Yan-yan Yu[1,2]**
Xiao-xiao Zheng[2]**
Ting-ting Bian[2]
Yin-jie Li[2]
Xiao-wen Wu[2]
Dong-zhi Yang[1,2]
Shui-shi Jiang[3]*
Dao-quan Tang[1,2]

[1]Department of Pharmaceutical Analysis, Xuzhou Medical College, Xuzhou, Jiangsu, China

[2]Key Laboratory of New Drug and Clinical Application, Xuzhou Medical College, Xuzhou, Jiangsu, China
[3]Nanjing Yoko Pharmaceutical Co. Ltd, Nanjing, Jiangsu, China

Received September 7, 2013
Revised September 30, 2013
Accepted October 2, 2013

Research Article

Development and application of a LC–MS/MS assay for the simultaneous quantification of edaravone and taurine in beagle plasma

An LC–MS/MS method was developed and validated for the simultaneous quantification of edaravone and taurine in beagle plasma. The plasma sample was deproteinized using acetonitrile containing formic acid. Chromatographic separations were achieved on an Agilent Zorbax SB-Aq (100 × 2.1 mm, 3.5 μm) column, with a gradient of water (containing 0.03% formic acid) and methanol as the mobile phase at a flow rate of 0.3 mL/min. The analyte detection was carried out in multiple reaction monitoring mode and the optimized precursor-to-product transitions of m/z $[M+H]^+$ 175.1 → 133.0 (edaravone), m/z $[M+H]^+$ 189.1 → 147.0 (3-methyl-1-*p*-tolyl-5-pyrazolone, internal standard, IS), m/z $[M-H]^-$ 124.1→80.0 (taurine), and m/z $[M-H]^-$ 172.0 → 80.0 (sulfanilic acid, IS) were employed to quantify edaravone, taurine, and their corresponding ISs, respectively. The LOD and the lower LOQ were 0.01 and 0.05 μg/mL for edaravone and 0.66 and 2 μg/mL for taurine, respectively. The calibration curves of these two analytes demonstrated good linearity ($r > 0.99$). All the validation data including the specificity, precision, recovery, and stability conformed to the acceptable requirements. This validated method has successfully been applied in the pharmacokinetic study of edaravone and taurine mixture in beagle dogs.

Keywords: Edaravone / LC–MS/MS / Pharmacokinetics / Taurine
DOI 10.1002/jssc.201300983

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Edaravone (MCI-186, 3-methyl-1-phenyl-pyrazolin-5-one), a newly synthesized antioxidant, has been proven to be capable of inhibiting both nonenzymatic peroxidation and lipoxygenase activity, as well as preventing the oxidative damage of vascular endothelial cells caused by hydroperoxyeicosatetraenoic acid *in vitro* [1–3]. In Japan, this compound has been used for the treatment of patients with acute ischemic stroke since 2001 [4]. In addition to its effect on hydroxyl radical removal, edaravone can also exert protective effects in a number of animal models of disease and tissue damage, including myocardial, lung, intestinal, liver, pancreatic, and renal injury [5]. Qi et al. have shown earlier that infarction volume and hemispheric swelling can be dose-dependently reduced when rats are treated with edaravone, and the effective doses for acute ischemia are from 3 to 10 mg/kg [6]. Hara et al. have also demonstrated the combined effects of normobaric hyperoxia and edaravone on focal cerebral ischemia-induced neuronal damage in mice [7]. These observations suggest that edaravone may be useful for the treatment of several non-neurological diseases and other clinical conditions.

The sulfur-containing amino acid, taurine (2-aminoethanesulphonic acid) is present in high concentration in the central nervous systems of mammals [8]. Recent evidence has suggested that taurine may be involved in various processes, such as modulating neuronal activity, participating in osmoregulation, regulating cellular Ca^{2+} fluxes and affecting protein phosphorylation and membrane ion channels [9–14]. Furthermore, over the past two decades, taurine has also demonstrated to be able to protect the neurons that were exposed to different cell-damaging conditions, including oxidants (such as hypochlorous acid and 1-methyl-4-phenylpyridinium), excitatory amino acids, hypoxia, and ischemic insults [15–17]. The intracellular

Correspondence: Dr. Dao-quan Tang, Department of Pharmaceutical Analysis, Xuzhou Medical College, Xuzhou, Jiangsu 221004, China
E-mail: tdq993@126.com
Fax: +86-0516-83262136

Abbreviations: IS, internal standard; **LLOQ**, lower LOQ; **MRM**, multiple reaction monitoring; **PK**, pharmacokinetics; **QC**, quality control

*Additional corresponding author: Dr. Shui-shi Jiang, E-mail: jiangshuishi@yoko-bio.com

**These authors have contributed equally to this work.

taurine level of myocardial cells in ischemic heart failure and hypoxia has been found to be lower [18], indicating that taurine may possess a neuroprotective effect against focal cerebral ischemia in human.

