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Enantioselective determination of dencichine in rabbit plasma by high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

An analytical method was developed for the determination of enantiomers of dencichine in plasma. Sample extraction from plasma was achieved by a solid-phase extraction (SPE) procedure using a C_{18} cartridge, with carbocisteine as the internal standard. Plasma was deproteinized using inorganic acid and derivatizated before the SPE. Chiral separation of dencichine enantiomers was achieved by pre-column derivatization using *o*-phthaldialdehyde (OPA) and the chiral thiol *N*-isobutanoyl-L-cysteine (NIBC) to form diastereoisomeric isoindole derivatives that were separable by ODS column using a gradient solvent programme. The column eluent was monitored using mass spectrometry (MS). The conditions of MS detection were optimized, and selected ion monitoring was used to selectively detect D-dencichine and its arrangement isomer. High sensitivity and selectivity were obtained using this method. The limit of detection was determined to be 10 ng/ml for D-dencichine and 8 ng/ml for L-dencichine in plasma. The linearity was demonstrated over a wide range of concentrations, from 0.5 to 50 μg/ml for both enatiomers. The intra- and inter-day precision (C.V.), studied at four concentrations, was less than 7.0%. No interferences from endogenous amino acids and isomers of dencichine were found. The method was suitable for pharmacokinetic studies of dencichine enantiomers.

Keywords: Dencichine; Eanatiomer separation; LC-ESI-MS

1. Introduction

Dencichine (Den, Fig. 1), named β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP), is the major non-protein amino acid that is isolated from the seeds of *Lathyrus sativus* and that is detected in several other plants, including other genera of leguminous and non-leguminous plants, such as *Panax ginseng*, *Panax notoginseng* and *Panax quinquefolius* [1–3]. Den has attracted scientists' attention for its particular neuro-excitatory properties. It has been found that the excessive consumption of *Lathyrus sativus* seeds can cause degeneration of the upper neurons (lathrism), which is believed to be caused by β-ODAP [4]. It has long been viewed as one of the neurotoxins. In the 1980s, the Japanese scientists Kosuge et al. [5] found that Den was the most effective compound in his pharmacology research of the homeostatic effects of *Panax notoginseng*. Zhao et al. [6], Zheng et al. [7] and Liu et al. [8] further studied the mechanism of Den's homeostatic action.

Den is a chiral molecule, as shown in Fig. 1, in which the original α-carbon atom represents the single optically active centre of the molecule. Den that is isolated from the seeds is an L-isomer. As shown by early stereoselective studies of Den enantiomers, D-Den demonstrated a different toxicological profile to L-Den. Rao [9] found that, unlike L-Den, the naturally occurring neurotoxin D-Den failed to produce any neurological or visible toxic symptoms when administrated to chicks, even at a high dose. As D-Den is not neurotoxic, it implies that whatever the mechanism of toxicity of the L-isomer is, it is the result of a stereospecific interaction. Kosuge's study [10] revealed that the D-isomer had the same homeostatic effect as the L-isomer, without neurotoxic effects. Therefore, to study further the different profiles of Den enantiomers in pharmacokinetics, metabolic

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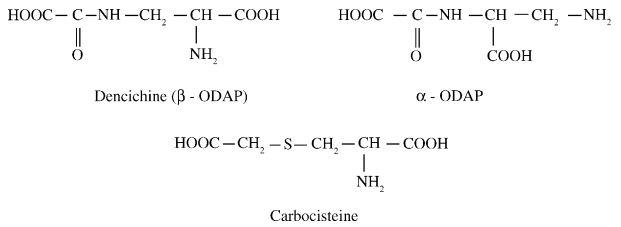

Fig. 1. Structures of Dencichine (β-ODAP), α-ODAP and Carbocisteine (I.S.).

pathways and toxicology, an enantioselective assay for Den is required.

