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Simultaneous and sensitive determination of xanthotoxin, psoralen, isoimpinellin and bergapten in rat plasma by liquid chromatography–electrospray ionization mass spectrometry

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ABSTRACT

A sensitive, specific and rapid liquid chromatography–mass spectrometry (LC–MS) method has been developed and validated for the simultaneous determination of xanthotoxin (8-methoxypsoralen), psoralen, isoimpinellin (5,8-dimethoxypsoralen) and bergapten (5-methoxypsoralen) in rat plasma using pimpinellin as an internal standard (IS). The plasma samples were pretreated by protein precipitation with methanol and chromatographic separation was performed on a C_{18} column with a mobile phase composed of 1 mmol ammonium acetate and methanol (30:70, v/v). The detection was accomplished by multiple-reaction monitoring (MRM) scanning via electrospray ionization (ESI) source operating in the positive ionization mode. The optimized mass transition ion-pairs (m/z) for quantitation were 217.1/202.1 for xanthotoxin, 187.1/131.1 for psoralen, 247.1/217.0 for isoimpinellin, 217.1/202.1 for bergapten, and 247.1/231.1 for IS. The total run time was 6 min between injections. The calibration curves were linear over the investigated concentration range with all correlation coefficients higher than 0.998. The lower limits of quantitation (LLOQ) of these analytes were less than 1.21 ng/ml. The intra- and inter-day RSD were no more than 9.7% and the relative errors were within the range of −8.1% to 4.5%. The average extraction recoveries for all compounds were between 90.7% and 106.2%. The proposed method was further applied to the determination of actual plasma samples from rats after oral administration of Radix Glehniae extract.

1. Introduction

Radix Glehniae (Beishashen) is an important member of traditional Chinese medicine (TCM) originated from the roots of *Glehnia littoralis* (G.) Fr. Schmidt ex Miq. (Umbelliferae). It has been recorded in Chinese Pharmacopoeia and frequently used in the clinic as tonic, antiphlogistic and mucolytic medicine for the treatment of respiratory and gastrointestinal disorders in China [1]. Modern pharmacological studies suggested that Radix Glehniae could relieve pain, mitigate, eliminate phlegm [2,3], as well as displayed outstanding antibacterial and antifungal bioactivities [4–6]. Recently, it was reported that Radix Glehniae was able to be a cheaper substitute for Panax quinquefolium with regard to its significant antioxidant effects [7].

Phytochemical investigation has found that Radix Glehniae contains many constituents such as coumarins, phenolic acids, adenosine, coumarin glycosides and flavonoids [8–10]. It has been reported that coumarins are important active constituents most contributing to the pharmacological efficacy of Radix Glehniae. Xanthotoxin (8-methoxypsoralen), psoralen, isoimpinellin (5,8-dimethoxypsoralen) and bergapten (5-methoxypsoralen) (see Fig. 1) are four major coumarins present in Radix Glehniae extract which demonstrated activities like photosensitization [11], anticancer [12–14], inducing hepatic drug-metabolizing enzymes [15] and inactivation of infectious pathogens and leukocytes in platelets and plasma [16]. Therefore, pharmacokinetic study of these coumarins is helpful to better understand the pharmacological and clinical effects of Radix Glehniae. And then a quantitative method for determining these components in plasma is demanded.

A number of analytical methods have been developed to determine the four compounds in plasma, urine and serum including fluorometric method [17], high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [18–20] or fluorometric detection (HPLC–FD) [21], thin layer chromatogra-