If combining two low-dose therapies with different neuroprotective actions was able to enhance the target effects and reduce the side effects, such combination therapy will be beneficial for stroke patients [7]. For instance, in clinical practice, puerarin, daio-orengedoku-to, or some other drugs are usually prescribed in combination with edaravone to obtain synergistic effects and diminish adverse reactions [19, 20]. Furthermore, it has been clarified the synergistic effects when combining taurine with edaravone for the treatment of stroke in previous reports, as both of which have proven to be safe and effective therapies against ischemia [21]. However, as far as we know, the pharmakinetics (KP) of edaravone co-administrated with taurine has not been focused on. Moreover, edaravone was metabolically unstable and quickly metabolized by pooled microsomes from noninduced male Sprague-Dawley rats with only 28% of parent remaining after a 30 min incubation period [22]. Thus, an effective sample preparation process devoted to maintain edaravone stable in plasma and a rapid, sensitive analytical method for the simultaneous quantification of edaravone and taurine are essential.

Currently, several analytical methods have been published for the quantification of either edaravone or taurine in biological matrices, such as chromatographic and chemiluminescent methods for edaravone [23–25], and ^{33}S NMR spectroscopy [26], LC with fluorescence detection upon derivatization with *o*-phthalaldehyde [27, 28], dinitrofluorobenzene [29] and fluorescamine [30] for taurine, but not by LC–MS/MS in plasma matrices. LC–MS has become a well-known technology with high specificity and sensitivity for the analysis of chemical components in biological matrices [31]. The availability of various atmospheric pressure ionization (API) methods in both positive and negative modes [e.g., ESI, atmospheric pressure chemical ionization, and atmospheric pressure photoionization] in MS enables the ionization of diverse classes of components. To the best of our knowledge, no published bioanalytical method has described a fully validated LC–MS/MS assay for the simultaneous determination of edaravone and taurine in beagle plasma.

Taken together, the main objectives of this study were (1) to develop a simple, sensitive, and reliable LC–MS/MS method for the simultaneous determination of edaravone and taurine in combination in beagle plasma and (2) to fully validate this method in preclinical PK studies and applied to the PK study of the two-drug combination with a single dose after intravenous administration to beagle dogs.

2 Materials and methods

2.1 Chemicals and reagents

High-purity reference standards including edaravone, taurine, and sulfanilic acid (≥ 98.0%) were all obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China). 3-Methyl-1-*p*-tolyl-5-pyrazolone was bought from Tokyo KaSei Industry (≥ 99%). Methanol of HPLC grade was obtained from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). Formic acid, acetonitrile, and other reagents were of at least analytical grade. Deionized (18 MΩ/cm) water was generated in-house using a Milli-Q water purification system from Millipore (Bedford, MA, USA). Active pharmaceutical ingredients of edaravone and taurine were provided by Nanjing Yoko Pharmaceutical and dissolved in water for injection to obtain a mixed solution at the concentration of 1 mg/mL for edaravone and 10 mg/mL for taurine.

2.2 LC–MS

Samples were analyzed on an Agilent 1260 HPLC–MS/MS system (Agilent Technologies, Santa Clara, CA, USA), consisting of a G1367E autosampler, two G1312B pumps, and a 6460 triple quadrupole mass spectrometer equipped with an ESI source. An Agilent Masshunter workstation was used for data acquisition and processing. For analysis, 2 μL of the extract was injected into the system. Chromatographic separations were carried out on an Agilent Zorbax SB-Aq (100 × 2.1 mm id, 3.5 μm) column with a column temperature set at 30°C. The autosampler temperature was kept at 4°C. Gradient elution was employed during 9 min analysis time using solvent A, 0.03% formic acid in water, and B, methanol, as mobile phases, which started at 10% B, increased linearly to 80% B over 1.0 min, and held constant for 2.0 min, then decreased linearly to 10% B over 3.0 min and held constant for the additional 6.0 min at a flow rate of 0.3 mL/min. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane filter.

All quantifications of analytes were performed in the multiple reaction monitoring (MRM) mode. The ESI source interface operated simultaneously in positive mode for edaravone and internal standard (IS) and negative ionization mode for taurine and IS was used in this study. The electrospray capillary voltage was set to 3.5 kV and the source temperature was set at 300°C. Nitrogen was used as a drying gas for solvent evaporation and the drying gas temperature was kept at 300°C. The optimized precursor-to-product transitions of *m/z* $[M+H]^+$ 175.1 → 133.0 (edaravone), *m/z* $[M+H]^+$ 189.1 → 147.0 (3-methyl-1-*p*-tolyl-5-pyrazolone, IS), *m/z* $[M-H]^-$ 124.1 → 80.0 (taurine) and *m/z* $[M-H]^-$ 172.0 → 80.0 (sulfanilic acid, IS) were employed to quantify edaravone, taurine, and their corresponding IS, respectively, for the highest sensitivity and minimum interference from matrix components (see Fig. 1).