Although the steroselective determination of Den has not been reported in the literature yet, the compound itself was already the subject of many analytical studies [11–20]. The most widely used method for determining Den utilizes the reaction of *o*-phthaldialdehyde (OPA) with 2,3-diaminopropionic acid (DAP), formed by hydrolysis of Den and measurement of the absorbance at 420 nm [11]. A few of high-performance liquid chromatography (HPLC) methods have been developed in recent years, including that using dansyl-Cl [12], 9-fluorenyl-methyl chloroformate [13], phenyl isothiocyanate [14] and 1-fluoro-2,4-dinitrobenzene [15] pre-column derivatization methods. Capillary zone electrophoresis (CZE) [16,17] and LC-biosensor methods [18,19] were also developed for the determination of Den. For the analysis of Den in biological fluids, the only method reported makes use of derivation HPLC with 2, 4-diphenylhydrazine as the derivate reagent [20].

This manuscript reports the development of the separation of Den enantiomers, which is based on pre-column derivation liquid chromatography–mass spectrometry (LC–MS) with selected ion monitoring (SIM). The lower limit of quantification (LLOQ) of the LC–MS method is 50 ng/ml per enantiomer and the lower limit of detection (LLOD) is 10 ng/ml. The method is reproducible and accurate and has been applied to the study of stereoselective pharmacokinetics of Den in rabbits after i.v. administration.

2. Experimental

2.1. Chemicals and reagents

DL-Den and L-Den were synthesized in our laboratory according to previously published methods [9,20]. The identity of the compounds was checked by means of melting point and optical rotation determination, MS and nuclear magnetic resonance spectroscopy. The internal standard carbocisteine was obtained from the National Institution for the control of Pharmaceutical and Biological Products (Beijing, China). The derivatizing reagents OPA and *N*-isobutanoyl-L-cysteine (NIBC) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). HPLC-grade methanol was supplied by Fisher ChemAlert (NJ, USA). All other chemicals used were of analytical-reagent grade.

Borate buffer (0.8 M, pH 12.5) was prepared by dissolving 2.47 g boric acid in 50 ml 1 M NaOH solution. Borate buffer (0.05 M, pH 10) was prepared by dissolving 0.15 g boric acid in 50 ml water. The pH was adjusted to 12.5 and 10 with NaOH solution. Stock solution of OPA was prepared by dissolving 0.268 g OPA in 10.0 ml methanol. The derivatizing reagent working solution was prepared by dissolving 56 mg NIBC in 1.5 ml borate buffer (0.05 M, pH 10) and then by adding 500 μl OPA stock solution. The storage life of the stock solution is at least 4 weeks at 4 °C; the working solution must be prepared daily.

2.2. Instruments

The analytical system consisted of a Series 1100 LC–MS system (Agilient Technologies, USA) that was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a thermostated column compartment (G1316A), a variable wavelength ultraviolet light detector (G1314A), a mass-selective detector (G1946B) supplied with an atmospheric pressure ionization electrospray (API-ES) interface and an online nitrogen generation system (Whatman, USA).

2.3. Chromatographic conditions

The separation of DL-Den was performed on an Intersil C_{18} column (250 mm × 4.6 mm I.D., 5 μm), supplied by GL Sciences (Tokyo, Japan), with a native C_{18} guard column (10 mm × 4.6 mm I.D., 10 μm). The mobile phase consisted of a mixture of methanol (A) and 20 mM ammonium acetate buffer (pH adjusted to 7.10 with ammonium hydroxide) (B), and was delivered as a gradient at a flow rate of 1.0 ml/min: 14% A (0 min); 14% A (12 min); 47% A (22 min); 90% A (22.01 min); 90% A (28 min); 14% A (28.01 min). The injection volume was 20 μl and the column temperature was operated at 25 °C. The post-column split of 1:2 was used. The smaller flow of the split was connected to the MSD and the larger flow was connected to an ultraviolet-light detector at 338 nm.

2.4. Optimization of the mass parameters

MS detection used an electrospray ionisation (ESI) source operating in the negative-ion mode. Mass spectra were recorded using a full-scan from 50 to 600 *m/z*. SIM was used to quantify the target compounds. The chromatograms were monitored at 174,219 *m/z* for Den enantiomers and Car (I.S.).

