bioactivities such as antidiarrheic (Sack et al., 1982; Taylor et al., 1989), antimicrobial (Kaneda et al., 1991; Yan et al., 2007), hypolipidemic (Kong et al., 2004; Doggrell et al., 2005; Brusq et al., 2006; Cicero et al., 2007), hypoglycemic (Yin et al., 2002; Pan et al., 2003; Lee et al., 2006; Turner et al., 2007; Zhou et al., 2007; Yin et al., 2008), antiarrhythmic (Wang et al., 1994; Law et al., 2001), anticancer (Inoue et al., 2005; Lin et al., 2006; Lin et al., 2007; Piyanuch et al., 2007; Serafim et al., 2007; Gansauge et al., 2007; Yu et al., 2007; Lanvers-Kaminsky et al., 2006), anti-inflammatory (Ckless et al., 1995; Lee et al., 2007), antiviral (Hayashi et al., 2007), antidepressant (Kulkarni et al., 2007; Peng et al., 2007) and hepatoprotective (Zhang et al., 2008) effects. Its chloride salt has been used for several decades in clinical situations to treat gastroenteritis and secretory diarrhoea in China. In recent years, berberine has also shown significant effects in treatment of diabetes mellitus (Ni, 1988), hyperlipemia (Kong et al., 2005), arrhythmia and heart failure (Zeng et al., 1999; Zeng et al., 2003; Law et al., 2001). However, pharmacokinetic studies have indicated that berberine has poor oral bioavailability (Shen et al., 1993; Yu et al., 2000; Zuo et al., 2006) , and a few of the metabolites have been identified in rats (Zuo et al., 2006) and in humans (Pan et al., 2002). To obtain more information about its metabolism in order to improve its clinical applications, we examined the biotransformation of berberine in rats and humans. The present paper describes the isolation and identification of urinary metabolites of berberine in these two species.

Materials and Methods

Materials. Berberine chloride (purity>99.5%) was supplied by the Northeast General Pharmaceutical Factory (Shenyang, China). Methanol was HPLC grade and water was double distilled in our laboratory. All other reagents were of analytical grade and purchased from Shenyang Chemical Company (Shenyang, China). Normal-phase and reverse-phase preparatory thin-layer chromatography was performed using products from Merck Company. Macroporous resin D101 was purchased from the Chemical Plant of Nankai University (Tianjin, China) and Diaion ion exchange resin HP20 from Mitsubishi Chemical Corporation (Japan). Sephadex LH-20 and ODS were obtained from Pharmacia Company (Freiburg, Germany). Spots on chromatograms were detected with Dragendorff's reagent (a solution of potassium bismuth iodide) which is usually used to visualize alkaloids that have been developed using TLC. Arylsulfatase (3.1.6.1) and β-Glucuronidase (3.2.1.31) were purchased from Sigma.

Subjects and Dosing Procedure. Eighty male Wistar rats, from eight to ten weeks old, weighing 200 to 250g, were used in the study of the urinary metabolites of berberine chloride in rats. Subjects were kept in the breeding room for 7 days before the study. Normal food and water were available at all times but withdrawn 12 hr prior to intragastric administration of berberine chloride. Each subject was given an oral dose, 100mg/kg (body weight), of berberine chloride and urine samples were collected for 48 hours.

For the study of the urinary metabolites of berberine chloride in humans, twelve healthy volunteers from 22 to 26 years of age and weighing 60 to 80 kg (all males) participated in this study. Subjects were judged to be in good health based on a medical history, physical examination, and laboratory profiles that were performed within a 2 week period before the start of the study. The study

followed the 1964 Principles of Helsinki for the ethical treatment of human subjects for biomedical research. Each subject was given an oral dose, 300mg, of berberine chloride, three times a day for 2 days, and the urine was collected between 0 and 72 h.

