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Two complementary liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods to study the excretion and metabolic interaction of edaravone and taurine in rats

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ABSTRACT

In this study, two independent and complementary liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were respectively developed and validated for the determination of edaravone or taurine in rat urine, feces and bile after intravenous administration, using 3-methyl-l-*p*-tolyl-5-pyrazolone and sulfanilic acid as the internal standards (IS). Edaravone was separated on an Agilent Eclipse Plus C_{18} column (100 × 2.1 mm, 3.5 μm) using methanol and water (containing 5 mM ammonium formate and 0.02% formic acid) as mobile phase, while taurine was performed on a Waters Atlantis HILIC Silica column (150 × 2.1 mm, 3 μm) using acetonitrile and water (containing 5 mM ammonium formate and 0.2% formic acid) as mobile phase. The mass analysis was performed in a Triple Quadrupole mass spectrometer via multiple reaction monitoring (MRM) with negative ionization mode. The optimized mass transition ion pairs (*m/z*) for quantification were 173.1→92.2 and 187.2→106.0 for edaravone and its IS, 124.1→80.0 and 172.0→80.0 for taurine and its IS, respectively. The validated methods have been successfully applied to the excretion and metabolism interaction study of edaravone and taurine in rats after independent intravenous administration and co-administration with a single dose. The results demonstrated that there were no significant alternations on the metabolism and cumulative excretion rate of edaravone and taurine, implying that the proposed combination therapy was pharmacologically viable.
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1. Introduction

Taurine, α-aminoethanesulfonic acid (molecular weight: 125.14), plays a very important role in several essential biological processes. This sulfur-containing amino acid is not incorporated into protein, and it is the most abundant free amino acid in heart, retina, skeletal muscle, leukocytes and brain [1,2]. Taurine has various important physiological functions such as membrane stabilization, osmoregulation and neuroprotection [3–5]. In addition, the intracellular taurine level of myocardial cells in ischemic heart failure and hypoxia has been found to be lower than that of healthy people [6], indicating that taurine may possess a neuroprotective effect against focal cerebral ischemia in human.

Edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one (molecular weight: 174.20), is a novel free-radical scavenger which has been studied for the treatment of cerebral stroke for many years. It has been utilized as a neuroprotective agent for the treatment of acute cerebral infarction by the Japanese Health Organization since 2001 [7]. Several studies have shown that it has anti-apoptotic, anti-necrotic and anti-cytokine effects [8–10].

The synergistic treatment effects of taurine combining with edaravone for stroke patients have clinically been clarified, as both of which have proven to be safe and effective therapies against ischemia [11]. However, commercial compound drug containing edaravone and taurine is still unavailable. For recognizing the pharmacokinetic interaction and exploring the potential possibility of new drug containing edaravone and taurine, non-clinical pharmacokinetic study of combination of two drugs is essential, which is the basis of their pharmacodynamic and toxicology.

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Recently, pharmacokinetic interaction of taurine co-administrated with edaravone in rat plasma has been reported by our group [12]. However, there is presently no available literature which describes excretion and metabolism interaction of taurine co-administrated with edaravone. The purpose of this study was to establish simple, quick, sensitive and reproducible methods for quantification of edaravone and taurine and apply the methods to the excretion and metabolism interaction studies.

Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been a useful tool to perform metabolism and excretion studies [13,14]. Furthermore, it has gained importance for the quantitative estimation of drugs in various biological matrices including plasma, feces, urine, bile, and ocular fluids, due to its high selectivity and reproducibility [15,16]. In fact, LC-MS/MS for the quantification of taurine in urine [17] or edaravone in plasma has been performed [18]. However, the former published the simultaneous determination of taurine and related biomarkers of bladder cancer in urine, so the analytical time is too long for the independent determination of taurine in urine, and this analytical method was also not suitable for the determination of taurine in bile and feces after our trial. The latter justly focused on the determination of edaravone in plasma, which was also been proven by our group to be not available for the determination of bile, feces and urine. When simultaneous LC-MS/MS determinations of edaravone and taurine was performed referring to our previous work [12,19], heavy interference and matrix effect from bile, feces and urine happed, which was difficult to be resolved by optimizing chromatographic condition and sample process after lots of experiments in our laboratory.

In the present study, two simple and sensitive LC-MS/MS methods were respectively developed to determine independently edaravone or taurine in rat bile, feces and urine. Due to the weak polarity of edaravone, its analysis was performed on a reverse phase (RP) chromatographic column, whereas hydrophilic interaction liquid chromatography (HILIC) was selected to improve the poor retention of taurine on traditional RP column. The methods were validated in terms of specificity, linearity, precision and accuracy, recovery, matrix effect and stability. They were successfully applied to excretion and metabolism interaction studies of edaravone and taurine in rats after independent intravenous administration or co-administration with a single dose. The results demonstrated that there were no significant alternations on the cumulative excretion rate of edaravone and taurine, and no new metabolites appeared, implying that the proposed combination therapy was pharmacologically feasible.

2. Experimental

2.1. Chemicals and reagents

The reference standards including edaravone and taurine (≥98.0%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China). The internal standards (IS), sulfanilic acid (≥98.0%, IS of taurine) and 3-methyl-1-p-tolyl-5-pyrazolone (≥99.0%, IS of edaravone), were respectively purchased from NICPP and Tokyo KaSei Industry Company. HPLC grade-methanol was obtained from Fisher Scientific (Fisher Scientific, USA). Formic acid and other reagents used in this study were of at least analytical grade. Purified water was used from a Milli-Q system (Millipore, Bedford, MA, USA). The deionized water was used for all solutions and dilutions. Active pharmaceutical ingredients of edaravone and taurine were provided by Nanjing Yoko Pharmaceutical Co., Ltd. and dissolved in water for prior administration to obtain the following solutions: a mixed solution containing 1 mg/mL edaravone and 10 mg/mL taurine, edaravone solution (1 mg/mL) and taurine solution (10 mg/mL).

2.2. Animals

Male Sprague-Dawley rats (220 ± 20 g) were obtained from the Laboratory Animals Center of Xuzhou Medical College. They were kept in an environmentally controlled breeding room for 7 days before starting the experiments and fed with standard laboratory food and water. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals and the related ethical regulations of the Laboratory Animals Center of Xuzhou Medical College. The study was also approved by the Animal Ethics Committee.

2.3. Instrumentation, chromatographic and mass spectrometric conditions

The analysis was performed using an Agilent 1260 series HPLC and an Agilent 6460 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, MA, USA).

For the quantitative analysis of edaravone or taurine, the HPLC separation was respectively performed on an Atlantis HILIC Silica column (150 mm × 2.1 mm, 3 μm, Waters, Ireland) using acetonitrile–water with 5 mM ammonium formate and 0.2% formic acid as mobile phase at 0.3 mL/min for taurine and an Eclipse Plus C_{18} column (100 mm × 2.1 mm, 3.5 μm, Agilent, MA, USA) using methanol–water with 5 mM ammonium formate and 0.02% formic acid as mobile phase at 0.4 mL/min for edaravone. Detailed settings of mass spectrometry and liquid chromatography conditions in this work were described in Table 1.

