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Simultaneous Quantification of Berberine Hydrochloride and Fibrauretine Tablets in Rat Urine, Feces, Bile and Plasma by HPLC/DAD

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Isoquinoline alkaloids *i.e.*, berberine and palmatine are bioactive components of berberine hydrochloride tablets and fibrauretine tablets, respectively. For pharmacological and pharmacokinetics purposes, a simple, rapid and sensitive HPLC/DAD method was employed to measure them in rat urine, feces, bile and plasma. After simple deproteinization by acetonitrile, the ethyl acetate/methanol extract was directly analyzed by HPLC/DAD using a kromasil C18 column (200 × 4.6 mm 5 µm) with UV detection at 265 nm, with the mobile phase comprising 0.2 % phosphoric acid buffer-acetonitrile (74:26, v/v), at a flow rate of 1 mL min^{-1}. Calibration curves of palmatine hydrochloride and berberine hydrochloride in rat urine, feces, bile and plasma were linear over the concentration range of 0.3-75 ng/mL. After oral administration of 30 mg/kg berberine hydrochloride tablets and fibrauretine tablets, cumulative excretion amount of palmatine hydrochloride and berberine hydrochloride in rat plasma were 10.14 ± 0.71 and 0.13 ± 0.06 ng at 24 h, respectively. The cumulative amounts of palmatine hydrochloride and berberine hydrochloride in rat urine reached 1.18 ± 0.28 and 16.32 ± 0.43 ng at 36 h after dosing, respectively. The results showed for their pharmacological differences study. Validation revealed that the method was specific, accurate and precise. The fully validated method was applied to the excretion study of berberine hydrochloride and palmatine hydrochloride in rats.

Keywords: HPLC/DAD, Berberine hydrochloride, Fibrauretine, Rat urine, Feces, Bile and plasma, Pharmacological profile study.

INTRODUCTION

Berberine hydrochloride tablets and fibrauretine tablets are two kinds of Chinese medicines, which have been used as an antiinflammatory for several decades in clinical situations in China[1]. Their main bioactive components are berberine hydrochloride and palmatine hydrochloride, respectively. Both are isoquinoline alkaloids and their chemical structures are shown in Fig. 1. Berberine can be found and extracted from several Chinese herbal medicines such as *rhizoma coptidis, cortex phellodendri and caulis mahoniae*[2-6]. Modern pharmacological studies have proven that berberine has a wide variety of bioactivity, including antidiarrheic, antimicrobial, hypolipidemic, hypoglycemic, antiarrhythmic, anticancer, antiinflammatory, antiviral, antidepressant and hepato-protective effects[7]. Its chloride salt tablet, as one of the most popular medicines, has been widely used in gastroenteritis and secretory diarrhea treatment in China[8-10]. Another isoquinoline alkaloid, palmatine, which is the main bioactive components of fibrauretine tablets, is found in the Chinese herb *fibaurea recisa* pierre[11]. Fibrauretine tablets stimulate an antibiotic activity against bacteria, fungi and viruses and exert vasodilatory, sedative, hepato-protective and antitumor effects[12]. In China, fibrauretine preparations have been widely used in the treatment of various inflammatory diseases, such as gynecological inflammation, bacillary dysentery, enteritis, respiratory tract infection, urinary infection, *etc*.[13,14]. Although berberine hydrochloride tablets and fibrauretine tablets are both anti-inflammatory medicines, their pharmacological applications are not the same. Berberine hydrochloride tablets are widely used in gastrointestinal inflammation and fibrauretine tablets are mainly used in gynecological inflammation. Their different clinical applications attracted our attention to study their *in vivo* distribution, metabolism and efficacy[15].

In this study, a high-performance liquid chromatography with diode array detection (HPLC/DAD) method was developed and validated for determination of berberine hydrochloride and palmatine hydrochloride in rat urine, feces, bile and plasma after the intragastric administration of 30 mg kg^{-1} berberine hydrochloride tablets and fibrauretine tablets simultaneously. Simultaneous determination of both tablets in biological samples would allow us to get more information

of their pharmacological and pharmacokinetics difference than separate assays. However, as to our best of knowledge, there has been no report for simultaneous administration and determination of these two kinds of Chinese medicine tablets in biological samples, even though several chromatographic methods have been reported for the analysis of either berberine hydrochloride or palmatine hydrochloride. The established HPLC method demonstrated good performance in terms of linearity, specificity, detection and quantification limits, precision and accuracy. This method could be successfully utilized to quantifiably compare pharmacological profiles of berberine hydrochloride and palmatine hydrochloride in rat urine, feces, bile and plasma. These studies might be helpful for further clinical applications of the two traditional Chinese medicine tablets.

