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Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous determination of D-amphetamine and diphenhydramine in beagle dog plasma and its application to a pharmacokinetic study

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Abstract

A new drug, quick-acting anti-motion capsule (QAAMC) composed of D-amphetamine sulfate, dimenhydrinate and ginger extraction has been studied for anti-motion-sickness use. We have developed a sensitive, specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the quantitative determination of D-amphetamine and diphenhydramine, the main effective components of the QAAMC, using pseudoephedrine as the internal standard. The analytes and internal standard were isolated from 200 μL plasma samples by a simple liquid–liquid extraction (LLE). Reverse-phase HPLC separation was accomplished on a Zorbax SB-C18 column (100 mm × 3.0 mm, 3.5 μm) with a mobile phase composed of methanol–water–formic acid (65:35:0.5, v/v/v) at a flow rate of 0.2 mL/min. The method had a chromatographic total run time of 5 min. A Varian 1200 L electrospray tandem mass spectrometer equipped with an electrospray ionization source was operated in selected reaction monitoring (SRM) mode with the precursor-to-product ion transitions *m/z* 136.0 → 91.0 (D-amphetamine), 256.0 → 167.0 (diphenhydramine) and 166.1 → 148.0 (IS) used for quantitation. The method was sensitive with a lower limit of quantitation (LLOQ) of 0.5 ng/mL for D-amphetamine and 1 ng/mL for diphenhydramine, with good linearity in the range 0.5–200 ng/mL for D-amphetamine and 1–500 ng/mL for diphenhydramine ($r^2 \geq 0.9990$). All the validation data, such as accuracy, precision, and inter-day repeatability, were within the required limits. The method was successfully applied to pharmacokinetic study of the QAAMC in beagle dogs.
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1. Introduction

Motion sickness, whether it occurs in a car, on a ship, in an aircraft or abroad a space vehicle, is induced through whole body vibrations by stimulation of the vestibular organ [1,2]. According to a theory of Wood and Graybiel, motion sickness is caused by an imbalance between the cholinergic and noradrenergic parts of the central nervous system [3]. Motion sickness is a very common disease characterized by various symptoms, e.g., pallor, cold sweating, nausea, vomiting, fatigue, peripheral vasoconstriction, or slowing of brain waves [4].

Various anti-motion-sickness medications, many of which are available over the counter, are commonly used to ameliorate motion sickness. Many antihistamines dimenhydrinate, meclizine and promethazine have been effective anti-motion-sickness drugs, however, these drugs are antihistamine-H_1 receptor antagonists that cause sedation as the most common

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subjective side effect [5]. Wood and Graybiel demonstrated that D-amphetamine improves tolerance to Coriolis stimulation of the vestibular system [6]. They found that the antihistamines produced an increase in treatment effectiveness and reduced sedation when D-amphetamine was added. However, many anti-motion-sickness drugs, alone or in combination are only partially effective, and their adverse effects cannot be ruled out. Therefore, it is highly desirable to look for a drug that is effective for prevention of motion sickness and which is relatively free of side effects. Traditional Chinese medicine has recommended ginger (*Zingiber officinale*) for over 2500 years. Water extract of the rhizome of ginger can mitigate symptoms of gastrointestinal distress, thus continuing a tradition that dates back at least as far as 1597, and the effect of the powered rhizome of ginger on the symptoms of motion sickness was compared with that of dimenhydrinate, and of the two, the former was superior [7]. It is widely accepted that the correct traditional Chinese medicine and western medicine combination can increase the therapeutic effects and reduce or even eliminate the side effects caused by western drugs. A new drug, quick-acting anti-motion capsule (QAAMC) composed of D-amphetamine sulfate, dimenhydrinate and ginger extraction has been studied for anti-motion-sickness use [8].

D-Amphetamine and diphenhydramine are the active moiety in the QAAMC. Therefore, to further understand and reveal the pharmacokinetic profile and mechanism of the QAAMC, sensitive and specific analytical methods for the simultaneous determination of D-amphetamine and diphenhydramine were urgently needed. Some procedures have been described for the determination of either D-amphetamine or diphenhydramine in biological fluids, such as gas chromatography–mass spectrometry [9–11], capillary electrophoresis with UV-absorbance detection [12–15], HPLC with UV-absorbance detection [16–18], however, these methods do not meet modern drug development needs with respect to an efficient extraction procedure, shorter run time and high sensitivity. Liquid chromatography coupled to tandem mass spectrometry has become a method of the choice for the determination of small molecules in biological matrices, including D-amphetamine and diphenhydramine [19–22], because of its superior LLOQ, sensitivity, and improved selectivity. We herein describe a simple, sensitive and high throughput method based on liquid–liquid extraction and LC–MS/MS for routine measurement of D-amphetamine and diphenhydramine using pseudoephedrine as the IS in beagle dog plasma in support of pharmacokinetic study. To the best of our knowledge, studies on LC–MS/MS for the simultaneous determination of D-amphetamine and diphenhydramine in biological sample have not yet been reported.