For the metabolic study, the HPLC separation was performed on an Atlantis HILIC Silica column (150 mm × 2.1 mm, 3 μm, Waters, Ireland). The mobile phase was composed of 95% acetonitrile water solution (A) and 50% acetonitrile water solution (B) (both containing 0.1% formic acid and 10 mM ammonium formate) at a flow rate of 0.2 mL/min in the following gradient elution mode: 0–10 min, 1% B; 10–11 min, 5% B; 11–30 min, 10% B; 30–60 min, 40% B; 60–70 min, 80% B; and 70–80 min, 1% B. The column temperature was set at 35 °C. The injected volume was 2 μL and the autosampler was set at 4 °C. The mass spectra were performed in a positive mode. Full scan data acquisition was performed from m/z 50 to 1000 in an MS scan mode. The capillary voltage was 3.0 kV and the ion spray temperature was 300 °C. The drying gas flow rate was 10 L/min and the nebulizer pressure was 38 Psi.

2.4. Preparation of standard and quality control samples

Stock solution of edaravone or taurine was separately prepared by dissolving 5 mg edaravone or 100 mg taurine in 5 mL methanol or water to give the final concentration of 1 and 20 mg/mL, respectively. A series of working standard solutions of edaravone or taurine were obtained by diluting mixed standard solution with methanol or 50% acetonitrile at appropriate concentrations. The IS stock solution of edaravone or taurine was prepared at a final concentration of 2 μg/mL for 3-methyl-1-p-tolyl- 5-pyrazolone and 800 μg/mL for sulfanilic acid in methanol or water, respectively.

The calibration standard samples were prepared by spiking 20 μL of working standard solutions into 100 μL blank biological sample to yield various concentrations of edaravone and taurine. Effective concentrations of edaravone in calibration standard samples were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 μg/mL for urine, bile and feces. Effective concentrations of taurine in calibration standard samples were 2, 5, 10, 20, 50, 100, 200, 500 μg/mL for urine, 1, 5, 10, 20, 50, 100, 200, 500 μg/mL for bile, 1, 2.5, 5, 10, 25, 50, 100, 250 μg/mL for feces. The quality control (QC) samples containing low, medium and high concentrations were prepared at the concentrations of edaravone (0.02, 0.2, 0.8 μg/mL for urine, bile and feces)

Table 1
Summary of the two LC-MS/MS conditions used for the determination of edaravone or taurine in rat urine, bile and feces.

Analytes	Edaravone			Taurine		
Sample	Urine	Feces	Bile	Urine	Feces	Bile
	HPLC conditions					
Column	Eclipse Plus C_{18} column (100 mm × 2.1 mm, 3.5 μm, Agilent Technologies, MA, USA)			Atlantis HILIC Silica column (150 mm × 2.1 mm, 3 μm, Waters, Ireland)		
Mobile phase	Methanol–water with 5 mM ammonium formate and 0.02% formic acid (55:45, v/v)	Methanol–water with 5 mM ammonium formate and 0.02% formic acid (70:30, v/v)		Acetonitrile–water with 5 mM ammonium formate and 0.2% formic acid (75:25, v/v)		
Flow rate	0.4 mL/min			0.3 mL/min		
Injection volume	2 μL			1 μL		
Column temperature		30 °C				
Autosampler temperature		4 °C				
	MS/MS conditions					
Internal standards (IS)	3-methyl-1-p-tolyl-5-pyrazolone			Sulfanilic acid		
Capillary voltage		3.5 kV				
Source temperature		300 °C				
Drying gas flow rate (N_2)		10 L/min				
Nebulizer pressure		38 psi				
Detect mode		negative ion mode				
Collision voltage (N_2) precursor-production pairs	30 v for edaravone and its IS m/z 173.1→92.2 for edaravone, m/z 187.2 → 106.0 for IS			17 v for taurine; 25 v for IS m/z 124.1 → 80.0 for taurine, m/z 172.0 → 80.0 for IS		

and taurine (2.5, 25, 250 μg/mL for urine and bile, 2, 20, 200 μg/mL for feces).

2.5. Sample processing

Referring to our previous study [19], 3-methyl-1-p-tolyl-5-pyrazolone and sulfanilic acid were respectively selected as the IS of edaravone and taurine and satisfactory results were obtained. It has been reported that the most suitable pH value for edaravone to keep stable is 3.0–4.5 whether in water solution or plasma [19–21]. The improved and favorable stability of edaravone for subsequent biomedical analysis was found when 20% formic acid was added into rat urine, bile and feces.

2.5.1. Urine sample

For the determination of edaravone, a two-step extraction was performed for urine samples. 100 μL urine sample was spiked with 20 μL IS, 10 μL 20% formic acid solution, 20 μL water (For QC or calibration samples, water was substituted with 20 μL edaravone standard solution). Then the mixture was vortexed for 5 s and extracted with 100 μL ethyl acetate by shaking on a vortex-mixer for 2 min at room temperature. After centrifugation at 4000 rpm for 10 min, the upper organic layer was transferred into another set of clean tube, and the lower layer was extracted again with 100 μL ethyl acetate. The two organic extracts were incorporated and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of 0.1% formic acid-methanol (80:20, v/v), and transferred to an autosampler vial after centrifugation at 14,000 rpm for 10 min. An aliquot of 2 μL was used for LC-MS/MS analysis.

For taurine, 100 μL urine sample was spiked with 20 μL IS and 20 μL water (For QC or calibration samples, water was substituted with 20 μL taurine standard solution). The mixture was vortexed, after which, 200 μL acetonitrile was added and further mixed vigorously for 2 min. After centrifugation at 14,000 rpm for 10 min, the supernatant (100 μL) was diluted with 900 μL 50% acetonitrile. An aliquot of 1 μL was used for LC-MS/MS analysis.

2.5.2. Bile sample

For the determination of edaravone or taurine, 100 μL bile sample was added 20 μL IS of edaravone or taurine, 10 μL 20% formic acid solution (just for edaravone), 20 μL water (For QC or calibration samples, water was substituted with 20 μL edaravone or taurine standard solution). Then the mixture was vortexed and 200 μL acetonitrile containing 0.1% formic acid was added and further mixed vigorously for 2 min before centrifugation at 14,000 rpm for 10 min. The supernatant (2 μL for the determination of edaravone, 1 μL for taurine) was injected into the LC-MS/MS system for analysis.

2.5.3. Feces sample

The feces samples were lyophilized and then pulverized in a mortar and pestle. Physiological saline was added into the pulverized feces sample in the ratio of 4:1 (v/w) and homogenized. The obtained feces homogenate was processed in a similar manner as the bile samples.

2.6. Method validation

The method was validated for specificity, linearity, precision, accuracy, extraction recovery, matrix effect and stability referring to the guidance of the Food and Drug Administration (FDA) for validation of bioanalytical methods.