2.3 Preparation of standard and QC samples

Stock solutions of edaravone and taurine were independently prepared by dissolving 5 mg edaravone and 100 mg taurine in 5 mL water to give the final concentration of 1 and

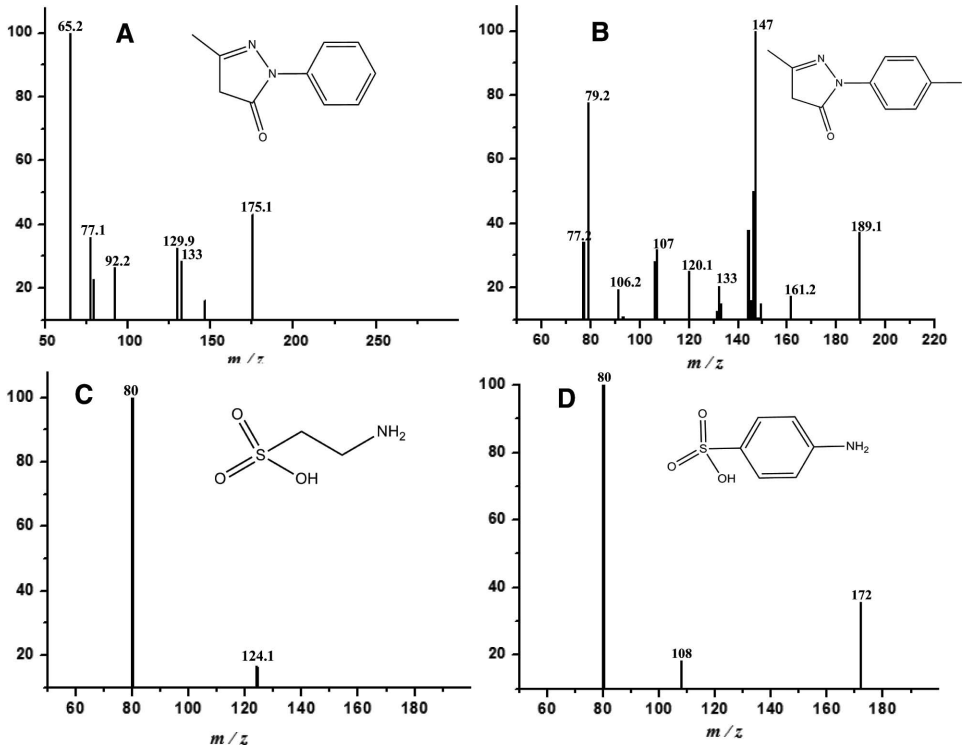

Figure 1. Structures and product ion spectra of edaravone (A), 3-methyl-1-*p*-tolyl-5-pyrazolone (IS) (B), taurine (C), and sulfanilic acid (IS) (D) in both positive and negative ionization modes.

20 mg/mL, respectively. A series of working standard solutions of edaravone ranging from 0.25 to 250 μg/mL and taurine from 10 to 5000 μg/mL were prepared by appropriate dilution of their respective stock solutions with water. The IS stock solutions were prepared at a final concentration of 1 mg/mL in water.

Calibration standards were prepared by spiking 100 μL blank beagle plasma with 20 μL working standard solutions to yield the final concentrations of 0.05–50 μg/mL for edaravone and 2–1000 μg/mL for taurine. The quality control (QC) samples were similarly prepared at three concentrations of 0.1, 4, and 40 μg/mL for edaravone and 4, 80, and 800 μg/mL for taurine, corresponding to low, medium, and high concentration QC samples, respectively. All the prepared solutions were kept at 4°C and brought to room temperature before use.

2.4 Sample extraction

A 100 μL plasma sample was spiked with 40 μL IS (including IS of edaravone and taurine 20 μL, respectively), 10 μL 20% formic acid, and 40 μL water (For QC or calibration samples, water was substituted with 20 μL edaravone and 20 μL taurine standard solution). The mixture was vortexed to mix, after which, 200 μL acetonitrile containing 0.1% formic acid was added and vortex-mixed vigorously for 2 min. Then, the sample was centrifuged at 12 000 rpm for 10 min, the supernatant (100 μL) was transferred to a sampler vial and diluted with 1 mL 0.1% formic acid/methanol (90:10, v/v). After that, 2 μL of the dilutions were injected into the HPLC–MS/MS system for analysis.

2.5 Method validation

2.5.1 Specificity

Six blank plasma samples from different sources were analyzed to check whether there were any signals interfering with the MRM transitions of the analytes or their IS. Blank plasma sample, blank plasma sample spiked with edaravone (0.05 μg/mL), taurine (2 μg/mL), and their IS (1 mg/mL), and plasma sample from a beagle dog after a single intravenous administration of the mixture of edaravone and taurine were analyzed to find out interferences from endogenous components.

2.5.2 Linearity of calibration and range, LOD, LLOQ

Linearity was assessed by assaying calibration curves at eight concentration levels ranging from 0.05–50 μg/mL for edaravone and 2–1000 μg/mL for taurine in beagle plasma. The curves were constructed by plotting the peak area ratios (analyte/IS) of plasma standards versus nominal concentrations. The best linear fit for the calibration curve was achieved with a $1/x^2$ weighting factor. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better. The LOD was defined for the analyte and background response level after the sample cleanup and was estimated to correspond to three times the baseline noise ($S/N \geq 3$). The lower LOQ (LLOQ) of the assay was assessed as the lowest concentration on the calibration curve that could be quantitatively determined with an acceptable precision <20% and accuracy

within ± 20%. Plasma samples containing 0.05 μg/mL edaravone and 2 μg/mL taurine were made and five replicates of each spiked sample were extracted.