2. Experimental

2.1. Chemicals and reagents

Capsule formulation of QAAMC (lot 20060308) with a declared content of 6.8 mg of D-amphetamine sulfate (corresponding to 5 mg D-amphetamine), 50 mg of dimenhydrinate (corresponding to 27.9 mg diphenhydramine) and 200 mg of ginger extraction was provided by Naval Medical Research Institute (Shanghai, PR China). D-Amphetamine sulfate, diphenhydramine hydrochloride and pseudoephedrine hydrochloride were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The purities of D-amphetamine sulfate, diphenhydramine hydrochloride and pseudoephedrine hydrochloride (IS) were >99.5%. Sodium hydroxide, ethyl acetate and *n*-hexane (analytical reagent grade) were purchased from Shanghai chemical reagent company (Shanghai, PR China). Formic acid (chromatographic grade) was purchased from Tedia (Fairfield, USA). Methanol (chromatographic grade) was purchased from Merck (Darmstadt, German). Deionized (18 MΩ/cm) water was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. LC–MS/MS instrumentation

A Varian HPLC–MS/MS system (Palo Alto, CA, USA) consisted of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source. Varian MS workstation version 6.3 software was used for data acquisition and processing.

2.3. Liquid chromatographic conditions

The chromatographic separation was performed on a Zorbax SB-C18 column (100 mm × 3.0 mm, 3.5 μm particle size, Agilent Technologies, Wilmington, DE, USA) at a column temperature of 30 °C. Autosampler temperature was kept at ambient temperature of 25 °C. A Security guard cartridge (4 mm × 2.0 mm, 5 μm particle size, Phenomenex, Macclesfield, Cheshire, UK) was used to extend the life of the analytical column. The mobile phase composed of methanol–water–formic acid (65:35:0.5, v/v/v) operated at a flow rate of 0.2 mL/min. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane filter. The injection volume was 20 μL and the analysis time was 5 min per sample.

2.4. Mass spectrometer conditions

The ESI-MS spectrometer was operated in the positive ion mode. The electrospray capillary voltage was set to 35 V. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 and 350 °C. Protonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas to yield product ions for each analyte and the IS. The collision energy was 16, 10 and 8 eV for D-amphetamine, diphenhydramine and IS, respectively. The scan time was 1 s and the detector multiplier voltage was set to 1330 V. Selected reaction monitoring of the precursor–product ion transitions *m/z* 136.0 → 91.0 for D-amphetamine, 256.0 → 167.0 for diphenhydramine and 166.1 → 148.0 for IS was used for quantitation. Product ion mass spectra for each analyte and IS are shown in Fig. 1.

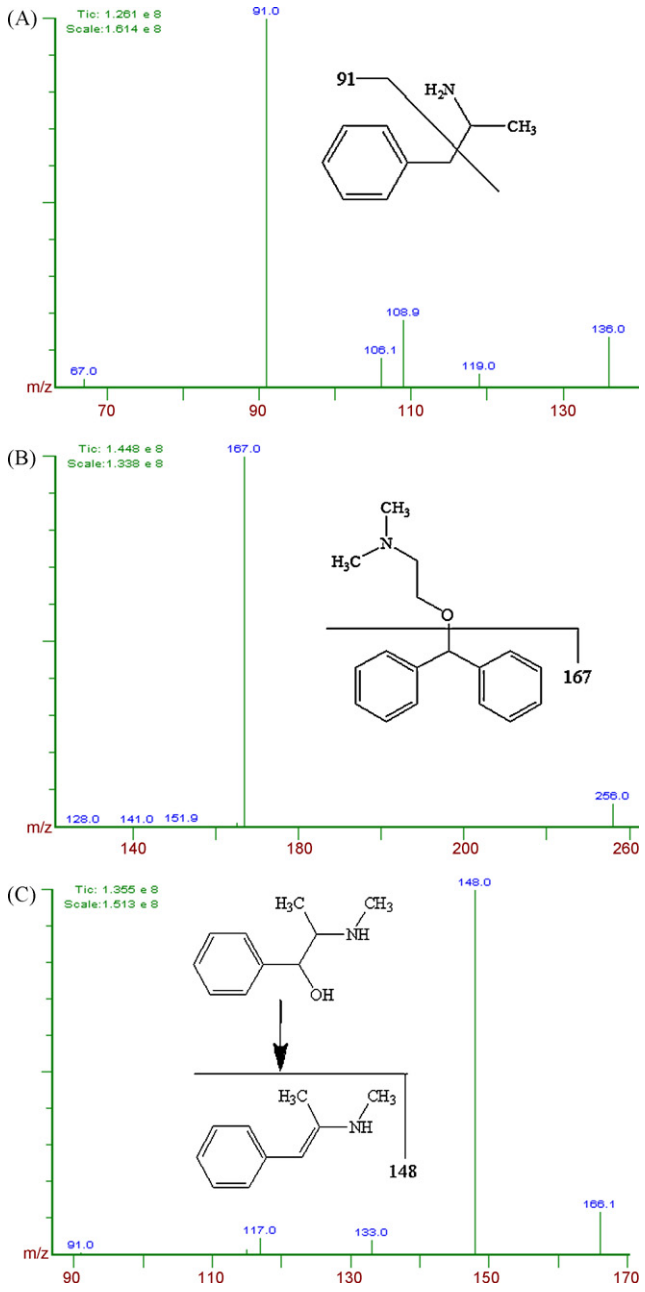

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of D-amphetamine (A), diphenhydramine (B) and IS (C).