EXPERIMENTAL

Standards of berberine hydrochloride (110713-200911, 86.8 %, Fig. 1a) and palmatine hydrochloride (110732-200907, 86.1 %, Fig. 1b) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Berberine hydrochloride tablets was purchased form Northeast General Pharmaceutical Factory (China) and Fibrauretine tablets was purchased form Hunan Dinuo Pharmaceutical Co., Ltd (Hunan,China).

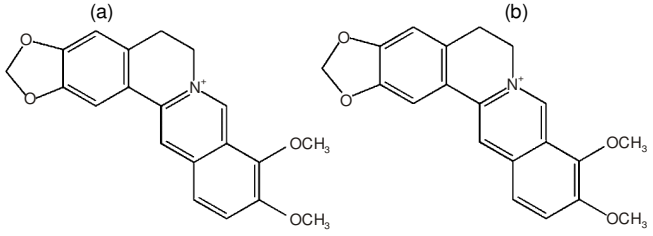

Fig. 1. Chemical structures of (a) palmatine and (b) berberine

HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Methanol was obtained from Fengchuan Chemical Co., Ltd (Tianjin, China). Ethyl acetate and phosphoric acid were obtained from Shenbo Chemical Co., Ltd (Shanghai, China). Drug-free blank urine, feces, bile and plasma were obtained by sacrificing male rats. HPLC-grade water from Hangzhou Wahaha Group Co., Ltd was used throughout the pre-study validation and analysis.

Chromatography was performed with a high performance LC (Agilent Technologies Series 1200) system comprising quaternary pumps, a diode-array detector, an auto injector (Waldbronn, Germany) and a chromatography workstation. Vortex machine from Hanuo (Shanghai, China) was used for vortex-mixing. An ultrasonic bath from Kunshan ultra-sonic instrument plant (Jiangsu, China) was used for extraction of samples. A HC-3018R centrifuge (Anhui, China) was used for complete phase separation.

Preparation of standard and solutions: Stock solutions of berberine hydrochloride and palmatine hydrochloride were both prepared in ethanol at concentrations of 1 mg mL^{-1}. These stock solutions were mixed and diluted with water to furnish solutions of six different concentrations for linear calibration assay. These standard solutions were stored at 4 °C in a brown glass volumetric flask.

Calibration standards and quality-control samples: The calibration standards of berberine hydrochloride and palmatine hydrochloride were prepared by diluting the standard working solution (10 µL) with drug-free rat urine (100 µL), feces (100 mg), bile (100 µL) and plasma (100 µL) to span a calibration standard range of 0.3-75 ng mL^{-1}. All quality-control (QC) samples used for validation and pharmacological study were prepared immediately before analysis and as the same way for the calibration standards.

Preparation of sample: Twenty tablets of berberine hydrochloride tablets and fibrauretine tablets were triturated to fine power, weighed accurately a quantity of each powder, equal to about 25 mg of berberine hydrochloride and palmatine hydrochloride, extracted with 25 mL water/methanol (75:25, v/v) and promoted by an ultrasonic bath under the frequency of 40 kHz for 0.5 h, respectively. 0.2 % hydrochloric acid was added to fibrauretine tablets dissolution to make sure that palmatine turn to palmatine hydrochloride.

Blank urine, feces, bile and plasma were collected before dosing. The feces samples were weighted, methanol (two-fold of feces sample weight) was added into feces samples and sonicated for 10 min. The volumes of urine and bile samples were recorded. The urine, feces and bile samples were centrifuged at 3,000 rpm for 10 min and then all supernatants were kept at -20 °C until use. The plasma sample (0.1 mL) was transferred to a 1 mL plastic test tube with 10 µL of 10 ng mL^{-1} standard solution. After vortexed-mixing for 10 s with vortexed-mixing for 1 min and centrifugation at 10,000 rpm for 10 min to separate the aqueous and immiscible organic layers. The clear organic layer was transferred to a 1 mL micro-centrifuge tube. Afterwards, ethyl acetate (150 µL) was added to the microcentrifuge tube, the upper layer was quantitatively transferred to a 1 mL glass tube and evaporated to dryness at 55 °C. The residue was reconstituted with 50 µL methanol, then vortex-mixed and transferred to a clean intubation in auto-sampler vial. An aliquot of 10 µL of mixture was injected into the HPLC system.