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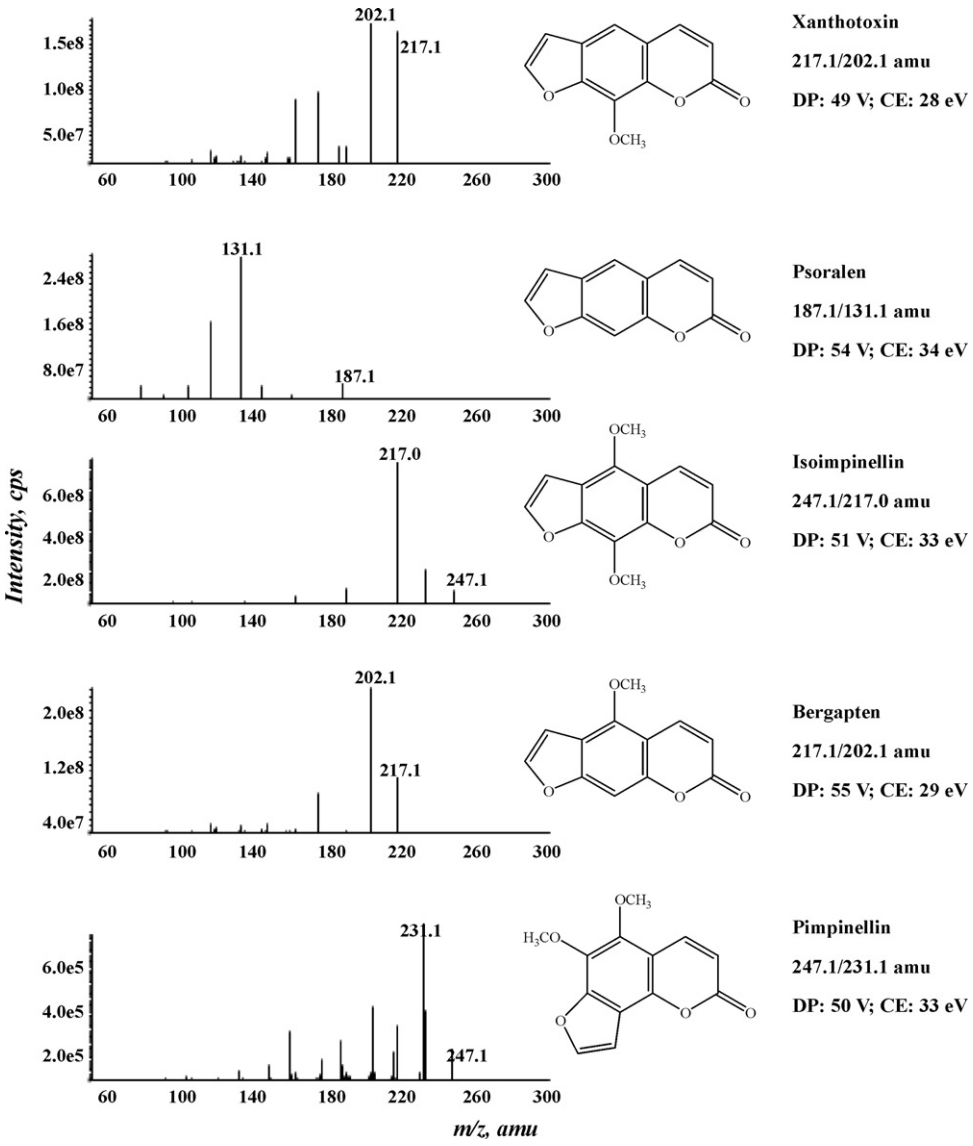

Fig. 1. The product ion scan spectra, chemical structures, monitored transitions, declustering potential (DP) and collision energy (CE) of xanthotoxin, psoralen, isoimpinellin, bergapten and IS.

phy (TLC) method [22], gas chromatography with electron capture detection (GC–ECD) [23], gas chromatography–mass spectrometry (GC–MS)[24,25], bioassay and competitive enzyme-linked immunosorbent assay (ELISA) [26,27]. Except for a HPLC–UV method simultaneously analyzing 9 coumarins in urine [18], the other assays mainly focused on the quantification of one or two coumarins in biological fluids. Furthermore, most of these assays required lengthy pre-treating procedures or a long chromatographic step to achieve better separation of analytes of interest from matrix and generally showed low sensitivity. Liquid chromatography coupled with mass spectrometry (LC–MS) is the current method of choice for the quantification of drugs in biological fluids for its high sensitivity and selectivity. But among the four compounds, only xanthotoxin in plasma has been determined with LC–MS [28]. It is also noted that in previous lectures, the influences of electrolytes in mobile phase on electrospray ionization efficiency of coumarins have never been described which are beneficial to improve sensitivity of the LC–MS method. And matrix effects have not been evaluated directly.

In the present study, we developed a sensitive and selective HPLC–ESI–MS method for simultaneous determination of psoralen, xanthotoxin, isoimpinellin and bergapten in rat plasma. The post-column infusion method was used to detect ion-suppression effects during method development. The ionization efficiency of the four coumarins was discussed in detail by comparing different electrolytes and LC mobile phases.

2. Experimental

2.1. Chemicals, reagents and materials

Psoralen (110739–200613) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Xanthotoxin, isoimpinellin, bergapten and pimpinellin (IS) with purity of greater than 98% were obtained from Shanghai Tauto Biotech Co., Ltd, China. HPLC grade methanol (Fisher, USA) was used for HPLC analysis and plasma sample preparation. Deionized water was produced by Heal Force-PWVF Reagent Water System (Shanghai CanRex Analyses Instrument Corporation Limited, China). Analytical grade dehydrated ethanol (Tianjin Chemical Corporation, China) was used for preparation of Radix Glehniae extract. Formic acid, acetic acid and ammonium acetate

were HPLC grade purchased from Diamond Technology Incorporation. Crude Radix Glehniae samples were collected from Anguo, Hebei province of China.