2.5. Preparation of standard and quality control (QC) samples

Primary stock solutions of D-amphetamine and diphenhydramine were prepared separately by dissolving the accurately weighed D-amphetamine sulfate and diphenhydramine hydrochloride in methanol to result in final base concentration of 1 mg/mL. The solutions were sonicated for 5 min to ensure complete dissolution. Following sonication, the solutions were allowed to equilibrate to room temperature after which they were diluted. Working standards of D-amphetamine and diphenhydramine in the concentration range 0.01–4 and 0.02–10 μg/mL, respectively, were prepared by independent dilution of 1 mg/mL stock solution with H_2O:methanol (55:45, v/v). The stock standard solution of IS was prepared by dissolving appropriate amounts of pseudoephedrine hydrochloride in methanol to give a final base concentration of 1 mg/mL. A 1 μg/mL internal standard working solution was obtained by diluting the stock solution of pseudoephedrine with H_2O:methanol (55:45, v/v). All the solutions were stored at 4 °C and brought to room temperature before use. Calibration standards were prepared daily by spiking 10 μL of the appropriate standard solutions to 200 μL of the blank beagle dog plasma. Plasma concentrations were 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL for D-amphetamine, and 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL for diphenhydramine, respectively. QC samples, which were used in the validation and during the pharmacokinetic study, were prepared from different sources by independent dilution at three levels for each analyte: 1.5, 15 and 150 ng/mL for D-amphetamine, 2, 40, 400 ng/mL for diphenhydramine, respectively. QC samples were aliquoted into 200 μL non-sterile eppendorf tubes and stored at −20 °C until analysis.

2.6. Extraction procedure

The plasma samples of beagle dog were taken out from −20 °C freezer and kept at room temperature for thawing. The samples were vortexed adequately before pipetting. To a 200 μL aliquot of plasma sample, 10 μL of IS working solution (1 μg/mL) and 100 μL of 1 M NaOH were added and vortexed to mix. The mixed sample was then extracted with 1 mL *n*-hexane:ethyl acetate (3:2, v/v), by vortex-mixing for 2 min. After centrifugation at 3500 rpm for 10 min, 0.8 mL of the upper organic layer was transferred to another tube. Extracts were concentrated to dryness at 40 °C under a gentle stream of nitrogen and reconstituted with 100 μL of H_2O:methanol (55:45, v/v). A 20 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.7. Pharmacokinetic study in beagle dogs

Six beagle dogs (both sexes), weighting 12.9 ± 0.3 kg (mean ± SD), were received an oral administration of four capsules of QAAMC after an overnight fast. Animal had access to water and food 4 h after drug administration. Blood samples (1 mL) were collected into heparinized tubes before administration and at different time points (0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h) after administration. The plasma was separated from heparinized blood by centrifugation and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Method development

3.1.1. Selection of internal standard

It is necessary to use an IS to get high accuracy when a mass spectrometer is equipped with HPLC as the detector. Pseudoephedrine, has a similar structure to D-amphetamine, was

adopted in the end because of its similarity of retention action, ionization and extraction efficiency as well as its less endogenous interference at *m/z* 166.1.

3.1.2. Sample pre-treatment

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. Currently, the most widely employed biological sample preparation methodologies are protein precipitation (PPT), solid phase extraction (SPE), and liquid–liquid extraction (LLE). As the PPT procedure has the advantages of simplicity and universality for drug molecules in plasma, our initial approach of developing an assay for D-amphetamine and diphenhydramine in plasma was based on PPT with methanol and acetonitrile. However, this technique resulted in strong interferences from the sample matrix and low recoveries of both analytes and IS. LLE was adopted in the end because this technique can not only purify but also concentrate the sample. Ethyl acetate, *n*-hexane, diethyl ether, *n*-hexane:isopropanol (95:5, v/v), ethyl acetate:*n*-hexane (1:1, v/v), ethyl acetate:*n*-hexane (2:1, v/v), ethyl acetate:*n*-hexane (1:2, v/v), were all tested, and finally ethyl acetate:*n*-hexane (2:3, v/v) was adopted because of its high extraction efficiency and less interference. Sodium hydroxide (100 μL × 1 M) was added into the plasma samples to accelerate the drugs' dissociation from the plasma and reduce interference from endogenous which were of acid nature.