Isolation of Metabolites. The urine samples (rats:3,800mL; human: 16,000mL) were chromatographed on macroporous adsorptive resin D101 and eluted with H_2O and 95% EtOH. The 95% EtOH eluate was subjected to HP 20 column chromatography using an H_2O/EtOH solvent system. The fractions eluted by 20% EtOH and 30% EtOH were then fractionated by chromatography on Sephadex LH-20 and RP-18 silica-gel columns with an MeOH-H_2O solvent system (0-30%). The fractions containing the metabolites underwent fractionation by Sephadex LH-20, RP-8 and RP-18 silica-gel column chromatography with an MeOH-H_2O solvent system (10-60%) and then purified by preparative HPLC.

Purification by HPLC. Preparative HPLC was performed with an ODS column (XTerra™ RP18, 7μm, 19×300mm) in a Waters 600 liquid chromatograph equipped with a Waters 490 UV detector. The usual detection wavelength was 340 nm. Elution was carried out with MeOH-H_2O at a flow rate of 10 ml/min. Elution with MeOH-H_2O (15:85) yielded RM1 (2.3 mg). Elution with MeOH-H_2O (16:84) yielded **HM2** (6mg), **HM3** (4mg) and **HM7** (1mg). Elution with MeOH-H_2O (22:78) yielded **HM1** (40mg), **HM4** (6.1mg), **HM5** (2.2mg) and **HM6** (1.2mg). Elution with MeOH-H_2O (55:45) yielded **RM5** (10 mg).

Spectroscopic Methods. Electrospray ion trap mass spectrometry in multi-stage full scan mode was performed on a Bruker esquire 2000 instrument with a mass range of 25 to 2200 (the mass was calibrated). The instrument was operated in the both positive and negative ion modes, using nitrogen as the nebulizing and dry gas. The CID of the quasi-molecular ion was achieved with helium as the

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collision gas. The ionization was carried out under the following conditions: capillary temperature, 250℃; capillary voltage, 3.0 kV. Sample solutions were directly introduced into the ESI source at a flow rate of 3uL/min by a syringe pump.

NMR spectra were measured on Bruker ARX-600 spectrometer and chemical shifts are given in ppm with tetramethylsilane as an internal standard. **HM1**-**HM7** and **RM5** were dissolved in DMSO-$d_{6.}$, and **RM1** was dissolved in D_2O:DMSO-d_6(1:2).

Enzymatic hydrolysis. The metabolites (**HM1**-**HM7** and **RM1**, each 0.2mg) were dissolved in 0.1M acetate buffer (pH5.0). Then, arylsulfatase (100 units) or β-glucuronidase (5000 units) was added and the solution incubated for 4h at 37°C. Following this the reaction solution was chromatographed on an ODS mini-column eluting with distilled water to remove any impurities. The subsequent MeOH eluate was evaporated to give a residue which was analysed by ESI–MS.

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Results

Isolation and Structure Elucidation of Berberine Metabolites.

By means of macroporous adsorptive resin chromatography, open ODS column chromatography, and preparative high performance liquid chromatography, five metabolites(**RM1**-**RM5**) from rat urine and seven metabolites(**HM1**-**HM7**) from human urine were isolated following oral administration of berberine chloride, and their structures were identified on the basis of enzymatic hydrolysis, ESI-MS, ^{1}H-NMR and NOESY spectra.

In the ESI-MS spectra (Fig. 1), removal of masses of 80 or 176 from the quasi-molecular ions $[M - Cl]^+$ of the metabolites showed that they were sulfate or glucuronide conjugates. The evidence provides useful information to help in the structural identification of the berberine metabolites.

Sulfate-conjugated Metabolites.

HM1(**RM3**) and **HM5** were identified as demethyleneberberine-2-O-sulfate and jatrorrhizine-3-O-sulfate, respectively, by enymatic hydrolysis, and further comparison of MS, and NMR data (Table 1 and 2)with those in the literature[Pan et al., 2002].

HM6(**RM4**): Yellow needle crystal, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **HM6** gave the quasimolecular ion $[M - Cl]^+$ at m/z 404 and $[M - Cl + Na - H]^+$ at m/z 426.Bombardment of m/z 426 afforded a fragment ion $[M - Cl + Na - H - SO_3]^+$ at m/z 346. Combined with enzymatic hydrolysis data, **HM6** was deduced to be a sulfate-conjugated metabolite.