2.2. Equipments and chromatographic conditions

2.2.1. Liquid chromatography

An Agilent 1200 liquid chromatography system (Agilent, USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment was used for all analysis. The chromatographic separation was performed on an Agilent Zorbax Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm), and the column temperature was kept at room temperature. The mobile phase consisted of 1 mmol ammonium acetate and methanol (30:70, v/v) with a flow rate of 0.8 ml/min. Total eluent flow from the HPLC was diverted directly into the turbo spray source without any splitting device. The total analysis time was just 6 min for each run.

2.2.2. Mass spectrometer

Detection was performed using a 3200 QTRAP™ system from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA), a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and TurboIonspray interface. The instrument was operated using electrospray ionization source in positive mode. The ion spray voltage was set to 5.5 kV, and the turbo spray temperature was maintained at 650 °C. Nebulizer gas (gas 1) and heater gas (gas 2) was set at 60 and 65 psi, respectively. The curtain gas was kept at 25 psi and interface heater was on. Nitrogen was used in all cases. Multiple-reaction monitoring (MRM) mode was employed for quantitation. The precursor-to-product ion pair, declustering potential (DP) and collision energy (CE) for each analyte are described in Fig. 1. All instrumentations were controlled and synchronized by Analyst software (versions 1.4.2) from Applied Biosystems/MDS Sciex.

2.3. Preparation of Radix Glehniae extract

Two hundred grams of Radix Glehniae chopped into pieces was extracted three times by decocting with boiling water (1:10, 1:10 and then 1:5, w/v) for 1 h per time. The extraction solutions were combined to be filtered and concentrated to 500 ml, and then precipitated with quadruple volumes of dehydrated ethanol. The precipitate was discarded after the solution was stored at 4 °C for 24 h, and then the ethanol was removed under reduced pressure. The residuary solution was then diluted with water to get the Radix Glehniae extract with a concentration equivalent to 2 g/ml of the raw Radix Glehniae material.

To calculate the administered dose, the contents of four coumarins in Radix Glehniae extract solution were quantitatively determined by external standard method using the same chromatography conditions as described above. The contents of xanthotoxin, psoralen, isoimpinellin and bergapten in the extract were 155, 82.2, 16.7 and 95.3 μg/ml, respectively.

2.4. Preparation of standard solution and quality control samples

The appropriate amounts of xanthotoxin, psoralen, isoimpinellin and bergapten were separately weighed and dissolved in methanol to make the stock solutions. Then, the four stock solutions were mixed and diluted with water to prepare a final mixed standard solution containing 3.40 μg/ml of xanthotoxin, 6.05 μg/ml of psoralen, 4.00 μg/ml of isoimpinellin, and 2.32 μg/ml of bergapten. A series of working solutions of these analytes were freshly prepared by diluting mixed standard solution with water at appropriate ratios to yield concentrations of 0.00340, 0.00850, 0.034, 0.170, 0.680 and 3.40 μg/ml for xanthotoxin, 0.00605, 0.0151, 0.0605, 0.302, 1.21 and 6.05 μg/ml for psoralen, 0.00400, 0.0100, 0.0400, 0.200, 0.800 and 4.00 μg/ml for isoimpinellin and 0.00232, 0.00580, 0.0232, 0.116, 0.464 and 2.32 μg/ml for bergapten, respectively. A quantity of pimpinellin was dissolved in deionized water to produce the IS solution with the concentration of 0.146 μg/ml. For the validation of the method, three concentration levels of standard solution containing xanthotoxin (0.00850, 0.170 and 2.72 μg/ml), psoralen (0.0151, 0.302 and 4.84 μg/ml), isoimpinellin (0.0100, 0.0400 and 3.20 μg/ml) and bergapten (0.00580, 0.116 and 1.85 μg/ml) were used for preparing QC plasma samples.

2.5. Preparation of plasma samples

To 50 μl of the plasma sample, 10 μl of IS solution, 10 μl of deionized water (volume of the corresponding working solution for calibration curve and QC samples) and 150 μl of methanol were added. The mixture was mixed for 1 min on a vortex-mixer and centrifuged at 12,000 rpm for 5 min to separate the protein. The supernatant was collected and centrifuged at 12,000 rpm for another 5 min. And then, 20 μl of this solution was injected into HPLC–MS system for analysis.

2.6. Method validation

The calibration plasma samples were prepared by adding 10 μl of working solution and 10 μl of IS solution to 50 μl of blank plasma, and then extracted as described in Section 2.5. The calibration curve consisted of six concentration levels, each level sample was prepared and assayed in duplicate on separate 3 days. The linearity was confirmed by using the ratio of the peak area of analytes to that of IS versus analytes concentrations with least-squares linear regression analysis, described in the form of $y = a + bx$ ($1/x^2$ weighted).