3.1.3. LC–MS/MS optimization

Selected reaction monitoring (SRM) conditions were established for each analyte and the internal standard by mixing 10 μg/mL of each compound (20 μL/min) with mobile phase (200 μL/min) and infusing the mixture via a tee-union into the mass spectrometer. Each compound was run separately. Parameters of MSD were tuned according to the MS signal response of the target compound and the results indicated that the positive mode was much more sensitive than the negative mode. In the positive ESI mode, the analytes and IS formed predominately protonated molecular ions $[M+H]^+$ (*m/z* 136.0, 256.0 and 166.1 for D-amphetamine, diphenhydramine and IS, respectively) in full scan mass spectra. In the product ion spectra, several fragment ions were obtained, but the ion at *m/z* 91.0, 167.0 and 148.0 were chosen because they displayed much greater intensity than the others in the acquisition of D-amphetamine, diphenhydramine and IS, respectively.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for each analyte and the IS, as well as a short run time. Modifiers, such as ammonium acetate and formic acid alone or in combination in different concentrations were added. It was found that a mixture of methanol–water–formic acid (65:35:0.5, v/v/v) could achieve this purpose and was finally adopted as the mobile phase. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in mass spectrometer. After careful comparison of many columns, a Zorbax SB-C18 column (100 mm × 3.0 mm, 3.5 μm) was finally selected with a flow rate of 0.2 mL/min to achieve an efficient chromatographic separation of the analytes and the endogenous plasma components for eliminating the matrix effects.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasmas and the spiked plasmas. Under the above conditions the retention time of D-amphetamine, diphenhydramine and IS was 2.5, 3.4 and 2.4 min, respectively. All plasma lots were found to be free of interferences with the compounds of interest. A representative chromatogram of a control plasma double blank is shown in Fig. 2A .

Ginger extraction is one of the indispensable components in the QAAMC. There are many saponins, flavonoids, tannins, phenols, coumarins, sterols and alkaloids in it, some of which can be absorbed as pharmacological activities [23]. Experiments in vitro and in vivo were designed to evaluate the interference from an oral administration of ginger extraction. In vitro experiment, 200 μL of the blank plasma spiked with ginger extraction at concentration of 20 μg/mL was extracted and analyzed. In the vivo experiment, a blank beagle dog was received an oral administration of 800 mg of ginger extraction after an overnight fast. Blood samples (1 mL) before administration and at different time points (0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h) after administration were collected, prepared and analyzed. Under the above LC–MS/MS conditions, All samples were found to be absent of interferences with the compounds of interest. A represent chromatogram is shown in Fig. 2B.

The LC–MS/MS system was also evaluated for the presence of "cross-talk" between the channels used for monitoring D-amphetamine, diphenhydramine and IS and no MS/MS response from the analytes into internal standard channel and *vice versa*.

3.2.2. Matrix effects

The matrix effects are generally due to the influence of coeluting compounds on the actual analyte ionization process. The importance of including the evaluation of matrix effects in any LC–MS/MS method is outlined in an excellent paper by Matuszewski et al. [24]. The effects of the plasma matrix on ionization efficiency were expressed as the ratio of the mean peak area of analytes spiked after extraction from five different lots of plasma (i.e. lots originating from five beagle dogs, respectively) to that of the neat standards at different concentrations. By the analyses of the samples at QC concentration levels, matrix effect values were calculated. Average matrix effect values obtained were 96.2% (CV = 2.6%, $n = 5$), 94.6% (CV = 2.1%, $n = 5$) and 97.1% (CV = 3.1%, $n = 5$) for D-amphetamine (1.5, 15 and 150 ng/mL), 100.3% (CV = 2.2%, $n = 5$), 97.6% (CV = 2.8%, $n = 5$) and 100.8% (CV = 2.1%, $n = 5$) for diphenhydramine (2, 40 and 400 ng/mL) and 98.5% (CV = 3.1%, $n = 5$) for IS (50 ng/mL). In addition, plasmas from the beagle dog received a single oral administration of ginger extraction and plasmas

added ginger extraction in vitro were also evaluated for matrix effects, which are due to the components in ginger extraction. No significant peak area differences were observed in all samples, and ion suppression or enhancement from matrix was negligible for the present method.

3.2.3. Sensitivity and linearity

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 15% of nominal and precision not exceeding 15%, was 0.5 and 1 ng/mL for D-amphetamine and