2.6.1. Specificity

The specificity of the method was determined by measuring the level of interfering components in six individual sources of blank biological matrix, for the exclusion of any endogenous co-eluting interference at the peak region of the analytes or IS.

2.6.2. Linearity and LLOQ

The linearity of the assay was assessed based on the relationship between the ratios of the peak area of analytes to that of IS and the theoretical concentration of analytes. The blank matrix was from pooled sample of 10 control rats. The calibrated peak area of taurine was the measured peak area minus the basal level in biomatrix, whereas for edaravone, the detected peak area just

represents the real value. The results were fitted to linear regression analysis using $1/x^2$ as weighting factor. Linearity of the method in each biological matrix was determined in five replicates of calibration standards at eight concentrations whereby a correlation coefficient ($r^2 \geq 0.99$) was considered satisfactory. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with acceptable accuracy within ±20% and precision below 20%.

2.6.3. Precision and accuracy

The intra- and inter-day precision and accuracy were evaluated by determining the edaravone or taurine concentration in five replicates of QC samples at three concentrations levels within a day or on three consecutive days. Precision was expressed as relative standard deviations (RSD, %) of measured concentration and accuracy values were calculated as accuracy (%) = (measured concentration/nominal concentration) × 100. The accepted values used for validation of accuracy were within ±15% and precision less than 15%.

2.6.4. Extraction recovery and matrix effect

Recoveries were evaluated by analyzing five replicates of QC samples at three concentration levels and calculating the ratio of the peak area of edaravone, taurine and their IS spiked in blank urine, bile and feces samples before extraction against post-extraction spiked edaravone, taurine and respective IS at corresponding concentration, which is also named the relative recovery.

The matrix effects of analytes were measured by comparing the standard analyte peak dissolved with blank matrix extract against those dissolved with mobile phase at three QC concentration levels. Each level was measured in five replicates. It was usually believed that a 15% deviation on a matrix effect could be a meaningful limit [22]. The extraction recovery and matrix effect of IS for urine, bile and feces samples at concentration of 800 μg/mL for sulfanilic acid, 2 μg/mL for 3-methyl-l-*p*-tolyl-5-pyrazolone were also evaluated using the same procedure.

2.6.5. Stability

The stabilities of analytes and IS in rat biological samples were investigated from QC samples at the three concentrations mentioned previously using five replicates for each concentration.

Freeze and thaw stability: the QC samples were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. After three cycles, the concentrations of edaravone or taurine were determined and the concentrations with those obtained before freezing.

Short-term temperature stability: the QC samples were thawed at room temperature and kept at room temperature for 8 h prior to analysis.

Long-term stability: the QC samples were kept at −20 °C for 15 days and thawed at room temperature prior to analysis.

Post-preparative stability: the post-preparative stability was conducted by repeatedly determining the processed QC samples which were kept in the autosampler (4 °C) for 7 days. The results were expressed as the percentage of initial content of edaravone or taurine in the freshly treated samples.

2.7. Application of the assay to the excretion and metabolism study

The rats were respectively administrated a single injection of edaravone at 1 mg/kg, taurine at 10 mg/kg or mixture (containing 1 mg/mL edaravone and 10 mg/mL taurine) at 11 mg/kg into the tail vein. For the urine and feces collection, six rats were then individually placed in a stainless-steel metabolic cage that allowed for the separate collection of urine and feces. The total quantities of excreted urine and feces samples were respectively collected from each rat at 0–2, 2–4, 4–8, 8–12, 12–24, 24–36 and 36–48 h. The fecal samples were collected and homogenized with physiological saline solution. Samples were stored at −20 °C until analysis. For the bile collection, six rats received a surgery under anaesthesia condition for cannulation of a polyethylene catheter with the bile fistulas. The bile was respectively collected at 0–2, 2–4, 4–6, 6–8, 8–12 and 12–16 h, the bile volumes were respectively calculated and then stored at −20 °C until analysis. The control samples were obtained from the rats received equal volume water.

The urine and bile samples were also used for metabolism studies. Sample was processed with acetonitrile (prechilled at 4 °C) at the ratio of 1:2 (v/v). After immediate oscillation for 2 min, the resultant mixtures were centrifuged at 14,000 rpm at 4 °C for 10 min. Then the supernatant was transferred to a sampler vial for LC-MS/MS analysis. The total ion chromatogram (TIC) was recorded. By comparing the TIC of blank urine and bile samples with that of various time periods' biological samples, the peak of metabolite was marked and further analyzed by MS/MS. The structure of metabolite was tentatively speculated according to the MS/MS spectra, literature data and metabolism rule.

3. Results and discussion

3.1. Optimization of MS conditions

Referring to previous study [12,18,19], good responses were obtained in positive ionization mode for edaravone and its IS, and negative ionization mode for taurine and its IS. In this study, the MS and MS/MS characterization of edaravone, taurine and their IS were carefully investigated by direct injection of the standard solution to respectively ascertain their precursor ions, select product ions in MRM, and optimize the MS parameters. The results showed that negative ion mode provided clean chromatographs compared with positive ion mode, and no significant interference was observed, especially for edaravone and its IS. Therefore, the deprotonated molecular ion $[M-H]^-$ of taure and sulfanilic acid (IS) were respectively at *m/z* 124.1 and *m/z* 172.0. Their product ion spectra all yielded high abundance fragment ions $[SO_3]^-$ at *m/z* 80.0 (Fig. 1a and b). On the other hand, the most abundant ions were deprotonated molecular ion $[M-H]^-$ *m/z* 173.1 and 187.2, high abundance fragment ions at *m/z* 92.2 and 106.0 for edaravone and IS (Fig. 1c and d), respectively. MS parameters such as the ESI source temperature, capillary voltage, nebulizer pressure, drying gas flow, drying gas temperature and collision energy were optimized to obtain the optimum response of analytes and listed in Table 1.

3.2. Optimization of chromatography conditions

Chromatographic conditions were optimized to obtain good separation, high sensitivity, good peak shape and short retention time. Our group [12,19] had developed the simultaneous determination of edaravone and taurine in beagle or rat plasma by LC-MS/MS. After lots of trials basing on our published methods, it was found that achieving simultaneous determination of edaravone and taurine in excretion samples was difficult because of big difference of their polarities and interference of endogenous substances. The high polarities of taurine and its IS resulted in their poor retention on conventional RP C_{18} or C_8 column with the capacity factor (k') less than 0.8 in isocratic mobile phase. Thus, Atlantis HILIC Silica column (150 mm × 2.1 mm, 3 μm) and Eclipse