The sensitivity of the Den signals was primarily dependent on the MSD experimental parameters. To identify the optimized conditions, the following MSD parameters were investigated: capillary voltage (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 kV); nebulizer pressure (20, 30, 35, 40, 45, 50 psig); drying gas temperature (200, 250, 300 and 350 °C); drying gas flow (7, 9, 10, 11 and 13 l/min) and fragmentation voltage (90, 100, 110, 120, 130 and 150 V). The optimized parameters were as follows: capillary voltage, 3.5 kV; nebulizer pressure, 35 psig; drying gas flow rate, 10.0 l/min; drying gas temperature, 350 °C; and fragmentor voltage, 110 V.

2.5. Sample preparation

Plasma samples (300 μl) were spiked with 50 μl of the internal standard solution (carbocisteine, 32 μg/ml) and 300 μl of a

6% solution of perchloric acid. The mixture was vortexed for 10 s and then centrifuged at 2000 × *g* for 5 min. The supernatants were transferred to conical tubes, and 200 μl borate buffer (0.8 M, pH 12.5) and 100 μl OPA-*N*-isobutyl-L-cysteine working reagent were added and mixed for 2 min. The resulting solution was acidified with 50 μl HAc, and added to a preconditioned 1 ml SPE cartridge. The cartridge was conditioned with 1 ml of methanol followed by 1 ml of water. After addition of the solution, the conditioned cartridge was washed with 2 ml of water and the retained compounds were eluted with 1 ml methanol–ammonia (99.5:0.5, v/v). The eluents were evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The resulting residues were dissolved in 100 μl methanol, transferred to a MS sample vial and stored at −20 °C prior to analysis by LC–MS.

2.6. Validation of the HPLC–MS method

The determination of Den was based on the internal standard method, using carbocisteine as the internal standard. Calibration curves were prepared by analysing 0.5 ml drug-free plasma samples that were spiked with a standard rac-Den solution in duplicate, resulting in plasma concentrations of 0.05, 0.08, 0.2, 1, 5, 10, 25 and 50 μg/ml of each enantiomer. Sample preparation and chromatographic conditions were as described previously. Plots of plasma concentrations versus peak height ratios, D- and L-Den/I.S., were constructed and the linear regression lines were used for the determination of enantiomer concentration in plasma samples. Linearity of the method was obtained by analysing calibration samples in the concentration range of 0.05–50 μg/ml for each Den enantiomer.

The precision and the accuracy of the method were evaluated by analysing the D- and L-Den enantiomers in plasma samples that had been spiked with four concentrations (0.1, 2.5, 12.5 and 40 μg/ml) of each enantiomer (quality-control (QC) samples). Aliquots of spiked plasma samples were stored at −20 °C and analysed in replicate experiments ($n = 6$) using a single calibration curve with intra-assay evaluation and in duplicate on five consecutive days for inter-assay evaluation. Accuracy was determined by comparing the observed concentrations of the QC standards that had been calculated from the calibration curve to their nominal concentrations.

Extraction efficiencies (% recovery) of D- and L-Den were determined by comparing peak heights for the QC standards to the peak heights that resulted from the chromatography of standard solutions containing the equivalent final concentrations.

Sensitivity was evaluated by determining the quantization limit (LOQ). The LOQ was obtained by the analysis of plasma samples that had been spiked with rac-Den. The LOQ was defined as the lowest plasma concentration for the calibration curve and analysed with an error ≤20% and a coefficient of variation ≤15%.

2.7. Application of the analytical method

Six Japanese white rabbits, weighting about 2.5 kg, were purchased from the Experimental Animal Center of Guiyang Medical School (Guizhou, China) and used in this study. All animals received water and food *ad libitum*. Before administration of Den, rabbits were fasted for 12 h. The rabbits were injected intravenously with a single dose of 30 mg/kg of Den racemate. Blood was sampled by using the marginal-ear-vein bleeding technique; the sampling time points were as follows: 0 (prior to the injection), 0.25, 0.5, 0.75, 1.5, 3, 5, 8, 12, 24 and 36 h after dosing. The blood samples (0.6 ml at each time point) were centrifuged, separated and frozen at −20 °C until analysis.

3. Results and discussion

3.1. Chiral separation of dencichine

In the past decades, a large number of methods, involving both the direct method using a chiral stationary phase (CSP) and an indirect method requiring derivatization with chiral reagents, have been developed for the resolution of amino-acid enantiomers. To establish accuracy, the simple method of enantiomer separation of Den, chiral gas chromatography, and direct and indirect HPLC, were investigated in this study.