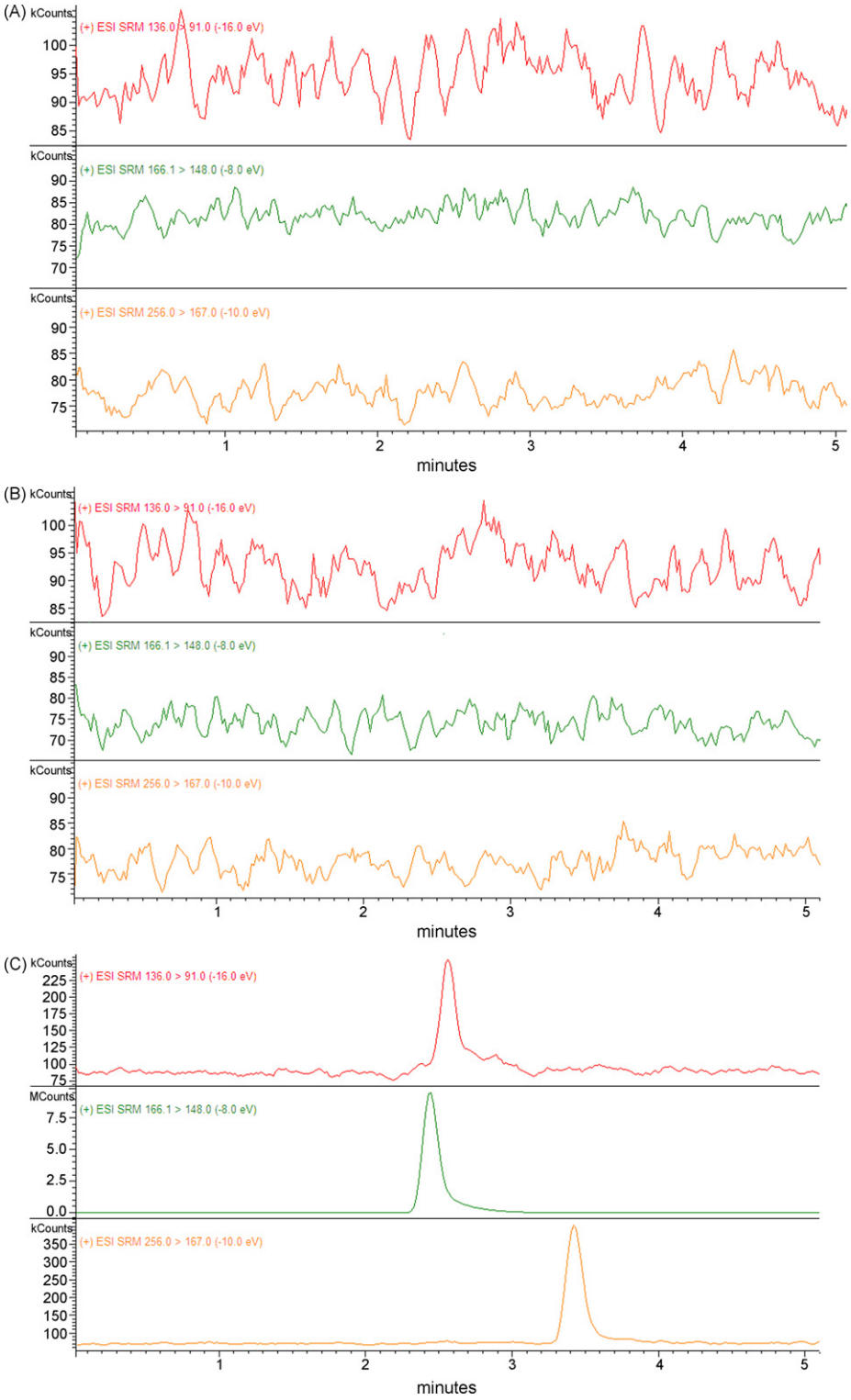

Fig. 2. Representative chromatograms: (A) control plasma double blank; (B) blank plasma spiked with 20 μg/mL of ginger extraction; (C) a blank plasma spiked with 0.5 ng/mL of D-amphetamine, 1 ng/mL of diphenhydramine and 50 ng/mL of IS; (D) plasma sample collected 24 h after an oral administration of four capsules of QAAMC. The measured concentration in this sample was 4.10 ng/mL for D-amphetamine and 14.05 ng/mL for diphenhydramine.

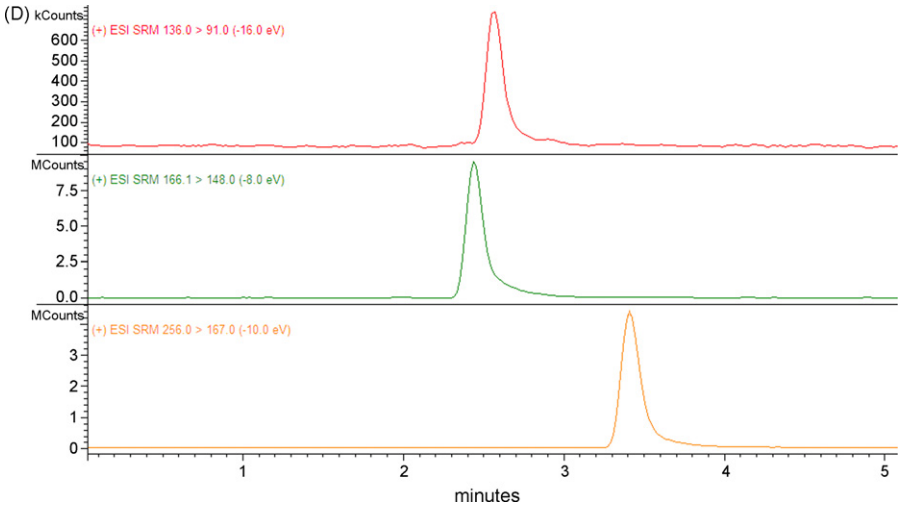

Fig. 2. (*Continued*).

diphenhydramine, respectively. The reproducibility of LLOQ was determined by examining five LLOQ samples independent from the standard curve. A typical chromatogram of an LLOQ sample is shown in Fig. 2C.