In the ^{1}H-NMR spectrum, the skeleton proton signals (Table 2)of the parent drug remained except for those of the substituent groups. The linked positions of two methoxyl groups were established by the NOESY spectrum: the methoxyl signal at δ4.24 correlated with that of H-8 at δ9.83, and the methoxyl group at δ3.95 correlated the H-1 at δ7.72, indicating that two methoxyl groups were linked at C-9 and

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C-2. Thus, the sulfate group must be linked to C-3 or C-10. Furthermore, 9-OCH_3 and H-11 shifted downfield by 0.1ppm, and H-4 hardly shifted at all, so that it was concluded that the sulfate group was located at C-10. Thus, **HM6** was identified as 3,10-Demethylpalmatine-10-O-sulfate.

Glucuronide-conjugated Metabolites.

HM2: Yellow powder, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **HM2** gave the quasi-molecular ion $[M - Cl]^+$ at m/z 514. Bombardment of 514 afforded the fragment ion $[M - Cl - C_6H_8O_6]^+$ at m/z 338, suggesting that **HM2** might be a glucuronide conjugate. This was further confirmed by enzymatic hydrolysis of **HM2**, in which the aglycone ion fragment at m/z 338 was detected by ESI-MS. Comparison of the ^{1}H-NMR data (Table 1) of the aglycone of **HM2** with those of berberine indicated many similarities except for the loss of the characteristic methylene signal and the appearance of a new methoxyl signal.

The linked positions of the newly formed methoxyl group and the glucuronic acid moiety were established by the NOESY spectrum, in which H-1(δ7.73) exhibited correlations with H-13 (δ9.04) and 2-OMe (δ3.93), H-4 (δ7.19) exhibited correlations with H-1'(δ5.14) and H-5(δ3.17), indicating that the glucuronic acid moiety was located at C-3 and the newly formed methoxyl group was located at C-2. The β-configuration of the glucuronic acid moiety was identified based on the coupling constants of the anomeric proton (δ5.14, J=7.0Hz). Therefore, the structure of **HM2** was identified as jatrorrhizine-3-O-β-D-glucuronide.

HM3: Yellow powder, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **HM3** gave the quasi-molecular ion $[M - Cl]^+$ at m/z 498. Bombardment of 498 afforded the fragment ion $[M - Cl - C_6H_8O_6]^+$ at m/z 322, suggesting that HM3 might be a glucuronide conjugate. This was further confirmed by enzymatic hydrolysis of **HM3**, in which the aglycone ion fragment at m/z 322 was

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detected by ESI-MS. In comparison with the ^{1}H-NMR spectrum of berberine, the other proton signals (Table 1) were observed in **HM2** except for the loss of one methoxy group. The linked positions of the remaining methoxyl group and the glucuronic acid moiety were established from the NOESY spectrum. In this, H-11(δ8.14) exhibited correlations with H-12(δ7.89) and H-1' (δ5.15), indicating that the glucuronic acid moiety was located at C-10 and the remaining methoxyl group was consequently located at C-9. The β-configuration of the glucuronic acid moiety was confirmed based on the coupling constants of the anomeric proton (δ5.15, J=7.5Hz). Thus, the structure of **HM3** was identified as thalifendine-10-O-β-D-glucuronide.

HM4 (**RM2**): Yellow amorphous powder, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **HM4** gave the quasi-molecular ion $[M - Cl]^+$ at m/z 498. Bombardment of 498 afforded the fragment ion $[M - Cl - C_6H_8O_6]^+$ at m/z 322, suggesting that **HM4** was a glucuronide conjugate. This was further confirmed by enzymatic hydrolysis of **HM4**. Comparison of the ^{1}H-NMR data (Table 1) of **HM4** with those of **HM3** indicated that **HM4** was an isomer of **HM3**. The linked positions of the methoxyl group were established from the NOESY spectrum, in which H-8(δ10.15) exhibited correlations with H-6(δ5.10,δ4.85), and H-11(δ8.19) exhibited correlations with H-12(δ8.04) and the methoxyl group (δ4.04). Therefore, the methoxyl group was located at C-10 and consequently the glucuronic acid moiety must be located at C-9. The β-configuration of the glucuronic acid moiety was confirmed based on the coupling constants of the anomeric proton (δ4.82, J=7.0Hz). Thus, the structure of **HM4** was identified as Berberrubine-9-O-β-D-glucuronide.