2.5.3 Accuracy and precision

QC samples were individually spiked at three different concentrations (0.1, 4, and 40 μg/mL for edaravone and 4, 80, and 800 μg/mL for taurine). The intraday precision and accuracy were estimated by determining QC samples at the three concentrations in five replicates. The interday precision and accuracy were assessed by determining QC samples at the three concentrations in five replicates on three consecutive days using calibration curves obtained daily. The precision, describing the uncertainty of measurements for the same sample at each QC concentration was expressed as the RSD. Accuracy values were calculated by accuracy (%) = (mean of measured concentration/nominal concentration) × 100.

2.5.4 Extraction recovery and matrix effect

The extraction recovery was determined at three concentration levels (0.1, 4, and 40 μg/mL for edaravone, 4, 80, and 800 μg/mL for taurine, $n = 5$ for each concentration). Percentage recovery for each analyte was estimated as the ratio of analyte/IS peak area spiked before extraction to analyte/IS spiked postextraction multiplied by 100. The matrix effect was investigated at eight calibration standard levels by comparing the peak response of blank plasma extracts spiked with the analytes (A) with that of pure standard solution containing equivalent amounts of the compounds (B). The ratio (A/B × 100)% was used to evaluate the matrix effect. As taurine in our study was present endogenously in beagle plasma, it was impossible to obtain "blank" plasma devoid of taurine. To neglect the endogenous occurrence of taurine, which could interfere with our evaluation of matrix effect, the peak area observed in the case of standard analyte spiked in matrix post extraction was corrected by subtracting the basal peak area of analyte in unspiked matrix.

2.6 Application of the method in a PK study

The method described above was applied to study the PKs in six male beagle dogs after a single intravenous administration of the mixture of edaravone and taurine. Beagle dogs (9.0–13.5 kg) were obtained from the Laboratory Animal Center of Xuzhou Medical College (Xuzhou, Jiangsu Province, China). They were kept in an environmentally controlled breeding room (temperature: 20 ± 2°C, humidity: 60 ± 5%, 12 h dark/light circle) for at least three days. Food was withheld 12 h before drug dosing while water was available *ad libitum*. A single administration of edaravone and taurine mixture (0.6 mg/kg for edaravone and 6 mg/kg for taurine) was given through the hind leg vein while the animals were awake. Blood samples (about 0.5 mL) were collected by a cannula into heparinized tubes prior to and at various times (0.03, 0.08, 0.17, 0.25, 0.42, 0.67, 1, 1.5, 2, 3, 4, 6, and 24 h) after administration. Then, the blood was immediately centrifuged at 4000 rpm for 10 min to obtain plasma. After that, 20% formic acid aqueous solution was successively added into the plasma with a volume ratio of 1:10 and stored at −20°C until analysis. The concentration versus time data were analyzed by a noncompartmental analysis using DAS 2.1 software package (Drug and Statistics for Windows). All the experiments were approved by institutional Animal Ethics Committee and followed the Guiding Principles for Care and Use of Laboratory Animals of Xuzhou Medical College.

3 Results and discussion

3.1 Method development

3.1.1 Selection of ISs for quantification

It is necessary to use an IS to get high accuracy when performing MS quantification. An ideal IS in the analysis of a biological sample should be a structurally similar analog of an analyte or a stable labeled compound. In our case, 3-methyl-1-*p*-tolyl-5-pyrazolone and sulfanilic acid were selected as the IS for edaravone and taurine, respectively, owing to their similarity with edaravone and taurine in structure, good chromatographic behavior, extraction conditions, reduced interference with the plasma matrix as well as stability (see Fig. 1).

3.1.2 Sample preparation

For the plasma sample preparation, the primary issue that should be addressed is the stability of edaravone. The stability of edaravone was found to be not very favorable when the plasma sample was spiked only with IS and water. It has been reported that the most suitable pH for edaravone to be stable is 3.0–4.5 [32]. Therefore, 20% formic acid was chosen as the acidification reagent and added into the plasma to make the spiked solution acidic. In addition, the volume ratio of formic acid to plasma was also optimized and finally, 1:10 (corresponding to the pH of the spiked solution at 3.8) was selected as the best proportion between formic acid and plasma as edaravone was observed to be mostly stable under these conditions.

A simple sample preparation procedure was required, considering the large batches of plasma samples during the experiment. Currently, protein precipitation has become one of the most widely employed biological sample preparation methodologies due to its simplicity and universality for drug molecules in plasma. It is a well-known nonselective purification method that introduces high amounts of endogenous components and can cause signal suppression or enhancement especially with an ESI source. In the present study, several organic solvents were compared as precipitation agents, such as methanol containing 0.1% formic acid, acetonitrile containing 0.1% formic acid, 10% perchloric acid, and 30%

trichloroacetic acid. The results showed that protein precipitation with acetonitrile containing 0.1% formic acid was the best choice for the simultaneous extraction of edaravone and taurine from beagle plasma, which produced a clean chromatogram for a drug-free plasma sample and offered satisfactory absolute recoveries for the two analytes. Meanwhile, as the existence of high amount of similar polar interferences can cause matrix effects to taurine and its IS, samples were diluted by ten times the volume of 0.1% formic acid/methanol (90:10) before injection. Consequently, matrix effects (i.e. ion suppression due to ionization competition) are suppressed as well as ensuring the requirement of LLOQ.