LLOQ is determined as the lowest concentration point of the standard curve, at which the concentration that can be reliably and reproducibly measured at least six replicates. The limit of detection (LOD) is defined as the amount that could be detected with a signal-to-noise ratio of 3.

Three validation batches, each contains six replicates of QC samples at low, medium and high concentration levels (1.70, 34.0 and 544 ng/ml for xanthotoxin, 3.02, 60.5 and 968 ng/ml for psoralen, 2.00, 40.0 and 640 ng/ml for isoimpinellin and 1.16, 23.2 and 371 ng/ml for bergapten), were assayed to assess the precision and accuracy of the method on 3 different days. The precision is expressed by relative standard deviation (RSD) between the replicate measurements. Accuracy is defined as relative error (RE) which is calculated using the formula RE% = [(measured value − theoretical value)/theoretical value] × 100.

The extraction recovery of analytes at three QC levels were determined by comparing the peak areas obtained from QC samples with those of analytes from neat standard samples (10 μl of required working solution and 10 μl deionized water spiked into 200 μl of pretreated blank plasma) at equivalent concentrations. The matrix effect was assessed by post-column infusion.

The stability of analytes in rat plasma was assessed by analyzing QC samples at three concentrations exposed to different conditions. The short-term stability was determined during the storage for 24 h at room temperature. The long-term stability was assessed after the QC samples had been stored at −20 °C for 30 days. The freeze–thaw stability was determined after three freeze–thaw cycles (−20 to 20 °C) on 3 consecutive days.

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry

Xanthotoxin, psoralen, isoimpinellin, bergapten and IS were at first characterized according to their mass spectra from syringe pump infusion analysis, respectively. Meanwhile, their precursor ions and product ions were ascertained for use in MRM. The electrospray interface (ESI) was employed for good sensitivity and fragmentation were obtained. It was also found that positive electrospray ionization could offer better sensitivity and reproducibility. In the full scan mass spectra, the protonated molecular ions $[M+H]^+$ of xanthotoxin, psoralen, isoimpinellin, bergapten and IS (m/z 217.2, 187.1, 247.1, 217.1 and 247.1) were stable and exhibited higher abundance. Under the product ion scan mode, the most intensive product ions were m/z 202.1 from m/z 217.1, m/z 131.1 from m/z 187.1, m/z 217.0 from m/z 247.1, m/z 202.1 from m/z 217.1 and 231.1 from m/z 247.1. The mass spectrometric parameters were optimized to obtain the higher signal for both precursor ions and product ions mentioned above. Fig. 1 shows the product ion scan spectrum of the analytes and IS.

3.1.2. Selection of electrolyte in mobile phase

Electrolyte modification of mobile phase can significantly improve the ESI efficiency resulting in enhanced analyte responses [29]. We first used an electrolyte-free mobile phase, water/methanol (30:70), and then tested different buffers including acetic acid (0.01%, 0.05% and 0.1%), ammonium acetate (0.2, 1 and 2 mmol) and formic acid (0.01%, 0.05% and 0.1%) to identify the optimal mobile phase which could produce the best sensitivity, efficiency and peak shape. The results are represented in Fig. 2. It was found that the four analysts showed strong resemblance in variation tendency of response. The addition of 0.1% acetic acid reduced about half of the response of the analytes comparing to electrolyte-free mobile phase, while formic acid did not produce obvious effect. Yet, the peak intensity of the four analytes enhanced when ammonium acetate was added to the mobile phase. The highest signal intensity was achieved when the concentration of ammonium acetate in the mobile phase reached to 1 mmol (approximately 1-fold higher than that in electrolyte-free mobile phase). The results demonstrated that appropriate ammonium acetate could increase ionization efficiency of the four coumarins and the content of ammonium acetate in mobile phase should be optimized to achieve the highest response. This rule could be also applicable to other furocoumarins.

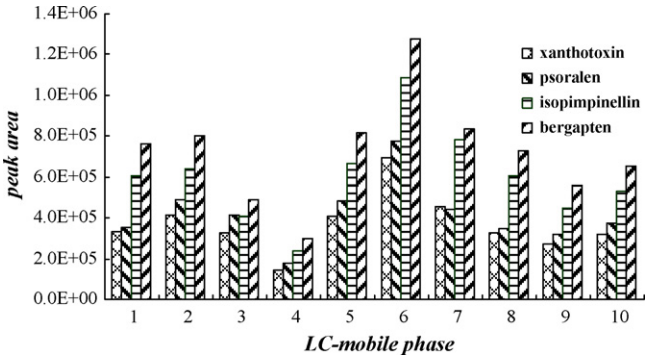

Fig. 2. Ionization efficiency of xanthotoxin, psoralen, isoimpinellin and bergapten using LC mobile phases (water/methanol, 30:70, v/v) containing no electrolyte (1), 0.01% acetic acid (2), 0.05% acetic acid (3), 0.1% acetic acid (4), 0.2 mmol ammonium acetate (5), 1 mmol ammonium acetate (6), 2 mmol ammonium acetate (7) 0.01% formic acid (8), 0.05% formic acid (9), 0.1% formic acid (10).