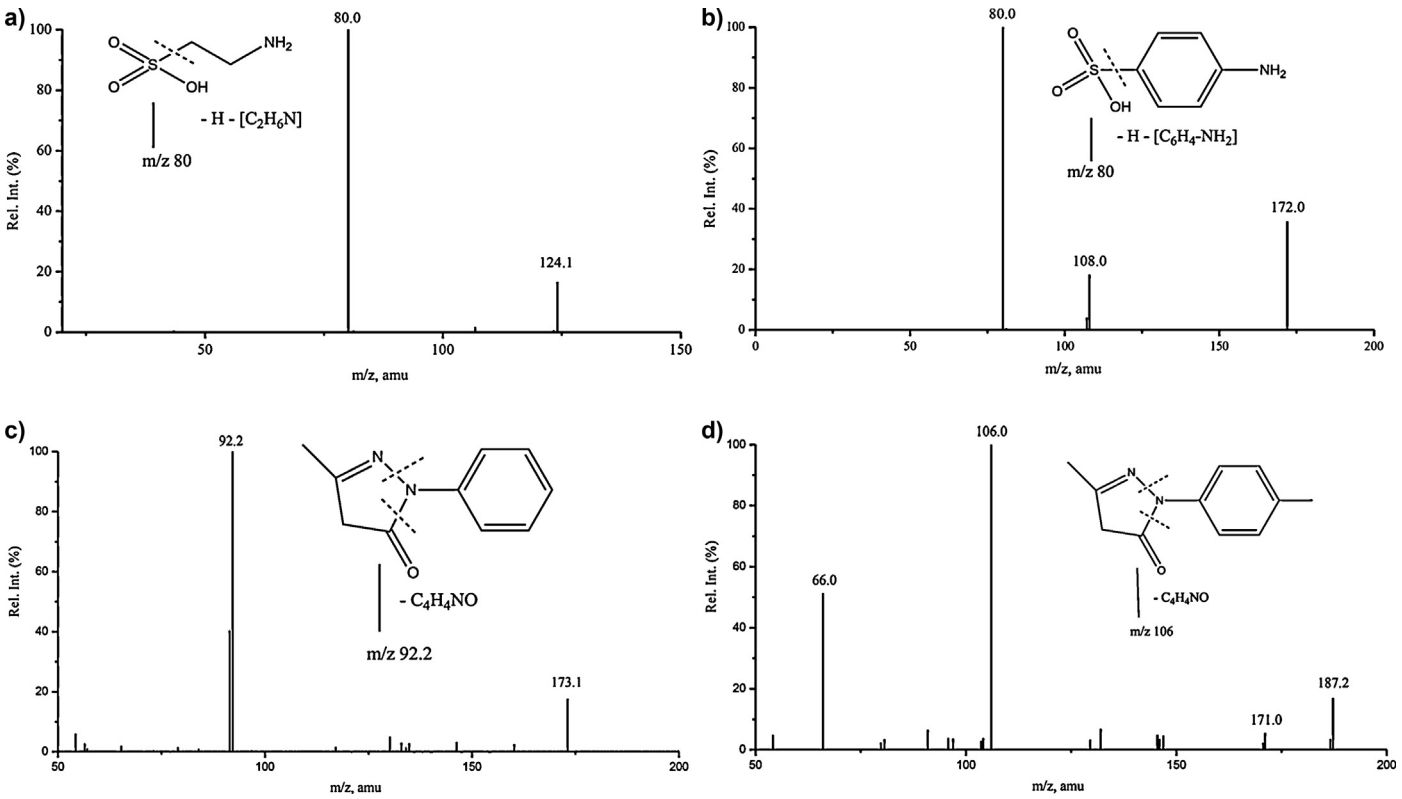

Fig. 1. Product ion scan spectra of $[M-H]^-$ of taurine (a) and sulfanilic acid (b), edaravone (c) and 3-methyl-1-p-tolyl-5-pyrazolone (d).

Plus C_{18} column (100 mm × 2.1 mm, 3.5 μm) column was respectively selected to separate taurine or edaravone. In the HILIC mode, acetonitrile was the weak solvent and water was the strong solvent. Strong retention can be achieved by increasing the ratio of acetonitrile, and acetonitrile-rich mobile phase can improve the sensitivity of ESI-MS [18,23]. In this work, good retention, high sensitivity and weak matrix effect were obtained when taurine was separated on an Atlantis HILIC Silica column using 75% acetonitrile as mobile phase. For the RP mode, good retention, high sensitivity and weak matrix effect were obtained when edaravone and its IS were analyzed on an Eclipse Plus C_{18} column using methanol as mobile phase.

In LC-MS/MS analysis, formic acid and ammonium formate are usually selected as addition in mobile phase for the sake of obtaining good peak shape and ion response. In this work, the concentration of formic acid and ammonium formate in mobile phase was investigated. For HILIC-MS/MS analysis of taurine and its IS, the concentrations of formic acid in aqueous phase were respectively set at 0.1%, 0.2% and 0.3% (v/v), whereas the concentrations of ammonium formate in aqueous phase were respectively set at 0, 5, and10 mM. Good results were obtained when 0.2% (v/v) formic acid and 5 mM ammonium formate were selected as additions. For the RPLC-MS/MS analysis of edaravone and its IS, the concentrations of formic acid in aqueous phase were respectively set at 0.01%, 0.02% and 0.03% (v/v), whereas the concentrations of ammonium formate in aqueous phase were respectively set at 0, 5 and 10 mM. At last, 0.02% (v/v) formic acid and 5 mM ammonium formate were the optimal choice.

3.3. Method validation

For specificity, edaravone, taurine and IS of rat urine, bile and feces could be detected on their own selected ion chromatograms without any significant interference from endogenous substances observed at the retention time of each analyte. Representative chromatograms for the analysis of taurine were presented using drug-free rat urine (Fig. 2a), LLOQ of taurine (2 μg/mL) (Fig. 2b) and rat urine during 0–2 h min after intravenous administration of 10 mg/kg taurine (Fig. 2c), whereas representative chromatograms for the analysis of edaravone were presented using (d) drug-free rat plasma, (e) LLOQ of edaravone (0.01 μg/mL) and (f) rat urine during 0–2 h after intravenous administration of 1 mg/kg edaravone.

The regression equation of calibration curves, their correlation coefficients (r^2), linear ranges and LLOQs obtained from typical calibration curves of urine, bile and feces were shown in Table 2. The correlation coefficients were greater than 0.9930 in all matrices. Those results revealed that the developed methods possessed good linearity in the entire analyzed concentration range for edaravone and taurine in rat urine, bile and feces.

The results of precision and accuracy measurements were assessed by analyzing QC samples at three concentrations presented in Table 3. Both the intra- and inter-day precision in different matrices was all less than 15%. The intra-day precision (RSD) ranged from 1.2 to 16.6% and the inter-day precision (RSD) from 3.7 to 14.2%. The intra-day accuracy ranged from 90.64 to 112.40% and the inter-day accuracy from 95.11to 106.72%. The data indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

The relative recoveries of edaravone, taurine and their corresponding IS in urine, bile and feces samples were summarized in Table 4. The results showed that the recoveries of edaravone, taurine and their corresponding IS in all these matrices were in the range of 69.21–83.77%, 71.50–85.39%, 71.26–87.87% and 76.30–89.02 respectively. These data indicated that extraction recovery of the method was consistent, reproducible, and acceptable.