3.1.3 Optimization of the LC–MS/MS system

For achieving a good resolution and symmetric peak shapes for each analyte and their IS as well as a short analysis time, the chromatographic conditions, including column type, composition of mobile phase, and mass detection were optimized through several trials.

Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. It was found that the peaks were more symmetrical and the signal intensities were higher when methanol was adopted. Adding formic acid into mobile phase can effectively overcome the peak tailing effect and further improve detection sensitivity. The percentage of formic acid (0.01, 0.03, and 0.05%) in the aqueous phase was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in mass spectrometer. At last, 0.03% formic acid in water was selected to be the mobile phase.

For taurine and its IS, one big problem is the matrix effect for analysis of plasma samples because of its extreme polarity, which brought about the coelution of other polar compounds in plasma with taurine and IS, causing signal suppression. Porous graphitic carbon columns are a good choice for reducing the matrix effect of taurine. Edaravone is of weak polarity that is suitable to be analyzed on RP columns. However, most RP columns can hardly retain polar taurine. Under such circumstances, after careful comparison among three kinds of columns, for example, Agilent Zorbax SB-Aq column (100 × 2.1 mm id, 3.5 μm), Agilent SB-C_{18} column (100 × 2.1 mm id, 5 μm), Agilent Eclipse plus C_{18} column (100 × 2.1 mm id, 5 μm), a Zorbax SB-Aq RP bonded column was finally selected, which can retain polar analytes when using highly aqueous mobile phases, and also can elute weak polar compounds with high organic phases. Meanwhile, matrix effects of taurine and its IS on this column were also reduced significantly compared with other two kinds of C_{18} columns.

As mentioned above, the polarity of taurine and its IS differs greatly from that of edaravone and its IS, therefore, the process of chromatographic elution must be optimized in order to separate the analytes completely from the coeluting interfering substances and reduce the matrix effect to a satisfactory level (within 75–125%) [33]. The chromatographic separation was realized by gradient elution using a mobile phase of 0.03% formic acid in water (A) and methanol (B) within 9.0 min at a flow rate of 0.3 mL/min. During the first 3 min of the gradient, solution B increased from 10 to 80%, and then decreased to 10% for over 3 min and kept to re-equilibrate the column until 9 min. A MRM chromatogram of edaravone, taurine, and their ISs in a plasma matrix is shown in Fig. 2.

Within this work, the MRM acquisition mode was employed for each analyte and IS. Analytes (50 ng/mL for edaravone and IS and 200 ng/mL for taurine and IS) were directly infused into the ion source of the mass spectrometer in both positive and negative ionization modes. Fragmentations were optimized by simultaneously applying both positive and negative ionization modes as well as different collision energies to break the precursor ions with consistent, stable responses and the highest intensity for quantifications. Good responses were obtained in positive ionization mode for edaravone and its IS, and negative ionization mode for taurine and its IS. In the positive ESI mode, the most abundant ions were protonated molecules $[M+H]^+$ m/z 175.1 and 189.1 for edaravone and its IS, respectively. The product ion spectra showed high-abundance fragment ions at m/z 133.0 and 147.0 for edaravone and its IS, respectively. In the negative ion mode, the deprotonated molecular ion of taurine ($[M–H]^-$ at m/z 124.1) yielded the $[SO_3]^-$ fragment at m/z 80.0 (ion transition 124.1→80.0). Sulfanilic acid ($[M–H]^-$ at m/z 172.0) also yielded the $[SO_3]^-$ fragment at m/z 80.0 (ion transition 172.0→80.0). The most suitable collision energy was also investigated by observing the response obtained versus the selectivity response for the fragmentation for each compound, and the results are shown in Supporting Information Table S1.

3.2 Method validation

Representative MRM chromatograms of edaravone, taurine, and their corresponding IS in beagle plasma are shown in Fig. 2. As can be clearly observed, under the above conditions, the typical retention time of edaravone, taurine, and their IS was 3.635, 1.006, 3.801, and 1.146 min, respectively. Moreover, in drug-free control plasma samples (A), no endogenous interference peaks were detected near the retention time of the analytes or their ISs, which thus verified that our proposed method is of high specificity.

The method exhibited a good linearity over the concentration range of 0.05–50 μg/mL for edaravone and 2–1000 μg/mL for taurine in beagle plasma. The correlation coefficient (r) of the two analytes both exceeded 0.99, showing a good linearity among the concentration range. Calibration curves, LLOQ and LOD of the assay are listed in Supporting Information Table S2.