Calibration curves were constructed by plotting the peak area ratios (analytes/IS) of plasma standards versus nominal concentration. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x$ weighting factor, giving a mean linear regression equation for the calibration curve of: $y = 1.36 \times 10^{-4}$ (CV = 8.9%, $n = 5$) + 1.85×10^{-2} (CV = 2.2%, $n = 5$) x, $r^2 = 0.9998$ for D-amphetamine and $y = -1.88 \times 10^{-4}$ (CV = 9.4%, $n = 5$) + 3.15×10^{-2} (CV = 2.7%, $n = 5$) x, $r^2 = 0.9995$ for diphenhydramine, where y represents the peak area ratios of analytes to that of IS, and x represents the plasma concentration of analytes in ng/mL. Calibration curves of five different lots of plasma were linear in the range 0.5–200 ng/mL for D-amphetamine and 1–500 ng/mL for diphenhydramine with $r^2 \geq 0.9990$. Unknown sample concentrations exceeding the range were diluted appropriately with control blank plasma and re-assayed. The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was varied from −3.7% to 8.3% for each point on the standard curve (CV varied from 1.4% to 13.7%) for D-amphetamine and −6.8% to 7.0% (CV varied from 2.0% to 12.6%) for diphenhydramine, respectively.

3.2.4. Accuracy and precision

Intra- and inter-day was assessed from the results of QCs. The mean values and RSD for QCs at three concentration levels were calculated over five validation days by using a one-way analysis of variance (ANOVA). The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (RE).

The method showed good accuracy and precision. Table 1 shows a summary of intra- and inter-day accuracy and precision for analytes from the QC samples, respectively. In this assay, the intra-day precision was less than 7.3% for each QC level of D-amphetamine and 8.4% for diphenhydramine. The inter-day precision was less than 8.7% for D-amphetamine and 8.7% for diphenhydramine. RE derived from QC samples was from −3.3 to 2.6% for D-amphetamine, and −4.3% to 7.0% for diphenhydramine.

Table 1
Summary of precision and accuracy from QC samples in beagle dog plasma ($n = 5$)

Nominal concentration (ng/mL)	Intra-day			Inter-day		
	Measured concentration (ng/mL) (mean ± SD)	CV (%)	RE[a] (%)	Measured concentration (ng/mL) (mean ± SD)	CV (%)	RE[a] (%)
D-Amphetamine						
1.5	1.5 ± 0.1	7.3	−0.5	1.5 ± 0.1	8.7	−1.6
15	14.5 ± 0.8	5.6	−3.3	15.4 ± 1.0	6.4	2.6
150	153.9 ± 3.5	2.3	2.6	150.7 ± 4.6	3.1	0.5
Diphenhydramine						
2	1.9 ± 0.2	8.4	−4.3	2.0 ± 0.2	8.7	−1.9
40	42.8 ± 1.6	3.7	7.0	42.1 ± 1.8	4.2	5.2
400	387.0 ± 7.9	2.0	−3.3	387.1 ± 8.2	2.1	−3.2

[a] RE is expressed as [(mean measured concentration)/(nominal concentration) − 1] × 100.

Table 2
Extraction recovery ($n = 5$)

Nominal concentration (ng/mL)	Peak area[a] (e^6) (A)	Peak area[b] (e^6) (B)	Extraction recovery[c] (%) (A/B)
D-Amphetamine			
1.5	2.4 ± 0.2	3.3 ± 0.1	72.0
15	22.6 ± 1.3	32.9 ± 0.7	68.6
150	224.9 ± 6.1	341.2 ± 10.7	65.9
Diphenhydramine			
2	5.0 ± 0.4	6.8 ± 0.2	74.3
40	100.8 ± 4.3	137.3 ± 3.8	73.4
400	998.8 ± 21.7	1442.0 ± 30.1	69.3
IS			
50	80.8 ± 2.5	110.7 ± 1.6	73.0

[a] Standards spiked before extraction.
[b] Standards spiked after extraction.
[c] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma pre-extraction (A) to the mean peak area of the analytes spiked into plasma post-extraction (B).

3.2.5. Extraction recovery

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) was prepared by spiking each analyte into plasma at QC concentration levels. Each of the samples was also spiked with IS at the working concentration of 50 ng/mL. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of the analytes and IS that actually existed in pre-extraction spiked samples (i.e. 80% of the concentration of the analytes and IS in the pre-extraction spiked samples). Extraction recovery values for each analyte and IS were determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to that of the samples spiked after extraction. The results are indicated in Table 2.

3.2.6. Stability

Bench-top stability was investigated to ensure that analytes were not degraded in plasma samples at room temperature for a time period to cover the sample preparation, and was assessed by exposing the QC samples to ambient laboratory conditions for 10 h. Freeze–thaw stability was assessed over three cycles. QC samples were thawed at room temperature and refrozen at −20 °C over three cycles and assayed. Due to the need for occasional delayed injection or reinjection of extraction samples, the stability of reconstituted samples in autosampler vials was assessed at ambient temperature for over 24 h. The freezer storage stability of the analytes in beagle dog plasma at −20 °C was evaluated by assaying QC samples at beginning and 2 weeks later. All stability QC samples were analyzed in five replicated. The result indicated that each analyte had an acceptable stability under those conditions, as shown in Table 3.