The matrix effects of the analytes for three different QC levels in urine, bile and feces were listed in Table 5. The

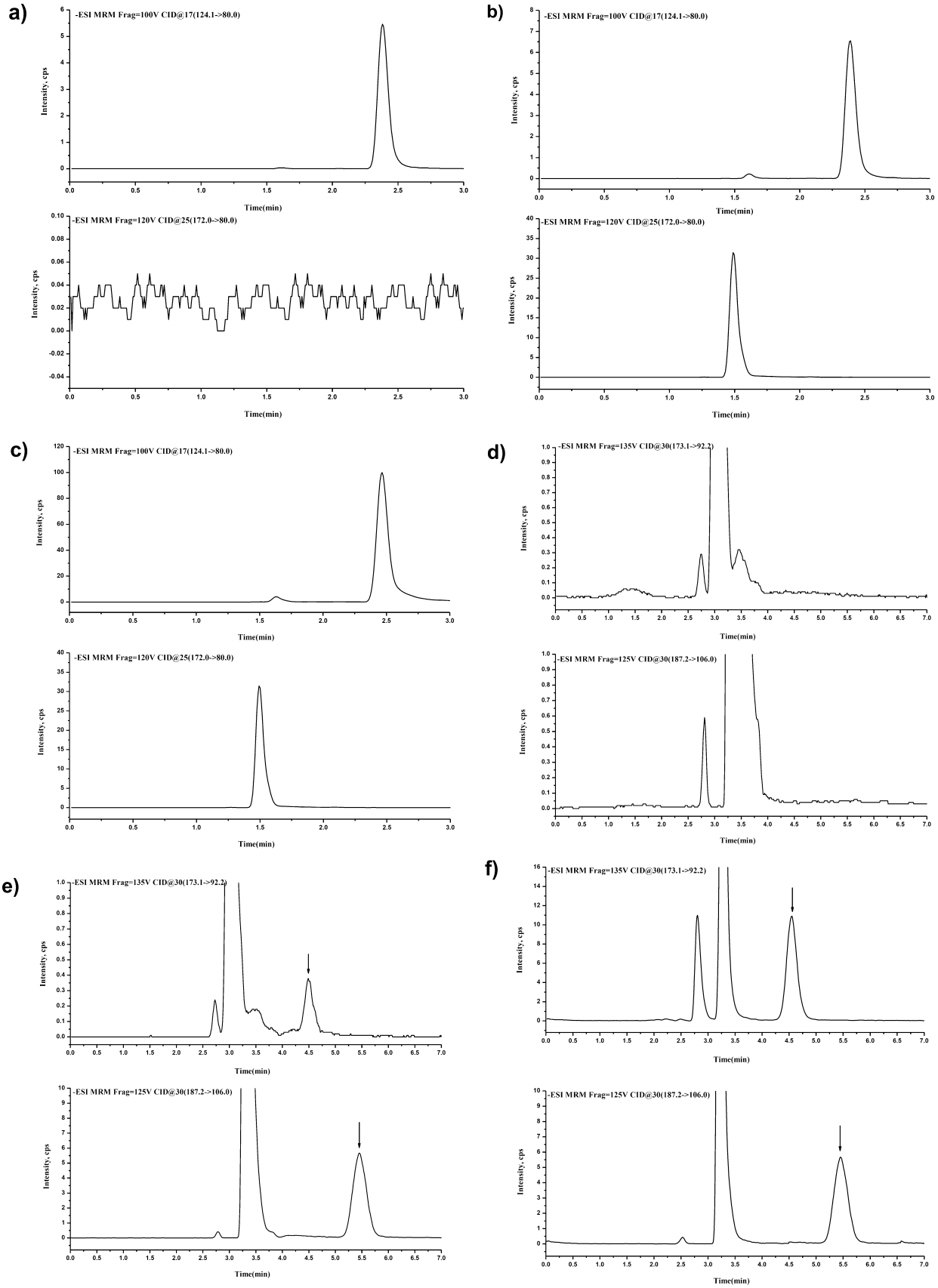

Fig. 2. The representative MRM (-) chromatograms of (a) drug-free rat urine, (b) LLOQ of taurine (2 μg/mL), (c) rat urine during 0–2 h min after intravenous administration of 10 mg/kg taurine, (d) drug-free rat plasma, (e) LLOQ of edaravone (0.01 μg/mL) and (f) rat urine during 0–2 h after intravenous administration of 1 mg/kg edaravone. The pane of (a), (b) and (c) from top to bottom respectively represents taurine (m/z 124.1→80.0) and sulfanilic acid (*m*/*z* 172.0 → 80.0), whereas the pane of (d), (e) and (f) from top to bottom respectively represents edaravone (*m*/*z* 175.1→133.0) and 3-methyl-1-p-tolyl-5-pyrazolone (*m*/*z* 189.2→147.0).

Table 2
Linear regression data, linear ranges and LLOQs of edaravone and taurine in rat urine, bile and feces (8 calibration points, 5 curves).

Biological sample	Compounds	Mean regression equation	r^2	Linear range/(μg/mL)	LLOQ/(μg/mL)
Urine	Edaravone	$y = 5.4075x - 0.0241$	0.9930	0.01–1	0.01
	Taurine	$y = 0.0091x + 0.0213$	0.9980	2–500	2
Bile	Edaravone	$y = 1.3483x + 0.0001$	0.9934	0.01–1	0.01
	Taurine	$y = 0.0234x + 0.0198$	0.9970	1–500	1
Feces	Edaravone	$y = 4.5604x - 0.0286$	0.9932	0.01–1	0.01
	Taurine	$y = 0.0178x + 0.0017$	0.9970	1–250	1

Table 3
Precision and accuracy summary for the analysis of edaravone and taurine in rat urine, bile and feces.