In this assay, the intraday precision was <8.54% for each QC level of edaravone and 12.81% for taurine. The interday precision was <9.45% for each QC level of edaravone and 13.57% for taurine. For the two compounds, the accuracy values were between 91.8 and 120.0%, which indicated that

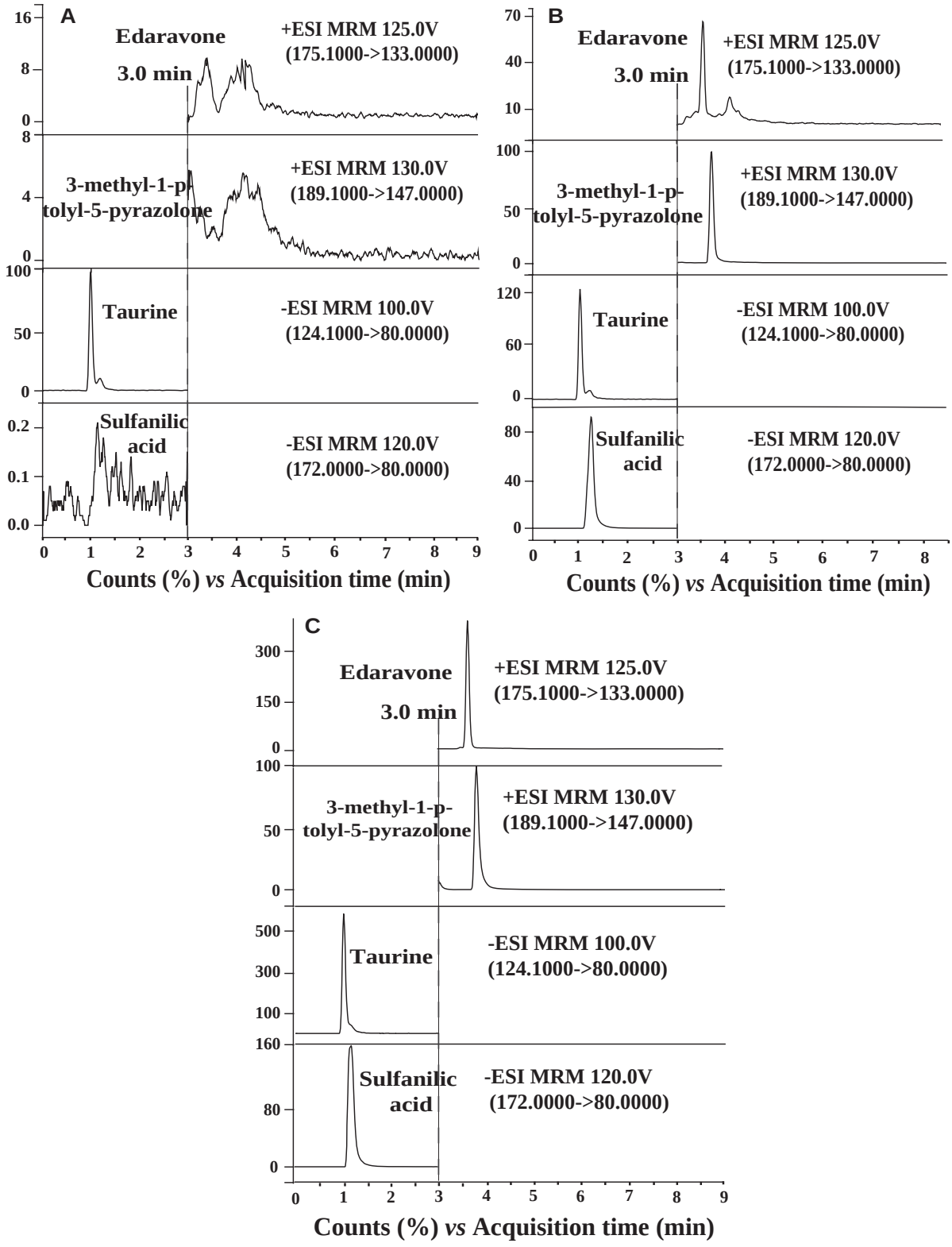

Figure 2. Representative MRM chromatograms of edaravone, taurine, and their IS in beagle plasma. (A) Blank plasma sample; (B) blank plasma spiked with edaravone at the LLOQ of 0.05 μg/mL, taurine at the LLOQ of 2 μg/mL, and IS of 1 mg/mL; (C) beagle plasma sample obtained at 25 min after an intravenous administration of the mixture of edaravone and taurine. The retention time for edaravone, taurine, and their IS were 3.635, 1.006, 3.801, and 1.146 min, respectively.

Table 1. Mean PK parameters after a single intravenous administration of the mixture of edaravone and taurine in six beagle dogs

Pharmacokinetic parameters	Edaravone	Taurine
T_{max} (h)	0.03 ± 0.000	0.03 ± 0.000
C_{max} (mg/L)	1.712 ± 0.312	37.242 ± 1.046
$t_{1/2}$ (h)	2.717 ± 0.652	1.819 ± 0.216
V (L/kg)	0.607 ± 0.223	0.51 ± 0.234
CL (L/h/kg)	0.151 ± 0.028	0.192 ± 0.056
$AUC_{0\text{-}t}$ (mg/L·h)	2.027 ± 0.313	32.818 ± 6.481
$AUC_{0\text{-}\infty}$ (mg/L·h)	2.031 ± 0.312	32.82 ± 6.480

the established method had a good accuracy and precision (Supporting Information Table S3).