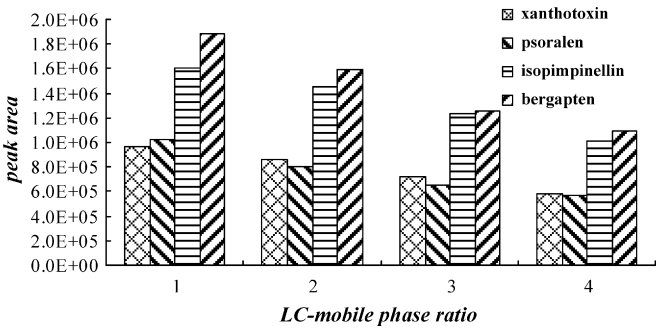

Fig. 3. Ionization efficiency of xanthotoxin, psoralen, isoimpinellin and bergapten in HPLC–MS with mobile phases of 1 mmol ammonium acetate and methanol at different ratio including 20:80 (1), 30:70 (2), 40:60 (3), 50:50 (4).

3.1.3. Matrix effect and mobile phase

Using mobile phase containing 1 mmol ammonium acetate, we also compared different ratio of water phase and methanol (20:80, 30:70, 40:60, 50:50, v/v) to recognize the optimal organic proportion. As shown in Fig. 3, with the increase of the ratio of water phase in HPLC–MS, the peak areas of four analytes decreased. Meanwhile, the retention time of those analytes increased dramatically. Taking bergapten (the last eluted analyte) as an example, its retention time changed from 3.00 to 16.5 min when the ratio of water phase changed from 20% to 50%. Therefore, lower ratio of water phase in mobile phase should be chosen to enhance signal and reduce chromatographic analysis time. And the ultimate ratio was ascertained combining with matrix effect described below.

Post-column infusion method was carried out to investigate matrix effect, for it could provide direct visualization of the nature (suppression or enhancement), chromatographic profile and the extent of the matrix effect [30]. It was found that the strong suppression occurred approximately from 1.5 to 3 min in the chromatogram of plasma sample using isocratic elution with mobile phase consisting of 1 mmol ammonium acetate and methanol at different ratio (20:80, 30:70, 40:60, 50:50, v/v). The ratio of water phase in mobile phase had insignificant effect on the suppression. However, the elution time of the analytes was exactly in the suppression window when the mobile phase consisting of 1 mmol ammonium acetate and methanol at the ratio of 20:80 was used. Considering the above-results that lower ratio of water phase in mobile phase should be chosen to enhance signal and reduce chromatographic analysis time, the eluent consisting of 1 mmol ammonium and methanol (30:70, v/v) was selected as the mobile phase. The matrix effect chromatograms are shown in Fig. 4.

3.1.4. Sample pretreatment

In the course of our method development, we found that liquid–liquid extraction technique had a low recovery when ethyl acetate or n-butanol was used as extraction solvents. Moreover, the procedure of the method was complicated. While, good sensitivity, acceptable recovery, clear supernatant and good separation from interfering peaks for four analytes were obtained by using methanol as the plasma protein precipitating reagent. Therefore, plasma samples were subjected to a simple protein precipitation procedure. It was proved that the present method considerably reduced the sample processing time without any loss of analytes.

3.1.5. Selection of IS

Several compounds served as suitable IS were investigated, such as isopsoralen, pimpinellin, osthole and so on. As these compounds are also coumarins, their structures, physicochemical features and ionization characteristics are similar to those of analytes. It was more essential that they were not found in the plasma samples after oral administration of Radix Glehniae extract. The result showed