Biological sample	Compounds	Nominal concentration (μg/mL)	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
			Found (μg/mL)	Accuracy (%)	Precision (%)	Found (μg/mL)	Accuracy (%)	Precision (%)
Urine	Edaravone	0.02	0.0189 ± 0.0008	94.64 ± 3.93	4.2	0.0203 ± 0.0016	101.28 ± 8.00	8.0
		0.2	0.2136 ± 0.0059	106.82 ± 2.96	2.8	0.2056 ± 0.0199	102.81 ± 9.94	9.7
		0.8	0.7776 ± 0.0158	97.20 ± 1.98	2.1	0.7937 ± 0.0286	99.22 ± 3.58	3.7
	Taurine	2.5	2.48 ± 0.41	99.28 ± 16.41	16.6	2.47 ± 0.35	98.67 ± 13.96	14.2
		25	25.37 ± 2.33	101.46 ± 9.31	9.2	24.93 ± 1.68	99.72 ± 6.73	6.8
		250	231.72 ± 3.30	92.69 ± 1.32	1.5	253.43 ± 17.58	101.37 ± 7.03	7.0
Bile	Edaravone	0.02	0.0215 ± 0.0017	107.53 ± 8.49	7.9	0.0201 ± 0.0022	100.33 ± 10.96	11.0
		0.2	0.2117 ± 0.0054	105.87 ± 2.68	2.6	0.2106 ± 0.0083	105.28 ± 4.16	4.0
		0.8	0.8590 ± 0.0103	107.38 ± 1.28	1.2	0.8051 ± 0.0565	100.64 ± 7.06	7.1
	Taurine	2.5	2.80 ± 0.22	111.84 ± 8.94	8.0	2.67 ± 0.27	106.72 ± 10.81	10.2
		25	27.10 ± 0.50	108.40 ± 2.00	1.9	26.32 ± 1.42	105.26 ± 5.68	5.4
		250	226.59 ± 3.05	90.64 ± 1.22	1.4	242.50 ± 18.74	97.00 ± 7.50	7.8
Feces	Edaravone	0.02	0.0211 ± 0.0007	105.56 ± 3.39	3.3	0.0208 ± 0.0010	104.10 ± 5.14	5.0
		0.2	0.2136 ± 0.0059	106.81 ± 2.96	2.8	0.2062 ± 0.0105	103.12 ± 5.27	5.2
		0.8	0.8643 ± 0.0121	108.03 ± 1.52	1.5	0.8355 ± 0.0525	104.43 ± 6.56	6.3
	Taurine	2	2.25 ± 0.16	112.40 ± 7.84	7.0	2.03 ± 0.24	101.60 ± 11.89	11.7
		20	19.54 ± 0.97	97.69 ± 4.84	5.0	19.68 ± 1.58	98.41 ± 7.88	8.1
		200	187.32 ± 7.41	93.66 ± 3.71	4.0	190.22 ± 13.09	95.11 ± 6.55	6.9

Table 4
The relative recoveries for edaravone and taurine and IS ($n = 5$).

Compounds	Urine			Bile			Feces		
	Concentration (μg/mL)	Mean ± SD	RSD (%)	Concentration (μg/mL)	Mean ± SD	RSD (%)	Concentration (μg/mL)	Mean ± SD	RSD (%)
Edaravone	0.02	78.83 ± 1.94	2.5	0.02	83.77 ± 7.63	9.2	0.02	69.21 ± 2.46	3.6
	0.2	77.18 ± 3.13	4.1	0.2	76.05 ± 2.75	3.7	0.2	73.83 ± 1.05	1.5
	0.8	74.20 ± 0.58	0.8	0.8	76.88 ± 1.54	2.1	0.8	71.13 ± 0.14	0.2
IS	2	77.79 ± 3.64	4.7	2	87.87 ± 5.22	6	2	71.26 ± 3.55	5
Taurine	2.5	75.51 ± 0.90	1.2	2.5	81.96 ± 1.27	1.6	2	76.08 ± 3.06	4.1
	25	75.23 ± 2.74	3.7	25	74.46 ± 1.81	2.5	20	83.15 ± 4.48	5.4
	250	71.50 ± 0.47	0.7	250	75.16 ± 2.35	3.2	200	85.39 ± 8.24	9.7
IS	800	76.30 ± 0.57	0.8	800	89.02 ± 1.04	1.2	250	82.31 ± 3.84	4.7

matrix effects for analytes and IS, calculated according to Section 2.6.4, were ranged from 85.80% to 108.11%. The results demonstrated that no significant matrix effect for analytes and IS was observed, indicating that the ionization competition between the analyte and the endogenous co-elution was negligible.

The results of the stability study showed there was no obvious substance loss under the conditions that biological samples might experience during this study, and these were summarized in Table 6. In addition, the extracted samples were stable in the autosampler at 4 °C for 7 days, and the accuracy and precision were within 15%.

Table 5
The matrix effects for edaravone and taurine and IS ($n = 5$).

Compounds	Urine			Bile			Feces		
	Concentration (μg/mL)	Mean ± SD	RSD (%)	Concentration (μg/mL)	Mean ± SD	RSD (%)	Concentration (μg/mL)	Mean ± SD	RSD (%)
Edaravone	0.02	108.11 ± 4.13	3.9	0.02	92.89 ± 8.08	8.7	0.02	103.49 ± 11.11	10.8
	0.2	95.09 ± 2.20	2.4	0.2	94.64 ± 11.22	11.9	0.2	92.02 ± 9.83	10.7
	0.8	89.33 ± 1.51	1.7	0.8	87.64 ± 12.39	14.2	0.8	91.33 ± 4.95	5.5
IS	2	91.16 ± 7.54	8.3	2	85.80 ± 6.72	7.9	2	92.37 ± 7.88	8.6
Taurine	2.5	91.79 ± 5.85	6.4	2.5	103.95 ± 7.53	7.3	2	86.05 ± 4.77	5.6
	25	88.71 ± 8.19	9.3	25	91.26 ± 6.60	7.3	20	94.97 ± 6.29	6.7
	250	93.84 ± 4.58	4.9	250	87.83 ± 8.50	9.7	200	95.66 ± 3.74	4.0
IS	800	100.25 ± 7.04	7.1	800	89.51 ± 3.07	3.5	250	87.29 ± 9.64	11.1

Table 6
The stability of edaravone and taurine in rat urine, bile and feces at different QC levels ($n = 3$).