HM7: Yellow powder, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **HM7** gave the quasi-molecular ion $[M - Cl]^+$ at m/z 514. Bombardment of 514 afforded the fragment ion $[M - Cl - C_6H_8O_6]^+$ at m/z 338, suggesting that **HM7** might be an isomer of **HM2**, This was further confirmed

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by enzymatic hydrolysis of **HM7**, in which the aglycone ion fragment at m/z 338 was detected by ESI-MS. A comparison of the ^{1}H-NMR spectrum of **HM7** with that of **HM2** showed that there were many similarities except for the downfield chemical shifts of H-1 by δ0.36(Table 1 and 2). The linked positions of the methoxyl group (δ3.88) and the glucuronic acid moiety were established from the NOESY spectrum, in which H-1(δ8.09) correlated with both H-13(δ9.15) and H-1'(δ5.06), and **H-4** (δ7.12) correlated with the methoxyl group (δ3.88), indicating that the glucuronic acid moiety was located at C-2 and the methoxyl group (δ3.88) was consequently located at C-3. The β-configuration of the glucuronic acid moiety was confirmed based on the coupling constants of the anomeric proton (δ5.06, J=7.5Hz). Therefore, the structure of **HM7** was identified as Columbamine-2-O-β-D-glucuronide.

RM1: Yellow powder, giving a positive reaction to Dragendorff's reagent. The ESI-MS of **RM1** gave a quasimolecular ion $[M - Cl]^+$ at m/z 676. Bombardment of 676 afforded the fragment ion $[M - C_6H_8O_6]^+$ at m/z 500, and consequent bombardment of 500 yeilded $[M - Cl - 2\times C_6H_8O_6]^+$ at m/z 324, suggesting that **RM1** might be a diglucuronide conjugate, This was further confirmed by enzymatic hydrolysis of **RM1**, in which the aglycone ion fragment at m/z 324 was detected by ESI-MS. In comparison with the ^{1}H-NMR spectrum of berberine, most of the skeleton proton signals (Table 2)remained in RM1 except for loss of the characteristic methylene signal. The linked positions of the two glucuronic acid moieties and the two methoxyl groups were established by NOESY. The two anomeric protons at δ5.12 (d,J=6.0Hz) and δ5.04(d,J=7.0Hz) correlated with H-1(δ 7.82) and H-4(δ 7.17), respectively, indicating that the two glucuronic acid moieties were located at C-2 and C-3, respectively. The two methoxyl groups at δ3.97(s) and δ4.01(s) correlated with H-11(δ8.03,d, J=9.0Hz) and H-8 (δ9.63), respectively, suggesting that they should be linked to C-10 and C-9, respectively. The

β-configurations of the two glucuronic acids moieties were confirmed from the coupling constants of the two anomeric protons(δ5.12, J=6.0Hz; δ5.04, J=7.0Hz). Therefore, the structure of **RM1** was finally determined as demethyleneberberine-2,3-di-O-β-D-glucuronide.

Unconjugated Metabolite.

RM5: red powder, giving a positive reaction to Dragendroff's reagent. The ESI-MS of **RM5** gave the quasimolecular ion $[M - Cl]^+$ at m/z 322 which is 14 mass units less than that of berberine. Comparison of the ^{1}H-NMR data (Table 2)with those of berberine showed there were many similarities except for the disapperance of one methyl group. The remaining methoxyl group was assigned to 9-OMe based on the NOESY spectrum, in which the methoxyl signal (δ4.05) showed a correlation with H-8 (δ9.71). Following comparison of the ^{1}H-NMR data with those in the literature (Niu et al., 2006), **RM5** was identified as thalifendine.