Chromatography: Chromatographic separations were achieved using a Kromasil 1 reversed-phase column (200 mm × 4.6 mm, 5 µm). The mobile phase consisted of 0.2 % phosphoric acid buffer-acetonitrile (74:26, v/v) and the flow rate was 1 mL min^{-1} (phosphate was provided by Chuandong Chemical Co., Ltd chongqing, China). The phosphate buffer was prepared fresh daily with HPLC-grade purified water. All solutions were filtered through a 0.45 µm membrane (Ø 50 mm, 0.22 u, Shanghai Xingya purifying material factory, China) and degassed prior to use.

The column was kept at 40 °C. The channel on the diode-array detector was carried out at 265 nm, 10 microliters of the samples, were injected into the HPLC for each chromatographic analysis. The total run time of each chromatographic analysis was 18 min.

Measurements of berberine hydrochloride in berberine hydrochloride tablets and palmatine hydrochloride in fibrauretine tablets: To calculate the administrated dose, the contents of berberine hydrochloride in berberine hydrochloride tablets and palmatine hydrochloride in fibrauretine tablets were

quantitatively determined. The LC analysis of berberine hydrochloride and palmatine hydrochloride were modified versions of a previously published methods. The concentrations of berberine hydrochloride and palmatine hydrochloride were determined by LC to be 891 and 795 mg/g in berberine hydrochloride tablets and fibrauretine tablets, respectively.

Method application: Animals, drug administration and the collection of urine, feces, bile and plasma: male rats (n = 20, weight = 160-180 g), were obtained from Laboratory Animal Centre of Kunming Medical University. All rats were kept in a controlled environment at 23 ± 2 °C and 50 ± 10 % relative humidity of a 12 h dark-light cycle. Food and water were allowed ad libitum. The rats had free access to water during the experiment. After a 12 h fast before the experiment.

Rats were divided into two groups randomly (n = 6) and after each rat was administered a 30 mg kg^{-1} single oral dose of berberine hydrochloride tablets and fibrauretine tablets, urine and feces samples were collected at 2, 4, 6, 12, 24, 48 and 72 h (merge to collected urine and feces, respectively). Bile samples were collected at 1, 3, 6, 12, 24 and 36 h (merge to collected bile). Plasma samples were collected in a sodium citrate tube *via* the heart of the rat after dosing with 2, 4, 8, 16, 24 h (merge to collected plasma) for analysis.

RESULTS AND DISCUSSION

Optimization of the separation conditions: During method development, several combinations of buffer and organic phase were tested. It was found that the mixture with phosphate buffer (adjusted to pH 2 with 0.02 % phosphoric acid)-acetonitrile (74:26, v/v) showed a good peak for berberine hydrochloride and palmatine hydrochloride. (Fig. 2.). The UV detector was operated at 265 nm. Potential interference from endogenous compounds were investigated by analyzing urine, feces, bile and plasma from six different rats.

Method validation

Specificity: The analytical methods had been validated for parameters such as linearity, repeatability, precision, accuracy and stability. The relative standard deviation (RSD %) was taken as a measure of precision, repeatability and stability. The retention times of berberine hydrochloride, palmatine hydrochloride were 9.5, 10.4 min, respectively. The peaks corresponded to berberine hydrochloride tablets and fibrauretine tablets and their standards were well resolved from instrumental noise and background interference. No significant interference peaks were observed at the retention times of the analyte and their standards in rat urine, feces, bile and plasma samples during the analysis.

Linearity: The calibration curves were constructed by analyzing at least six different concentrations of standard solutions. As a result, good linearity ($r^2 > 0.99$) of the investigated concentration ranges was observed.

The injection concentration, which could be detected at the signal-to-noise ratio of 3, was considered to be the limit of detection (LOD). Limit of quantification (LOQ) was the injection concentration corresponding to the peak heights with a signal-to-noise of 10. The concentration ranges of palmatine hydrochloride and berberine hydrochloride in urine, feces, bile and plasma samples were shown in Table-1. The linear regression of the curve for the peak area ratio (y) *versus* concentration (x) was plotted for each type of sample. The R values for the standard curves and the LOQ are also listed in Table-1.