Compounds	Storage condition	Urine			Bile				Feces			
		Added (μg/mL)	Detected (μg/mL)	Accuracy (%)	RSD (%)	Added (μg/mL)	Detected (μg/mL)	Accuracy (%)	RSD (%)	Added (μg/mL)	Detected (μg/mL)	Accuracy (%)	RSD (%)
Edaravone	Freeze-thaw (2 cycles)	0.02	0.0202 ± 0.0029	100.88 ± 14.36	14.3	0.02	0.0200 ± 0.0016	100.24 ± 8.05	8.1	0.02	0.0213 ± 0.0012	106.26 ± 6.14	5.8
		0.2	0.2047 ± 0.0231	102.37 ± 11.53	11.3	0.2	0.2086 ± 0.0143	104.29 ± 7.13	6.9	0.2	0.2046 ± 0.0105	102.32 ± 5.25	5.2
		0.8	0.8096 ± 0.0222	101.20 ± 2.78	2.8	0.8	0.8162 ± 0.0269	102.03 ± 3.36	3.3	0.8	0.8290 ± 0.0354	103.63 ± 4.42	4.3
	Short-term (8 h, 25 °C)	0.02	0.0225 ± 0.0005	112.26 ± 2.56	2.3	0.02	0.0205 ± 0.0013	102.56 ± 6.26	6.2	0.02	0.0197 ± 0.0017	98.32 ± 8.29	8.5
		0.2	0.2204 ± 0.0106	110.21 ± 5.30	4.9	0.2	0.1990 ± 0.0042	99.51 ± 2.09	2.2	0.2	0.2075 ± 0.0050	103.75 ± 2.48	2.4
		0.8	0.8287 ± 0.0215	103.59 ± 2.69	2.6	0.8	0.8194 ± 0.0282	102.42 ± 3.52	3.5	0.8	0.8594 ± 0.0197	107.42 ± 2.46	2.3
	Long-term (15 d, -20 °C)	0.02	0.0199 ± 0.0020	99.64 ± 9.94	10.0	0.02	0.0200 ± 0.0017	100.06 ± 8.54	8.6	0.02	0.0191 ± 0.0021	95.46 ± 10.64	11.2
		0.2	0.1934 ± 0.0159	96.70 ± 7.96	8.3	0.2	0.2152 ± 0.0110	107.60 ± 5.51	5.2	0.2	0.2037 ± 0.0123	101.85 ± 6.15	6.1
		0.8	0.7986 ± 0.0451	99.83 ± 5.63	5.7	0.8	0.7866 ± 0.0720	98.33 ± 9.00	9.2	0.8	0.8127 ± 0.0535	101.59 ± 6.68	6.6
	Auto-sampler (7 d, 4 °C)	0.02	0.0200 ± 0.0022	99.90 ± 11.10	11.2	0.02	0.0203 ± 0.0020	101.35 ± 10.05	10.0	0.02	0.0199 ± 0.0020	99.72 ± 10.21	10.3
		0.2	0.1947 ± 0.0167	97.37 ± 8.35	8.6	0.2	0.2123 ± 0.0122	106.17 ± 6.09	5.8	0.2	0.2041 ± 0.0117	102.06 ± 5.87	5.8
		0.8	0.8037 ± 0.0362	100.46 ± 4.53	4.6	0.8	0.7984 ± 0.0275	99.80 ± 3.44	3.5	0.8	0.8072 ± 0.0435	100.90 ± 5.43	5.4
Taurine	Freeze-thaw (3 cycles)	2.5	2.65 ± 0.24	105.93 ± 9.61	9.1	2.5	2.59 ± 0.31	103.56 ± 12.46	12.1	2	2.05 ± 0.25	102.52 ± 12.62	12.4
		25	25.83 ± 1.70	103.32 ± 6.80	6.6	25	25.89 ± 1.12	103.54 ± 4.48	4.4	20	20.47 ± 1.79	102.36 ± 8.97	8.8
		250	254.74 ± 22.89	101.90 ± 9.15	9.0	250	244.60 ± 15.20	97.84 ± 6.08	6.3	200	195.28 ± 11.74	97.64 ± 5.87	6.1
	Short-term (8 h, 25 °C)	2.5	2.47 ± 0.27	98.77 ± 10.62	10.8	2.5	2.50 ± 0.36	100.18 ± 14.47	14.5	2	2.02 ± 0.17	101.18 ± 8.66	8.6
		25	26.57 ± 0.98	106.26 ± 3.93	3.8	25	26.24 ± 1.20	104.95 ± 4.81	4.6	20	19.26 ± 0.80	96.30 ± 4.01	4.2
		250	223.58 ± 6.67	89.43 ± 2.67	3.0	250	234.65 ± 8.45	93.86 ± 3.38	3.7	200	188.18 ± 15.30	94.09 ± 7.65	8.2
	Long-term (15 d, -20 °C)	2.5	2.59 ± 0.26	103.76 ± 10.40	10.1	2.5	2.55 ± 0.33	102.06 ± 13.37	13.2	2	2.05 ± 0.25	102.25 ± 12.36	12.1
		25	24.79 ± 1.93	99.17 ± 7.74	7.8	25	25.26 ± 1.49	101.02 ± 5.97	6.0	20	20.26 ± 1.03	101.30 ± 5.13	5.1
		250	253.19 ± 21.94	101.28 ± 8.78	8.7	250	239.50 ± 12.85	95.80 ± 5.14	5.4	200	193.42 ± 12.56	96.71 ± 6.28	6.5
	Auto-sampler (7 d, 4 °C)	2.5	2.58 ± 0.34	103.13 ± 13.49	13.1	2.5	2.50 ± 0.33	100.16 ± 13.34	13.4	2	1.99 ± 0.23	99.72 ± 11.69	11.8
		25	25.27 ± 1.39	101.08 ± 5.56	5.6	25	26.29 ± 0.98	105.15 ± 3.92	3.8	20	19.90 ± 1.60	99.49 ± 7.99	8.1
		250	259.99 ± 20.20	103.99 ± 8.08	7.8	250	243.22 ± 14.89	97.29 ± 5.96	6.2	200	196.06 ± 17.54	98.03 ± 8.77	9.0

Table 7
Recovery cumulative excretion of edaravone and taurine in urine, bile and feces (%, Mean ± SD, $n = 6$).

Matrix	Time (h)	Edaravone co-administrated taurine		Edaravone alone	Taurine alone
		Edaravone	Taurine		
Urine	0–2	0.16 ± 0.02	9.17 ± 1.73	0.21 ± 0.07	8.31 ± 0.81
	0–4	0.21 ± 0.03	15.29 ± 3.34	0.24 ± 0.06	14.41 ± 1.39
	0–8	0.25 ± 0.03	21.74 ± 4.12	0.27 ± 0.06	20.32 ± 2.43
	0–12	0.26 ± 0.03	26.11 ± 4.77	0.29 ± 0.08	24.94 ± 3.53
	0–24	0.28 ± 0.04	32.25 ± 5.88	0.30 ± 0.07	29.95 ± 5.00
	0–36	0.28 ± 0.04	33.22 ± 5.85	0.30 ± 0.07	30.96 ± 5.48
	0–48	0.28 ± 0.04	33.29 ± 5.88	0.30 ± 0.07	31.06 ± 5.48
Bile	0–2	0.29 ± 0.04	1.01 ± 0.20	0.26 ± 0.09	0.92 ± 0.22
	0–4	0.47 ± 0.11	2.28 ± 0.34	0.39 ± 0.13	2.05 ± 0.41
	0–6	0.52 ± 0.12	3.08 ± 0.41	0.47 ± 0.15	2.96 ± 0.54
	0–8	0.53 ± 0.12	3.54 ± 0.51	0.48 ± 0.15	3.45 ± 0.46
	0–12	0.53 ± 0.12	3.80 ± 0.59	0.48 ± 0.15	3.81 ± 0.47
	0–16	0.53 ± 0.12	3.81 ± 0.59	0.48 ± 0.15	3.86 ± 0.46
Feces	0–2	ND	0.33 ± 0.15	ND	0.20 ± 0.15
	0–4	0.004 ± 0.002	0.81 ± 0.31	0.005 ± 0.002	0.85 ± 0.48
	0–8	0.010 ± 0.005	1.33 ± 0.29	0.014 ± 0.007	1.41 ± 0.26
	0–12	0.011 ± 0.005	1.70 ± 0.44	0.017 ± 0.011	1.74 ± 0.27
	0–24	0.011 ± 0.005	1.80 ± 0.46	0.017 ± 0.011	1.88 ± 0.25

ND, less than the limit of quantification.