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Discussion

Structural elucidation of metabolites is one of the most challenging tasks in drug metabolism studies. In recent years, comparisons of ESI-MS^n data and HPLC retention times with synthetic standards are usually used to identify the structures of metabolites. However, when the standards are difficult to synthesize, some metabolite structures deduced only from LC/MS^n data may not be correct, especially in the case of the presence of isomeric metabolites. In our study, three groups of isomers (**HM1** and **HM6**; **HM2** and **HM7**; **HM3** and **HM4**) were obtained, and they had identical LC/MS^n data. Therefore, their exact structures could not be identified from only LC/MS^n data (Zhu et al., 2007). In these cases, preparation of metabolites and further identification based on NMR data is needed. Of course, the direct isolation of the metabolites from urine, bile, or feces of humans or animals can be difficult, but it is the most reliable method for the identification of metabolites. We have determined the definitive structures of nine metabolites of berberine by enzymatic hydrolysis, and examination of MS and NMR spectra. These results are important for a better understanding its in vivo metabolic fate and disposition in rats and humans.

In this study, nine urinary metabolites of berberine in rats and humans were isolated and identified. Based on the metabolites, it is suggested that berberine is metabolized in both rats and humans mainly involving 2,3-OCH_2O and 9,10-OCH_3 by cleavage of the dioxymethylene five-membered ring or demethylation to form the corresponding phase 1 metabolites, some of which then underwent conjugation with glucuronic acid or sulphuric acid to form phase II metabolites. Most of the final metabolites were sulfate or glucuronide conjugates. These metabolites are very polar and are easily excreted.

In order to examine the possible site of metabolism, we analyzed the gastrointestinal contents of rats

4 hours after oral administration of berberine, and could not detect any metabolites, indicating that berberine is relatively stable in the gastrointestinal tract. However, we also investigated the urinary metabolites of berberine after intraperitoneal injection, and obtained the same metabolites as those after oral administration, implying that the metabolites of berberine are formed after absorption rather than in gastrointestinal tract. Based on the structures of the metabolites isolated from the urine, possible metabolic pathways in rats and humans are proposed (Fig. 2).

Although berberine exhibits a variety of pharmacological effects in clinical situations, its poor oral bioavailability makes it hard for us to understand its clinical application for treatment of diabetes mellitus (Ni, 1988), hyperlipemia (Kong et al., 2005), arrhythmia and heart failure (Zeng et al., 1999; Zeng et al., 2003; Law et al., 2001). This study demonstrated that the absorbed berberine is easily metabolized in vivo and its an extensive biotransformation may be one of the main reasons for its low oral bioavailability

Several reports have showed that a high concentration is necessary for berberine to display its in vitro bioactivities (Kong et al., 2005; Yin et al., 2005; Piyanuch et al., 2007). However, the blood-berberine concentration in animals or humans was very low when given orally in experimental or clinical doses (Shen et al., 1993; Yu et al., 2000), suggesting that the berberine metabolites might be responsible for the pharmacological effects. The bioactivities of the berberine metabolites including the corresponding phase I metabolites are now under investigation.

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Footnotes

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Legends for Figures

Fig.1. ESI/MS spectra of $[M-Cl]^+$ ion of berberine metabolites **HM1**(A), **HM2**(B), **HM3**(C), **HM5**(D), **RM1**(E) and **RM5**(F) in rat and human urine

Fig.2. Structures of berberine metabolites in rat or human urine and possible metabolic pathways for their production

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Table 1 ^{1}H-NMR data of berberine and its metabolites **HM1-4** in humans and rats