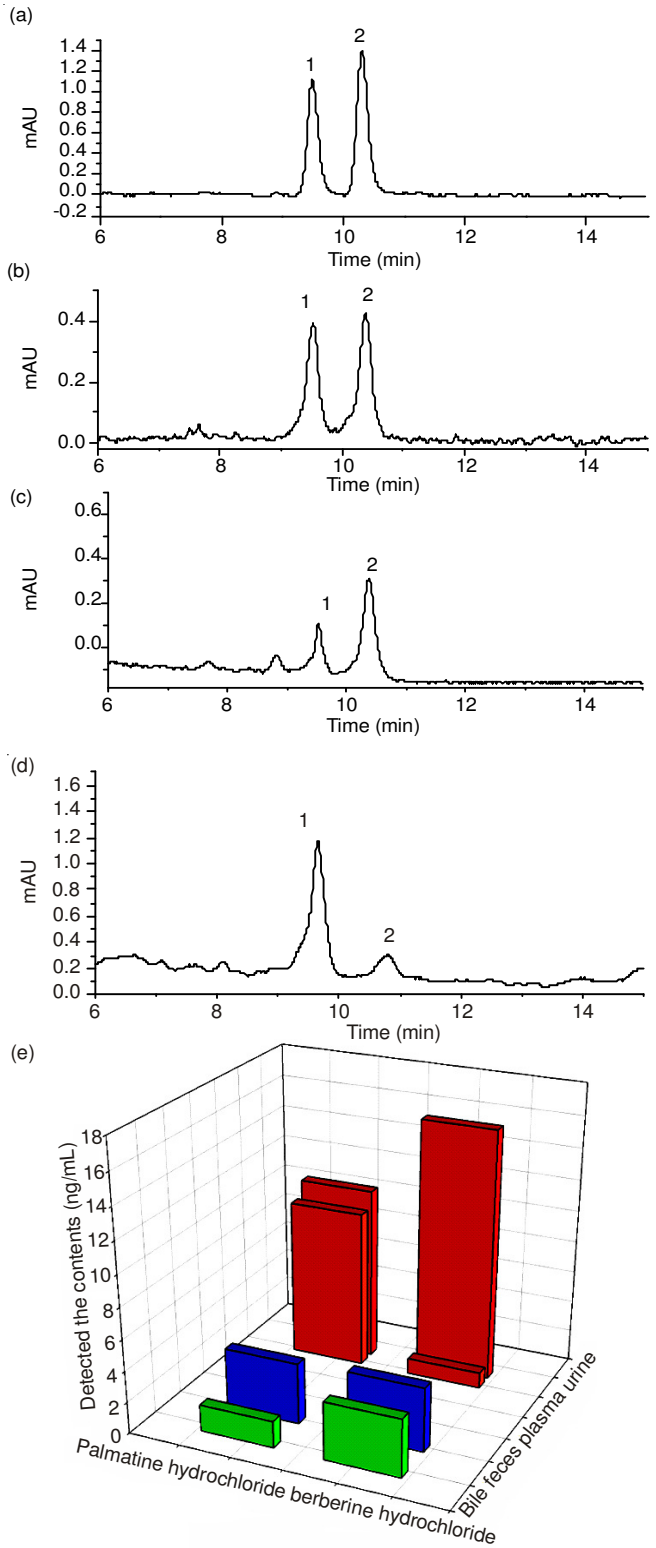

Fig. 2. Chromatograms of (a) a urine sample obtained with 72 h after the intragastric administration of 30 mg kg^{-1} berberine hydrochloride tablets (Peak 2) and fibrauretine tablets (Peak 1); (b) a feces sample obtained with 72 h after the intragastric administration; (c) a bile sample obtained with 36 h after the intragastric administration; and (d) a plasma sample obtained with 24 h after the intragastric administration: (e) the contents of berberine hydrochloride and palmatine hydrochloride in different samples

TABLE-1
CALIBRATION RANGES, EQUATIONS, CORRELATION COEFFICIENTS, AND LOQ OF PALMATINE HYDROCHLORIDE AND BERBERINE HYDROCHLORIDE IN ALL BIOLOGICAL MATRICES

Matrix	Analyte	Calibration range (ng mL^{-1})	Calibration equation	Correlation coefficient (r^2)	LOQ (ng mL^{-1})
Urine	Palmatine hydrochloride	0.3-30	y=10.487x+5.563	0.9980	0.3
	Berberine hydrochloride	0.3-30	y=11.394x+8.516	0.9962	0.3
Feces	Palmatine hydrochloride	0.6-30	y=12.745x+4.277	0.9942	0.6
	Berberine hydrochloride	0.6-30	y=15.586x+19.63	0.9962	0.6
Bile	Palmatine hydrochloride	0.8-75	y=13.190x+7.095	0.0905	0.8
	Berberine hydrochloride	0.8-75	y=19.632x+6.108	0.9969	0.8
Plasma	Palmatine hydrochloride	0.5-70	y=17.481x+23.88	0.9977	0.5
	Berberine hydrochloride	0.5-70	y=20.384x+29.98	0.9983	0.5