3.4. Excretion and metabolism study

The endogenous concentration of taurine was determined by randomly selecting 12 rats and keeping them in an environmentally controlled breeding room, with food and water normally supplied for three days. Then urine and feces were respectively collected at 0–2, 2–4, 4–8, 8–12, 12–24, 24–36 and 36–48 h, while bile was respectively collected at 0–2, 2–4, 4–6, 6–8, 8–12 and 12–16 h on that day and processed as described above. The results showed that the deviation value of taurine concentration in rat urine, bile and feces from the different time periods of intra-individual was within 15% within one day.

The excretion samples of rats that were intravenously administrated edaravone or taurine alone or together were respectively analyzed under corresponding LC-MS/MS methods described above. The concentrations of edaravone and taurine were calculated from their corresponding calibration curves. It should be stressed herein that as taurine is present endogenously, the real concentration of taurine in biomatrix at different time points after administration should be expressed as the measured concentration minus the basal level of biomatrix, whereas for edaravone, the detected concentration just represents the real concentration at different time points after administration. The recovery cumulative excretion of edaravone and taurine in rats' urine,

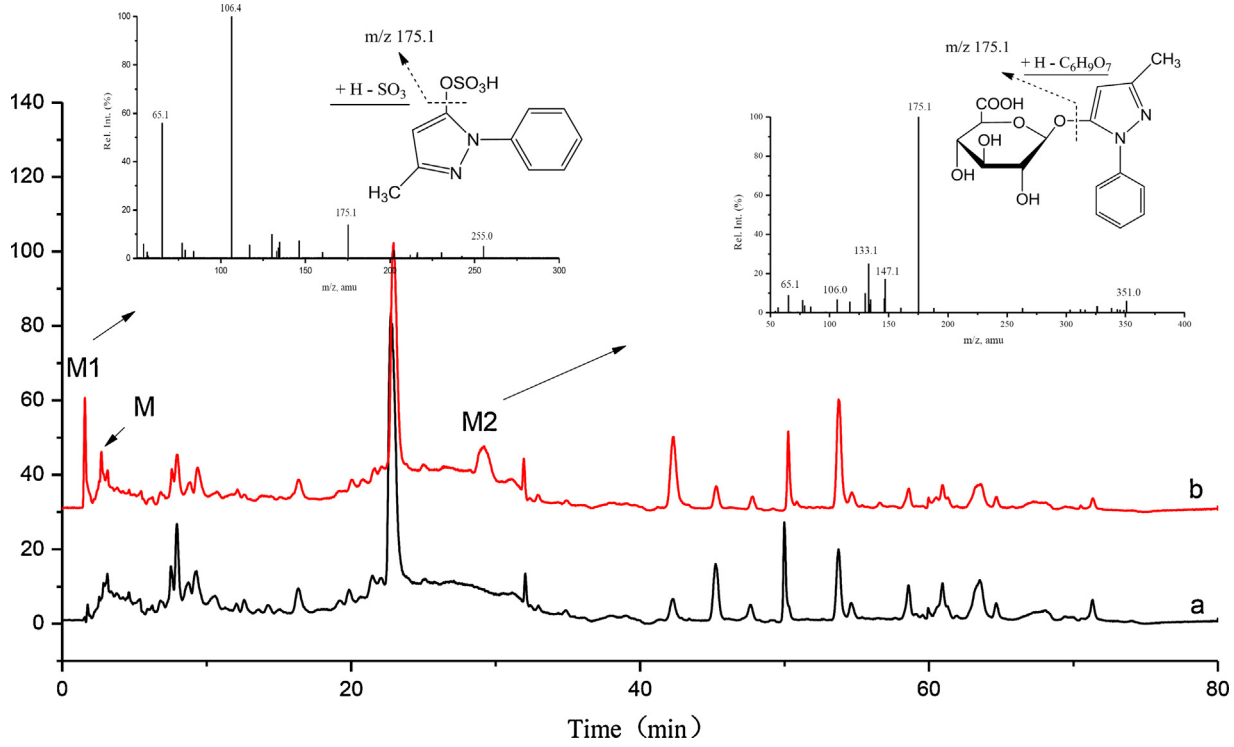

Fig. 3. The representative total ion chromatograms (TIC) of (a) drug-free rat urine, (b) rat urine during 0–2 h after intravenous administration of edaravone injection (1 mg/kg) and the product ion scan spectra of M1 and M2.

bile and feces after intravenous administration were shown in Table 7.

As shown in Table 7, no significant difference between administration alone and co-administration of edaravone and taurine was observed, whether for the pharmacokinetic parameters of edaravone or those of taurine, indicating that there were no pharmacokinetic interactions between both drugs. After independent intravenous administration, 0.28–0.30% of the administered edaravone was excreted in urine within 48 h, 0.48–0.53% was recovered in bile within 16 h, and 0.011–0.017% was recovered in feces within 24 h. Low recoveries of edaravone in urine, bile and feces indicated that few unchanged form of edaravone was excreted by kidney or biliary, suggesting edaravone undergoes extensive hepatic metabolism in rat following intravenous administration. Thus, monitoring of the liver function may become necessary and dose adjustment is needed in patients whose liver metabolic function is impaired. Table 7 also showed that taurine, as unchanged drug, was mainly excreted during 48 h after intravenous administration via urine, 31.06–33.29% of the total amount. The excretion data of taurine in bile and feces indicated that less than 1.80% (3.81–3.86% and 1.80–1.88%, respectively) of the dose administered was excreted as unconverted form. This means the urinary excretion was a major pathway comparing with biliary and fecal excretion.

The metabolism of taurine is dominated by its synthesis via oxidation of the sulfhydryl group of cysteine and the formation of cysteine sulphinic acid, which undergoes oxidation to cysteic acid followed by decarboxylation to taurine, or decarboxylation to hypotaurine followed by oxidation to taurine [24]. It is use to catabolize by forming the bile acid conjugates taurocholate and taurochenodeoxycholate, which can be excreted in the bile. By comparing the chromatograms of blank urine and bile samples to those of urine and bile samples after administration, we did not find any new chromatogram peaks, hinting that the metabolism of taurine had no obvious difference between administration of taurine alone and co-administration.

As it was known from literature, edaravone sulfate (M1) and glucuronate (M2) were the main metabolite of edaravone in biology [25]. In this study, M1 and M2 metabolites were detected in rats' urine and bile after intravenous administration edaravone and co-administration of edaravone and taurine (Fig. 3). No new metabolites were detected in rat urine and bile in the present experiment, indicating that combination of these two drugs did not change the metabolism in urine and bile.

4. Conclusion

In conclusion, two independent and complementary LC-MS/MS methods were respectively developed for the determination of edaravone or taurine in rat bile, urine and feces. Good linearity, precision and accuracy, stability and recovery were consistently achieved. All validated parameters met the criteria set in FDA guidelines for bioanalytical methods. The validated method was also successfully applied to the excretion and metabolism investigation of edaravone and taurine in rat. This assay demonstrated that there were no observable pharmacokinetic and metabolism interaction between edaravone and taurine in rats after separate intravenous administration or with co-administration, implying that combination therapy of them may be viable and pharmacologically feasible.

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