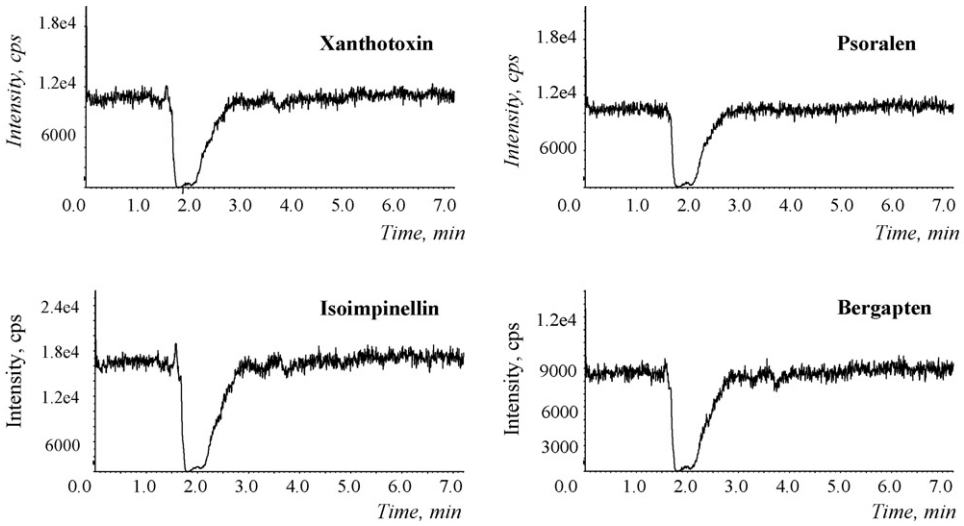

Fig. 4. Post-column infusion chromatograms of xanthotoxin, psoralen, isoimpinellin and bergapten for LC–ESI–MS/MS analysis of blank plasma. The mobile phases used in the experiments were 1 mmol ammonium and methanol (30:70, v/v) with a flow rate of 0.8 ml/min.

Table 1
The regression equations, linear range, LLOQ and LOD of the four coumarins.

Compound	Slope ± SD	Intercept ± SD	*r*	Linear range (ng/ml)	LLOQ (ng/ml)	LOD (ng/ml)
Xanthotoxin	0.0238 ± 0.0001	0.0124 ± 0.0003	0.9994	0.680–680	0.680	0.510
Psoralen	0.0300 ± 0.0011	0.0199 ± 0.0004	0.9988	1.21–1210	1.21	0.726
Isoimpinellin	0.0328 ± 0.0009	0.0037 ± 0.0000	0.9985	0.800–800	0.800	0.436
Bergapten	0.0694 ± 0.0028	0.0414 ± 0.0028	0.9991	0.464–464	0.464	0.232

pimpinellin was more appropriate for IS, because it provided clean chromatographs and shorter retention time and no significant direct interference was observed.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method towards endogenous plasma matrix was evaluated in six rat plasma. The typical chromatograms of blank plasma, plasma sample spiked with xanthotoxin, psoralen, isoimpinellin, bergapten and IS, and the plasma sample from a rat after oral administration of Radix Glehniae extract are shown in Fig. 5. Blank rat plasma yielded relative clean chromatograms without interfering peaks both to the analytes or IS. The retention times of xanthotoxin, psoralen, isoimpinellin, bergapten and IS were 3.22, 3.24, 3.76, 4.05 and 4.71 min, respectively. Bergapten and xanthotoxin, isoimpinellin and IS were isomers, but they were not mutual interference because of different retention times. All the peaks of the analytes and IS in plasma samples were unambiguously identified by comparison of retention time, parent and product ions with reference standards.

3.2.2. Linearity and sensitivity

Table 1 lists the linearity parameters and LLOQ of the four analytes. The correlation coefficients of these calibration curves were all higher than 0.998. Both precision and accuracy of the four ana-

Table 2
The intra- and inter-day accuracies and precisions of the four coumarins in rat plasma at low, medium, high concentration levels ($n = 6$).

Compounds	Intra-day ($n = 6$)			Inter-day ($n = 6$)		
Spiked conc. (ng/ml)	Measured conc.[a] (ng/ml)	Accuracy (%)	Precision (%)	Measured conc.[a] (ng/ml)	Accuracy (%)	Precision (%)
Xanthotoxin						
1.70	1.77 ± 0.10	4.2	5.5	1.69 ± 0.16	−0.6	9.7
34.0	33.0 ± 1.6	−2.9	4.9	33.6 ± 2.6	−1.3	7.9
544	538 ± 35	−1.1	6.5	540 ± 48	0.7	8.9
Psoralen						
3.02	3.02 ± 0.10	−0.1	3.2	3.06 ± 0.21	1.3	6.9
60.5	63.2 ± 2.1	4.5	3.6	61.4 ± 4.2	1.4	6.8
968	935 ± 49	−3.4	5.3	909 ± 63	−6.1	6.9
Isoimpinellin						
2.00	2.03 ± 0.16	1.6	7.8	2.00 ± 0.17	0.0	8.4
40.0	37.0 ± 1.9	−7.5	5.2	36.8 ± 0.8	−8.1	2.2
640	611 ± 17	−4.6	2.8	599 ± 25	−6.4	4.2
Bergapten						
1.16	1.19 ± 0.08	2.7	6.3	1.15 ± 0.10	−1.2	8.5
23.2	24.0 ± 1.1	3.3	4.8	23.3 ± 2.0	0.2	8.4
371	353 ± 16	−4.9	4.6	346 ± 17	−6.6	4.9

[a] Mean ± standard deviation.

lytes at LLOQ were less than 15%. The detailed LOD data of the four analytes are also listed in Table 1. The high sensitivity made this method advantageous to measure the trace concentration of these analytes in plasma.