Precision and accuracy tests: Different quality-control samples were used to evaluate the accuracy and precision of the assay. Quality-control samples for palmatine hydrochloride in urine, feces, bile and plasma were 0.15, 1.0 and 10 ng/mL, respectively. For berberine hydrochloride in urine, quality-control samples were 0.30, 1.0 and 10 ng/mL. Quality-control sample for feces were 0.60, 1.2 and 10 ng/mL. Quality-control samples with low, medium and high concentrations were 0.8, 1.6 and 10 ng/mL, respectively for berberine hydrochloride in bile and plasma. The accuracy for the assay of palmatine hydrochloride in urine, feces, bile and plasma were less than ±4.9 %. The accuracy of berberine hydrochloride in all samples ranged from -3.5 to 2.3 %.

For assessment of the intra-day precision, CVs of two analytes were below 4.9, 5.9 and 6.2 % for the quality-controls in urine, feces, bile and plasma, respectively. For inter-day precision, CVs were below 7.1 % at three quality-control levels for palmatine hydrochloride and berberine hydrochloride in all matrices. Both accuracy and precision results were satisfactory.

Recovery test: Extraction recovery was calculated by comparing the observed concentrations with the spiked concentrations. The mean recoveries of palmatine hydrochloride and berberine hydrochloride from rat urine ranged over 85.1 and 96.4, respectively. The mean recoveries of palmatine hydrochloride and berberine hydrochloride from rat feces were between 85.9 and 97.6 %. The mean recoveries of palmatine hydrochloride and berberine hydrochloride from bile were higher 83.3 %. The mean recoveries of palmatine hydrochloride and berberine hydrochloride from rat plasma were 95.9 and 101.5 %. The RSD values of each concentration level were < 7.73 %.

Repeatability and stability: Stability of palmatine hydrochloride and berberine hydrochloride in urine, feces, bile and plasma were evaluated. After three freeze-thaw cycle, palmatine hydrochloride and berberine hydrochloride in all samples were found to be rather stable at the three observed concentrations. The relative errors (RE) varied between -3.29 and 5.31 % for palmatine hydrochloride and berberine hydrochloride, respectively. The results from storage stability at -20 °C, 7 days and at room temperature showed that palmatine hydrochloride and berberine hydrochloride were stable (RE of ± 5.87 % or less) under the above two storage conditions.

Berberine hydrochloride tablets are poorly absorbed from the gut after oral administration. This may be because of the rapid metabolism in the liver and intestine. For these reasons berberine hydrochloride is unlikely to be high concentrations in urine, feces, bile and plasma samples. Compared to berberine hydrochloride tablets, we have less *in vivo* information about fibrauretine tablets. In this study we investigated berberine hydrochloride and palmatine hydrochloride profile after oral administration berberine hydrochloride tablets and fibrauretine tablets to rats. The LOQs for both of this method were = 0.8 ng mL^{-1}, which was sufficient for this study. Simple sample-preparation procedures and short analysis times are crucial for such studies. Berberine hydrochloride and palmatine hydrochloride have two absorption wavelengths, both in the ultraviolet at 265 nm and in the visible region at 345 nm. To obtain good sensitivity, in this study 265 nm was selected as the detection wavelength.

Conclusion

Urine, feces, bile and plasma concentrations of berberine hydrochloride and palmatine hydrochloride were determined after simultaneously oral administration of the same quantity of Berberine hydrochloride tablets and fibrauretine tablets. Although berberine hydrochloride tablets and fibrauretine tablets are both anti-inflammatory medicines, their pharmacological applications are not the same. We found the significant difference between the two compounds in rat plasma and bile. (Fig. 2e) palmatine hydrochloride had much higher level plasma content than berberine hydrochloride and palmatine hydrochloride much lower than berberine hydrochloride in bile. Berberine hydrochloride in berberine hydrochloride tablets which can hardly be absorbed in intestine and result in a low bioavailability and a relatively high concentration of berberine hydrochloride detected in the urine and feces. But palmatine hydrochloride in fibrauretine tablets can be retained in plasma. It suggested the different antiinflammatory mechanism and pharmacological of berberine hydrochloride and palmatine hydrochloride. Unlike western medicine, traditional Chinese medicine concentrates on the overall functional state of the patient, which is becoming a trend in modern medicine for treating complicated diseases. Therefore, it is valuable to pointed out the different tissue distribution of berberine hydrochloride and palmatine hydrochloride after oral administration.

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