NO	**berberine**	**HM1(RM3)**	**HM2**	**HM3**	HM4(**RM2**)
1	7.79(*1H*,s)	8.02(*1H*,s)	7.73(*1H*,s)	7.82(*1H*,s)	7.77(*1H*,s)
4	7.09(*1H*,s)	6.92(*1H*,s)	7.19(*1H*,s)	7.09(*1H*,s)	7.10(*1H*,s)
5	3.22 (*2H*, t ,5.6Hz)	3.19 (*2H*, t ,5.6Hz)	3.17(*2H*,o)	3.21(*2H*,o)	3.24(*2H*,o)
6	4.95 (*2H*, t ,5.6Hz)	4.93 (*2H*, t ,5.6Hz)	4.94 (*2H*,m)	4.92 (*2H*,m)	5.10(*1H*,m);4.85(*1H*,m)
8	9.91(*1H*,s)	9.85(*1H*,s)	9.93(*1H*,s)	9.84(*1H*,s)	10.15(*1H*,s)
11	8.20 (*1H*, d ,8.7Hz)	8.19 (*1H*, d ,9.2Hz)	8.21(*1H*, d ,9.2Hz)	8.14(*1H*, d ,9.0Hz)	8.19(*1H*, d ,9.2Hz)
12	8.01 (*1H*, d ,8.7Hz)	8.11(*1H*, d ,9.2Hz)	8.04(*1H*, d ,9.2Hz)	7.89(*1H*, d ,9.0Hz)	8.04(*1H*, d ,9.2Hz)
13	8.96(*1H*,s)	8.81(*1H*,s)	9.04(*1H*,s)	9.01(*1H*,s)	8.89(*1H*,s)
2,3-OCH_2O	6.17(*2H*,s)			6.18(*2H*,s)	6.18(*2H*,s)
2-OCH_3			3.93(*3H*,s)		
9-OCH_3	4.10(*3H*,s)	4.09(*3H*,s)	4.10(*3H*,s)	4.14(*3H*,s)	
10-OCH_3	4.07(*3H*,s)	4.06(*3H*,s)	4.08(*3H*,s)		4.04(*3H*,s)
3-OH		9.78(*1H*,s)			
GlcUA-1'			5.14(*1H*, d ,7.0Hz)	5.15(*1H*, d ,7.5Hz)	4.82(*1H*, d ,7.0Hz)

Notes: a) all spectra were recorded on a AV-600 spectrometer, in DMSO-d_6.

b) s,singlet; d,doublet; t,triplet; m, multiplet; o, overlapped

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Table 2 ^{1}H-NMR data of berberine metabolites **HM5-7**, **RM1** and **RM5** in humans and rats

NO	**HM5**	**HM6**(**RM4**)	**HM7**	**RM1**	**RM5**
1	7.73(*1H*,s)	7.72(*1H*,s)	8.09(*1H*,s)	7.82(*1H*,s)	7.52(*1H*,s)
4	7.57(*1H*,s)	6.83(*1H*,s)	7.12(*1H*,s)	7.17(*1H*,s)	7.06(*1H*,s)
5	3.20 (*2H*, t ,6.2Hz)	3.13 (*2H*, t ,6.0Hz)	3.23(*2H*,o)	3.15(*2H*,o)	3.18 (*2H*, t ,6.0Hz)
6	4.95 (*2H*, t ,6.2Hz)	4.88 (*2H*, t ,6.0Hz)	4.96(*2H*,m)	4.76(*2H*,m)	4.89 (*2H*, t ,6.0Hz)
8	9.91(*1H*,s)	9.83(*1H*,s)	9.87(*1H*,s)	9.63(*1H*,s)	9.71(*1H*,s)
11	8.22(*1H*, d ,9.2Hz)	8.30(*1H*, d ,9.0Hz)	8.20(*1H*, d ,9.0Hz)	8.03(*1H*, d ,9.0Hz)	7.84(*1H*, o)
12	8.05(*1H*, d ,9.2Hz)	7.84(*1H*, d ,9.0Hz)	8.02(*1H*, d ,9.0Hz)	7.99(*1H*, d ,9.0Hz)	7.84(*1H*, o)
13	9.05(*1H*,s)	8.93(*1H*,s)	9.15(*1H*,s)	8.72(*1H*,s)	8.84(*1H*,s)
2,3-OCH_2O					6.15(*2H*,s)
2-OCH_3	3.93(*3H*,s)	3.95(*3H*,s)			
3-OCH_3			3.88(*3H*,s)		
9-OCH_3	4.10(*3H*,s)	4.24(*3H*,s)	4.09(*3H*,s)	4.01(*3H*,s)	4.05(*3H*,s)
10-OCH_3	4.08(*3H*,s)		4.06(*3H*,s)	3.97(*3H*,s)	
GlcUA-1’			5.06(*1H*, d ,7.5Hz)	5.12(*1H*, d ,6.0Hz)	
GlcUA-1’’				5.04(*1H*, d ,7.0Hz)	

Notes: a) all spectra were recorded on a AV-600 spectrometer, in DMSO-d_6 except that RM1 were dissolved in D_2O:DMSO-d_6(1:2).