The results from Supporting Information Table S4 indicate that the recoveries of edaravone and taurine, and their IS were consistent and not concentration dependent. No significant matrix effect was observed in beagle plasma for the two analytes at the inspected QC levels and IS, indicating that ion suppression or enhancement from beagle plasma could be negligible under the conditions used.

3.3 Application to a PK study

The endogenous concentration of taurine was determined by randomly selecting 12 beagle dogs and keeping them in an environmentally controlled breeding room, with food and water normally supplied for three days. After this, blood samples were collected at 9:00, 12:00, 15:00, 18:00, 21:00, and 24:00 on that day and processed as described above. The results showed that the deviation value of taurine concentration in beagle plasma was within 15% in one day.

The above method was then applied for the PK study of edaravone and taurine in plasma from six beagle dogs following a single intravenous administration of the mixture of edaravone and taurine (0.6 mg/kg for edaravone and 6 mg/kg for taurine). The mean plasma concentration (± SD) ($n = 6$) versus time profiles of edaravone and taurine are shown in Fig. 3. It should be stressed herein that as taurine is present endogenously, the real plasma concentration of taurine at different time points after administration should be expressed as the measured concentration minus the basal level in beagle dogs, whereas for edaravone, the detected concentration just represents the real plasma concentration at different time points after injection. The main PK parameters of edaravone and taurine in beagle dogs after a single intravenous administration are presented in Table 1, which are reported for the first time. The maximum plasma concentration (C_{max}) was 3.908 ± 4.209 mg/L for edaravone and 54.577 ± 5.317 mg/L for taurine. The area under the plasma concentration versus time curve from zero to the time of last measurable concentration ($AUC_{0\text{-}\infty}$) was 2.761 ± 0.712 mg h/L for edaravone and 53.639 ± 8.353 mg h/L for taurine. The terminal phase half-life ($t_{1/2}$) was 2.966 ± 0.394 h for edaravone and 1.138 ± 0.718 h for taurine. All of these data indicated the applicability of this method to the PK study of edaravone and taurine.

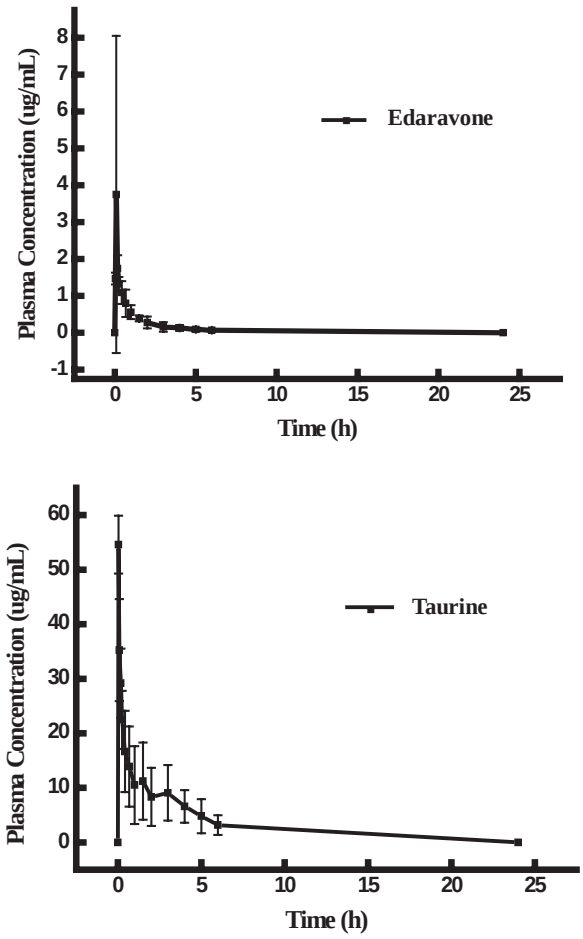

Figure 3. Mean plasma concentration versus time profiles of edaravone and taurine after a single intravenous administration of the mixture of edaravone and taurine in six male beagle dogs. Data are expressed as means and error bars represent SDs.

4 Conclusions

This paper presents the first validated method for the simultaneous quantification of edaravone and taurine in plasma samples using LC–MS/MS. The validation using beagle plasma confirmed the linearity, acceptable accuracy, and precision over a wide concentration range (0.05–50 μg/mL for edaravone and 2–1000 μg/mL for taurine) and the high sensitivity (LOD at 0.01 and 0.66 μg/mL for edaravone and taurine) of the assay using 100 μL plasma. The established method was then successfully applied to the analysis of beagle plasma samples for a PK study of a mixture of edaravone and taurine. With the above promising observations, the LC–MS/MS method developed herein should be useful for clinical and PK studies, to explore potential metabolic interactions in case of edaravone–taurine association.

The authors acknowledge support for this work by a Blue Project of Young Academic Leader, Jiangsu Overseas Research & Training Program for University Prominent Young & Middle-Aged Teachers and Presidents, Programs of the National Natural Science Foundation of China (No. 21205102) and the Scientific & Research Foundation for Outstanding Talents of Xuzhou Medical College.

The authors have declared no conflict of interest.