3.2.7. Sample dilution

To demonstrate the ability to dilute and analyze samples containing D-amphetamine and diphenhydramine at concentration above the assay upper limit of quantitation, a set of plasma samples was prepared containing D-amphetamine and diphenhydramine at a concentration of 800 and 2000 ng/mL, respectively, and placed in a −20 °C freezer overnight prior to analysis. After thawing, a 20 μL aliquot was withdrawn for analysis ($n = 5$), diluted with 80 μL of control beagle dog plasma, and prepared and analyzed. The results of this experiment are shown in Table 4.

3.3. Comparison of methods

Two references on LC–MS/MS determination in vivo for diphenhydramine have been published, Han et al. [19] and Kumar et al. [20]. In Han's report, the LLOD was 2 ng/mL and 2 mL organic solvent was used to extract 0.5 mL plasma with extraction recovery was 58.5%. The LLOD was 0.2 ng/mL in Kumar's report, while 6 mL ethyl acetate was used to extract 1 mL plasma, and the extraction recovery was 76.6%. The larger volume of the organic solvent is harmful to the environment. No matrix effects and "cross talk" were evaluated in both Han's and Kumar's report. There are some references on determination of amphetamine in biological matrix including hair [25], urine [26], meconium [27], oral fluid [22], serum and plasma [21,22,28–32]. The methods on determination of

Table 3
Stability of D-Amphetamine and diphenhydramine in beagle dog plasma ($n = 5$)

Sample condition	Nominal concentration (ng/mL)											
	D-Amphetamine						Diphenhydramine					
	1.5		15		150		2		40		400	
	Assayed (ng/mL)	RSD	Assayed (ng/mL)	RSD	Assayed (ng/mL)	RSD	Assayed (ng/mL)	RSD	Assayed (ng/mL)	RSD	Assayed (ng/mL)	RSD
Bench top stability[a]	1.5	7.6	15.0	5.2	153.4	3.4	2.0	8.8	42.1	4.7	389.0	1.8
Autosampler stability[b]	1.5	7.0	15.5	5.3	153.9	3.4	2.0	8.1	41.3	5.9	390.7	2.3
Freeze–thaw stability[c]	1.5	7.2	15.4	5.5	150.9	4.2	2.0	7.3	40.2	5.8	393.2	2.1
2-week storage stability[d]	1.5	7.3	15.5	6.1	150.8	3.7	2.1	7.5	40.7	4.6	394.8	2.8

[a] Exposed at ambient temperature (25 °C) for 10 h.
[b] Kept at ambient temperature (25 °C) for 24 h.
[c] After three freeze–thaw cycles.
[d] Stored at −20 °C.

Table 4
Sample dilution and precision

D-Amphetamine		Diphenhydramine	
Assayed concentration (ng/mL)	Reported concentration (ng/mL)	Assayed concentration (ng/mL)	Reported concentration (ng/mL)
167.3	836.5	379.1	1895.5
162.2	811.0	384.0	1920.0
161.9	809.5	396.2	1980.9
167.7	838.5	398.4	1991.9
158.8	794.0	386.7	1933.5
Mean	817.9		1944.4
CV (%)	2.3		2.1
Accuracy (%)	102.2		97.2

Nominal concentration: 800 ng/mL for D-Amphetamine and 2000 ng/mL for diphenhydramine. Dilution factor: 5.

amphetamine by LC–MS/MS in serum and plasma were summarized and compared, as shown in Table 5. Compared with the methods listed in Table 5, our method is sensitive for simultaneously determination of D-amphetamine and diphenhydramine, with LLOQ of 0.5 ng/mL for D-amphetamine and 1 ng/mL for diphenhydramine using 200 μL plasma sample, 1 mL ethyl acetate:*n*-hexane (2:3, v/v) was used in the process of one-step liquid–liquid extraction, and no interference was found from plasma matrix and herb constituents.

3.4. Application of the assay

The method described above was applied to study pharmacokinetics in six beagle dogs after an oral administration of four capsules of QAAMC. A representative chromatogram from a post-dose sample is shown in Fig. 2D. The mean plasma concentrations–time profiles of D-amphetamine and diphenhydramine after an oral administration are shown in Fig. 3. The concentration–time data were analyzed by non-compartmental method using the Bioavailability Program Package (BAPP, Version 2.0, Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the concentration–time data. Area under the plasma concentration–time curve from time zero to the last sampling time ($AUC_{0-\tau}$) was calculated by the trapezoidal rule. $AUC_{0-\infty}$ values were estimated by the combination of $AUC_{0-\tau}$ and $AUC_{\tau-\infty}$, where $AUC_{\tau-\infty}$ was calculated by dividing the last plasma concentration value by the elimination rate constant. Mean residence time (MRT) was estimated from AUMC/AUC. The pharmacokinetic parameters are shown in Table 6.