3.2.3. Precision, accuracy and extraction recovery

Table 2 summarizes the intra- and inter-day precisions and accuracies of xanthotoxin, psoralen, isoimpinellin, bergapten at three concentration levels (low, middle and high). The intra- and inter-day precisions (RSD) of these analytes were all less than 7.8% and 9.7%. The extraction recoveries of xanthotoxin, psoralen, isoimpinellin, bergapten in rat plasma are shown in Table 3. At three concentration levels of these analytes, the extraction recoveries were all between 90.7% and 106.2%. The results demonstrated that the values were all within the acceptable range and the method was accurate and precise.

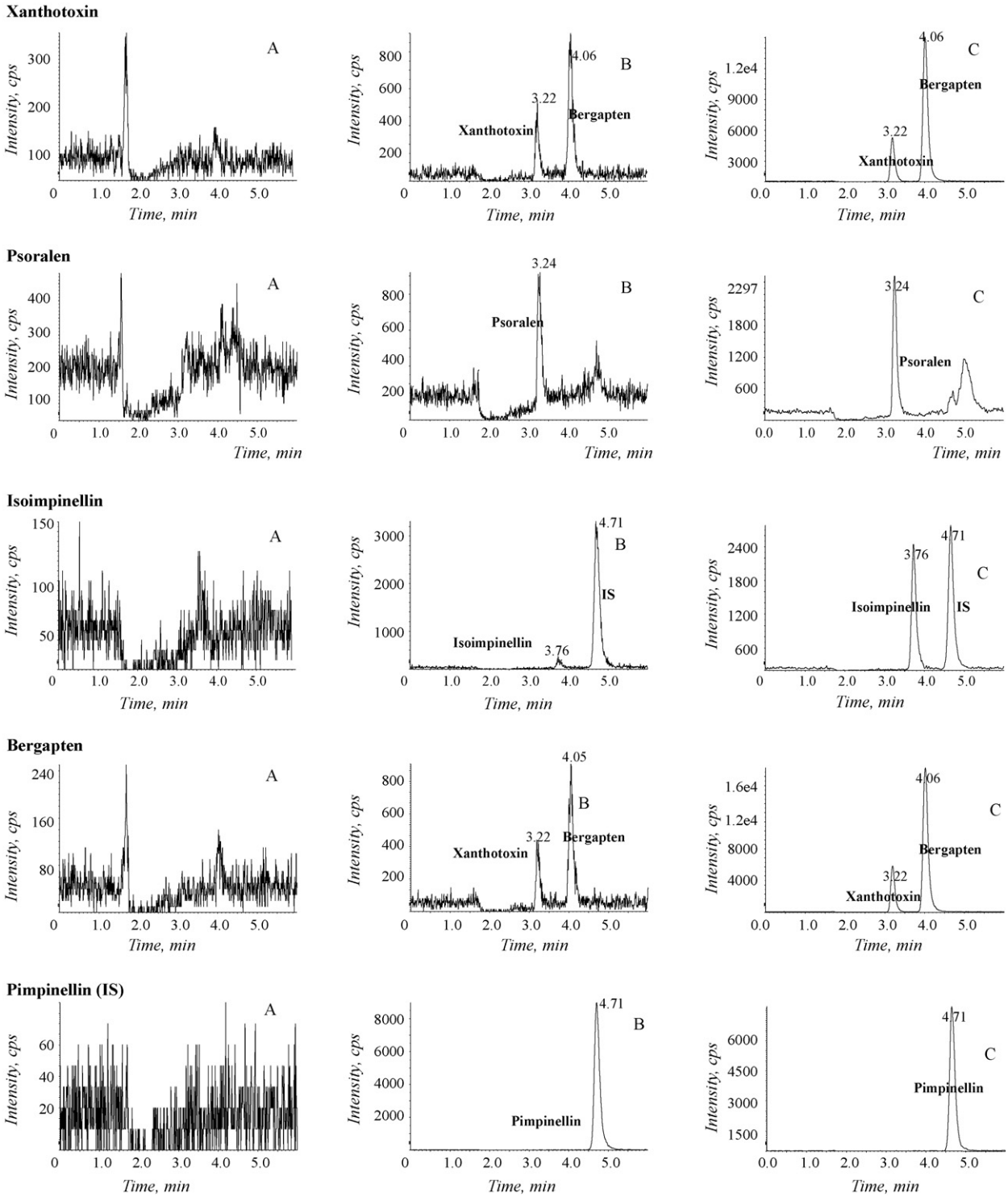

Fig. 5. Representative MRM chromatograms of xanthotoxin, psoralen, isoimpinellin, bergapten and pimpinellin (IS): (A) blank plasma, (B) blank plasma spiked with the four analytes at LLOQ and IS (C) sample plasma 4 h after a single oral administration of Radix Glehniae extract.