5 References

[1] Watanabe, T., Yuki, S., Egawa, M., Nishi, H., *J. Pharmacol. Exp. Ther.* 1994, *268*, 1597–1604.

[2] Watanabe, T., Morita, I., Nishi, H., Murota, S., *Prostaglandins Leukot. Essent. Fatty Acids* 1988, *33*, 81–87.

[3] Tada, S., Nakamoto, N., Kameyama, K., Tsunematsu, S., Kumagai, N., Saito, H., Ishii, H., *J. Gastroenterol. Hepatol.* 2003, *18*, 851–857.

[4] Zhang, N., Komine-Kobayashi, M., Tanaka, R., Liu, M., Mizuno, Y., Urabe, T., *Stroke* 2005, *36*, 2220–2225.

[5] Kikuchi, K., Uchikado, H., Miyagi, N., Morimoto, Y., Ito, T., Tancharoen, S., Miura, N., Miyata, K., Sakamoto, R., Kikuchi, C., Iida, N., Shiomi, N., Kuramoto, T., Kawahara, K., *Int. J. Mol. Med.* 2011, *28*, 899–906.

[6] Qi, X., Okuma, Y., Hosoi, T., Nomura, Y., *J. Pharmacol. Exp. Ther.* 2004, *311*, 388–393.

[7] Nonaka, Y., Shimazawa, M., Yoshimura, S., Iwama, T., Hara, H., *Neurosci. Lett.* 2008, *441*, 224–228.

[8] Huxtable, R. J., *Prog. Neurobiol.* 1989, *32*, 471–533.

[9] Lombardini, J. B., *Adv. Exp. Med. Biol.* 1994, *359*, 9–17.

[10] Schaffer, S., Takahashi, K., Azuma, J., *Amino Acids* 2000, *19*, 527–546.

[11] Foosand, T. M., Wu, J. Y., *Neurochem. Res.* 2002, *27*, 21–26.

[12] El Idrissi, A., Trenkner, E., *Adv. Exp. Med. Biol.* 2003, *526*, 527–536.

[13] Albrechtand, J., Schousboe, A., *Neurochem. Res.* 2005, *30*, 1615–1621.

[14] Molchanova, S. M., Oja, S. S., Saransaari, P., *Brain Res.* 2007, *1130*, 95–102.

[15] Kearns, S., Dawson, R., *Adv. Exp. Med. Biol.* 2000, *483*, 563–570.

[16] Louzada, P. R., Paula Lima, A. C., Mendonca-Silva, D. L., Noel, F., De Mello, F. G., Ferreira, S. T., *FASEB J.* 2004, *18*, 511–518.

[17] Saransaari, P., Oja, S. S., *Amino Acids* 2000, *19*, 509–526.

[18] Kramer, J. H., Chovan, J. P., Schaffer, S. W., *Am. J. Physiol.* 1981, *240*, H238–246.

[19] Zhu, L., *Zhejiang J. Integr. Trad. Chin. West. Med.* 2006, *16*, 157–158.

[20] Cho, K. H., Oh, J. K., Jang, Y. S., Jung, J. W., Oh, H. R., Park, E. K., Kim, D. H., Moon, S. K., Kim, D. H., Ryu, J. H., *Meth. Find. Exp. Clin. Pharmacol.* 2008, *30*, 443–450.

[21] Hao, X., Li, X. L., Liu, Y. B., Shen, H. T., Yue, L. Q., *Chin. J. Misdiagn.* 2012, *12*, 1597–1598.

[22] Lapchak, P. A., *Transl. Stroke Res.* 2010, *1*, 96–107.

[23] Fu, G. Y., Wen, M. L., Jia, L. H., Zuo, X. P., *Pharm. J. Chin. PLA* 2006, *22*, 458–460.

[24] Hong, Z. Y., Le, J., Lou, Z. Y., Wu, X. F., Chai, Y. F., *Pharm. J. Chin. PLA* 2004, *20*, 29–31.

[25] Liao, P., Yan, Z. Y., Xu, Z. J., Sun, X., *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 2009, *72*, 1066–1070.

[26] Musioand, R., Sciacovelli, O., *J. Magn. Reson.* 2001, *153*, 259–261.

[27] Meek, J. L., *Anal. Chem.* 1976, *48*, 375–379.

[28] Porter, D. W., Banks, M. A., Castranova, V., Martin, W. G., *J. Chromatogr A* 1988, *454*, 311–316.

[29] Chen, Z., Xu, G., Spechi, K., Yang, R., She, S., *Anal. Chim. Acta* 1994, *296*, 249–253.

[30] McMahon, G. P., O'Kennedy, R., Kelly, M. T., *J. Pharm. Biomed. Anal.* 1996, *14*, 1287–1294.

[31] Toyo'oka, T., *J. Pharmaceut. Biomed.* 2012, *69*, 174–184.

[32] Fu, G. Y., Wen, M. L., Jia, L. H., *China. New Drug. J.* 2005, *14*, 726–728.

[33] Zhao, X., Zhao, Y. L., Liu, X. M., Han, W., Yu, Z. G., *J. Sep. Sci.* 2012, *35*, 984–993.