b) s,singlet; d,doublet; t,triplet; m, multiplet; o, overlapped

Figure 1

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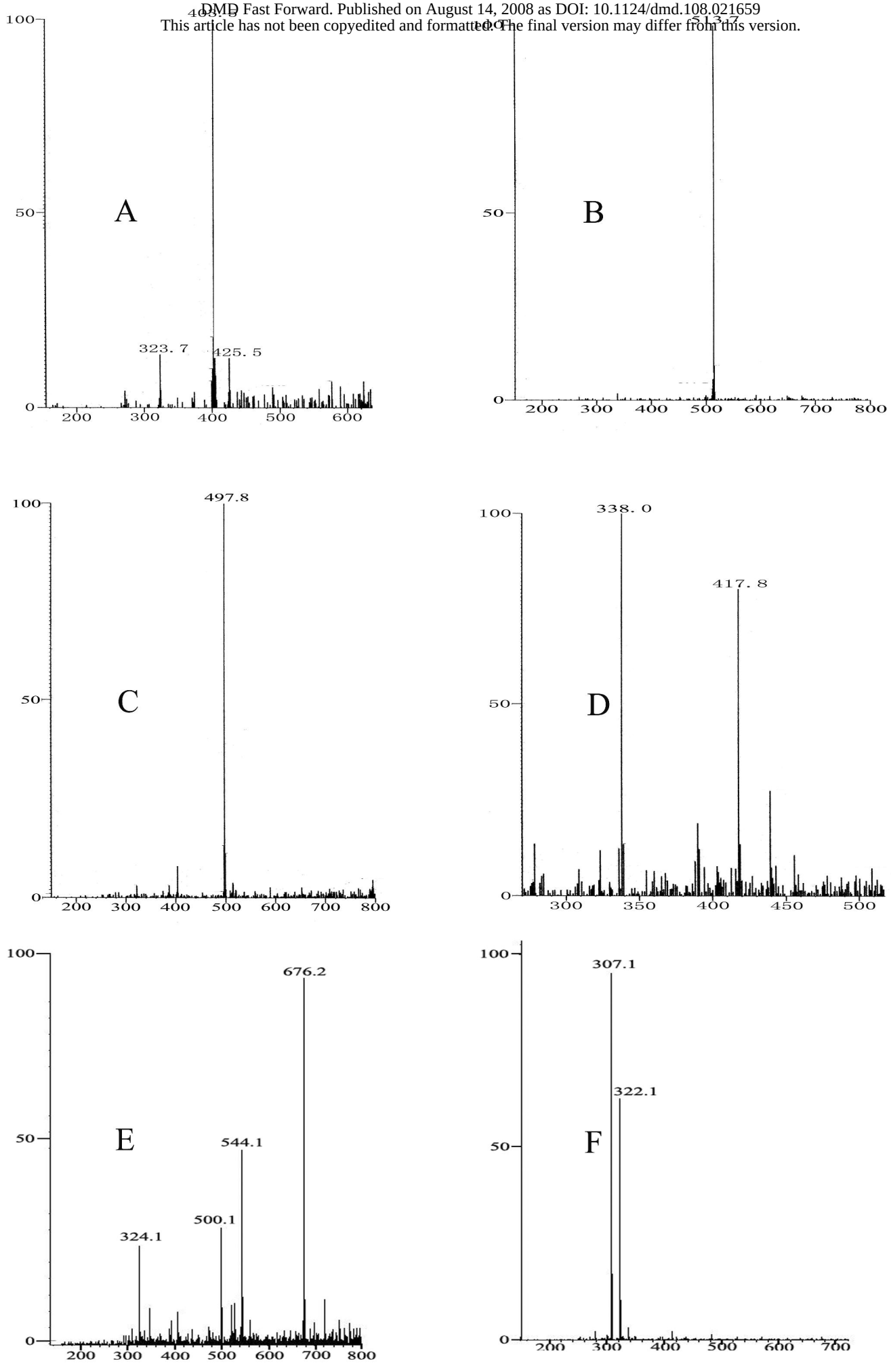

Figure 2

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