Table 3
The mean recoveries of the four coumarins in rat plasma ($n = 5$).

Components	Spiked conc. (ng/ml)	Recovery (%)	RSD (%)
Xanthotoxin	1.70	101.0 ± 3.9	3.9
	34.0	102.1 ± 4.6	4.5
	544	97.2 ± 1.0	1.0
Psoralen	3.02	90.7 ± 4.5	5.0
	60.5	101.0 ± 5.8	5.8
	968	96.3 ± 1.9	2.0
Isoimpinellin	2.00	105.6 ± 7.1	6.7
	40.0	106.2 ± 7.2	6.8
	640	96.7 ± 1.9	1.9
Bergapten	1.16	101.7 ± 6.4	6.3
	23.2	102.1 ± 5.7	5.6
	371	97.1 ± 1.0	1.0

Table 4
Stability of the four coumarins in rat plasma ($n = 3$).

Compound	At room temperature for 24 h in plasma		After three freeze–thaw cycles in plasma		At −20 °C for 30 days in plasma	
Spiked conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (%)	Measured conc. (ng/ml)	Accuracy (%)	Measured conc. (ng/ml)	Accuracy (%)
Xanthotoxin						
1.70	1.65 ± 0.11	−2.7	1.84 ± 0.04	8.5	1.85 ± 0.01	8.9
34.0	32.8 ± 0.6	−3.6	32.6 ± 0.5	−4.0	34.4 ± 1.9	1.2
544	579 ± 18	6.3	564 ± 29	3.7	570 ± 23	4.8
Psoralen						
3.02	2.86 ± 0.16	−5.4	3.11 ± 0.13	2.9	3.18 ± 0.14	5.2
60.5	60.0 ± 0.2	−0.8	58.9 ± 0.3	−2.7	61.6 ± 3.5	1.9
968	933 ± 28	−3.7	904 ± 60	−6.7	949 ± 5	−1.9
Isoimpinellin						
2.00	1.99 ± 0.05	−0.6	2.04 ± 0.14	2.2	2.15 ± 0.00	7.7
40.0	39.0 ± 1.3	−2.4	39.1 ± 2.1	−2.2	40.0 ± 2.0	0.0
640	636 ± 12	−0.6	613 ± 37	−4.2	624 ± 24	−2.4
Bergapten						
1.16	1.13 ± 0.02	−2.3	1.14 ± 0.02	−1.5	1.13 ± 0.05	−2.4
23.2	22.4 ± 0.2	−3.6	21.5 ± 1.4	−7.2	21.7 ± 1.5	−6.5
371	355 ± 20	−4.4	351 ± 13	−5.4	358 ± 21	−3.4

3.2.4. Stability

The results of stability are shown in Table 4. It was indicated that the analytes in rat plasma were all stable for 24 h at room temperature, three cycles of freeze–thaw, 30 days at −20 °C with accuracy in the rang between −7.2% and 8.9%.

3.3. Application

To demonstrate the applicability of the method for real sample, plasma concentrations of xanthotoxin, psoralen, isoimpinellin and bergapten from 6 healthy male Sprague–Dawley rats (250 ± 20 g) were determined after a single oral administration of Radix Glehniae extract (10 ml/kg). The rats were supplied by Lab Animal Center of Hebei Medical University. It was found that the plasma concentrations of the analytes 4 h after administration were all above the LLOQ (the mean concentration ($n = 6$): xanthotoxin, 36.8 ng/ml; psoralen, 2.48 ng/ml; isoimpinellin, 14.1 ng/ml; bergapten, 31.9 ng/ml). The MRM chromatograms obtained from one rat plasma sample are shown in Fig. 5(C). It suggested that the method could be applied to the pharmacokinetic research of Radix Glehniae. Meanwhile the research is on conducting in our laboratory.

4. Conclusion

A sensitive and selective LC–ESI–MS method for the simultaneous determination of xanthotoxin, psoralen, isoimpinellin and bergapten in rat plasma was firstly developed and validated. The simple protein precipitation procedure during the pretreatment of samples and fast LC–MS determination allowed high-throughput analysis with a short run time of 6 min. The proposed method showed appropriate accuracy and precision and was successfully applied for analysis of the four analytes in rat plasma after oral administration of Radix Glehniae extract, which indicated that the method should be suitable for pharmacokinetic study of the four compounds.

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