Studies on the pharmacokinetics of diphenhydramine have been reported in rabbits [9], sheep [20], human beings [19], camels and horses [33], except beagle dogs. There is only one literature on the pharmacokinetics of amphetamine in beagle dogs, reported by March et al. [11], and the pharmacokinetic parameters after an oral administration of 10 mg of 'immediate-

Table 5
Comparison of LC–MS/MS methods for amphetamine and diphenhydramine in serum and plasma

No.	Biological matrix	Sample volume (μL)	Extraction procedure	LLOQ (ng/mL)	Reference
Diphenhydramine					
1	Human plasma	500	LLE	2	[19]
2	Ovine plasma	1000	LLE	0.2	[20]
3	Dog plasma	200	LLE	1	Present method
Amphetamine					
4	Human plasma	50	PP	0.5	[22]
5	Rat plasma	100	PP	1	[28]
6	Human plasma	1000	SPE	2	[29]
7	Human serum	3000	SPME	0.3	[30]
8	Human plasma	200	SPE	2	[31]
9	Rat serum	150	SPE	0.3	[21]
10	Human serum	1000	SPE	1.4	[32]
11	Dog plasma	200	LLE	0.5	Present method

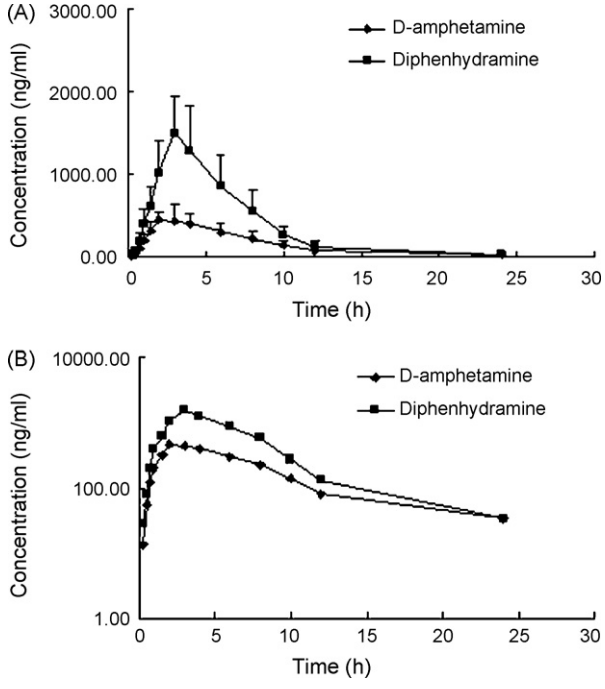

Fig. 3. Mean plasma concentration vs. time after an oral administration of four capsules of QAAMC in six beagle dogs. (A) Linear and (B) log-transform scale.

Table 6
Mean pharmacokinetic parameters after oral administration of four capsules of QAAMC in six beagle dogs

Pharmacokinetic parameters	D-Amphetamine	Diphenhydramine
T_{max} (h)	2.3 ± 0.5	3.2 ± 0.4
C_{max} (ng/mL)	450.1 ± 95.0	1501.0 ± 452.9
$t_{1/2}$ (h)	5.6 ± 2.4	4.4 ± 0.9
MRT (h)	11.6 ± 3.9	6.5 ± 0.8
$AUC_{0-\tau}$ (μg h/mL)	3.8 ± 1.6	9.2 ± 3.1
$AUC_{0-\infty}$ (μg h/mL)	4.1 ± 2.1	9.5 ± 3.2

release' amphetamine dose are as follows: C_{max} = 86.6 ng/mL, T_{max} = 1 h, $AUC_{0-\tau}$ = 0.56 μg h/mL, $AUC_{0-\infty}$ = 0.67 μg h/mL. In our study, the concentration of D-amphetamine increasd gradually up to 450.1 ng/mL at 2.3 h after an oral administration of 4 QAAMCs, corresponding to 20 mg D-amphetamine, $AUC_{0-\tau}$ and AUC $_{0-\infty}$ are 3.8 and 4.1 μg h/mL, respectively. These discrepancies of pharmacokinetic parameters of D-amphetamine should be mainly due to drug formulation difference and origination variability of beagle dogs. Ginger extraction, the main moiety of QAAMC, was co-administrated with D-amphetamine, raising the potential of herb–drug interaction. It is important to state that herb–drug interaction is widely existed, useful literature reviews on this topic was reported by Z. Hu et al. [34]. Therefore, the interaction between ginger extraction and D-amphetamine should be paid much attention to in further study.

4. Conclusion

An LC–MS/MS method was developed and validated for the simultaneous determination of D-amphetamine and diphenhydramine in beagle dog plasma. The method is very sensitive with an LLOQ of 0.5 ng/mL for D-amphetamine and 1 ng/mL for diphenhydramine using small sample volume (200 μL). Good linearity, precision and accuracy were achieved. The method was suitable for the pharmacokinetic studies of QAAMC containing D-amphetamine and diphenhydramine.

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