3.1.1. GC separation of enantiomers

Den is a non-protein amino acid. Separation of Den enantiomers by chiral gas chromatography requires derivatization of the polar groups in order to increase volatility and to introduce suitable functions for additional chiral association. The *N*-acetylated isopropyl esters of D- and L-Den were prepared by reaction with isopropanol in 3 M HCl, following reaction with acetic anhydride [21]. The separation of derivatives was carried out on hydrogen-bonding CSP, L-Chirasil-Val (*tert*-butylamide-linked polydimethyl-siloxane).

However, the derivatization of Den was not a single reaction that produced a couple of enantiomeric peaks. Two side-reactions occurred and two groups of new enantiomeric peaks of side-products appeared in the chromatogram. As the reproducible and quantitative derivatization of Den was difficult to achieve, chiral gas chromatography cannot be applied to quantitative determination of Den enantiomers.

3.1.2. HPLC separation of enantiomers

3.1.2.1. Direct separation during the chiral stationary phase. In the initial experiments for the HPLC enantioseparation of Den, a chiral crown ether-based column, Crownpak CR (+) (150 mm × 4 mm I.D., 5 μm), was applied. This column can resolve compounds that bear a primary amino group near the chiral centre. The experiments were carried out at 25 °C with perchloric acid (pH 2.0) as the mobile phase, at a flow rate of 0.5 ml/min. Under these conditions, the stereoisomers obtained partial separation. According to the optimization of chromatography conditions, the resolution can be improved but the optimal $Rs \geq 1.5$ was not reached. Den underwent partial separation on this CSP.

3.1.2.2. Indirect HPLC separation. Indirect separation of amino-acid enantiomers is based on the use of chiral derivati-

zation reagents to yield diastereoisomeric derivatives that differ in their chemical and physical behaviour and, therefore, can be separated during the achiral stationary phase. In our trials, Den enantiomers were derivatized with OPA and *N*-acyl-L-cysteine to form diastereoisomic isoindols that were efficiently resolved on conventional reversed-phase columns.

It has been reported that the chiral thiol that is used for derivatization greatly influences the chromatographic behaviour of derivatives formed, and plays an important role in the resolution of amino-acid enantiomers. The homologous series of *N*-acyl-L-cysteines–including *N*-acetyl, *N*-propyl, *N*-butyl and *N*-isobutyl-L-cysteine–were synthesized according to previously published methods [22–24] and were systematically investigated for their abilities to separate D- and L-Den under the same chromatographic conditions. Rs values were 0.923, 1.83, 3.28 and 5.03 in *N*-acetyl and *N*-isobutyl derivatives. Among the various reagents tested for the suitability for separating D- and L-Den, NIBC was selected as the most suitable reagent to obtain the best resolution in a shorter retention time period.

The elution order of Den enantiomers by this method is L-Den followed by D-Den. The peak assignment was performed by comparing the retention times of enantiomerically pure L-Den standard with those of racemate.

3.2. Optimization of the mass spectrometric detection

3.2.1. Selection of the monitored ions

It is already documented in the literature that Den (β-ODAP) is not stable in aqueous solution. It undergoes a transformation to α-ODAP until an equilibrium mixture of β-ODAP to α-ODAP (3:2 ratio) is reached at an elevated temperature of about 55 °C [25,26]. Furthermore, Mehta et al. [20] found the presence of α-ODAP in extracts of monkey tissues during their studies on tissue distribution and metabolism of Den. α- and β-ODAP have similar structures and differ in the position of the oxalyl moiety (see Fig. 1). A preliminary investigation of a mixture of L-Den and α-isomers (obtained by heating the L-Den standard solution at 55 °C for 8 h) showed that the α-isomer has the same retention time as D-Den under the described chromatographic conditions, with ultraviolet light detecting (338 nm) and interfering with the quantification of D-Den. The separation of α-isomer and D-Den cannot be achieved through the optimization of chromatographic conditions. The ultraviolet light or fluorescence detector does not discriminate between D-Den and the α-isomer. The SIM technique of MS has been used to resolve this problem.

In the collision-induced dissociation (CID) zone, the energy of the collision can be conveniently controlled by changing the

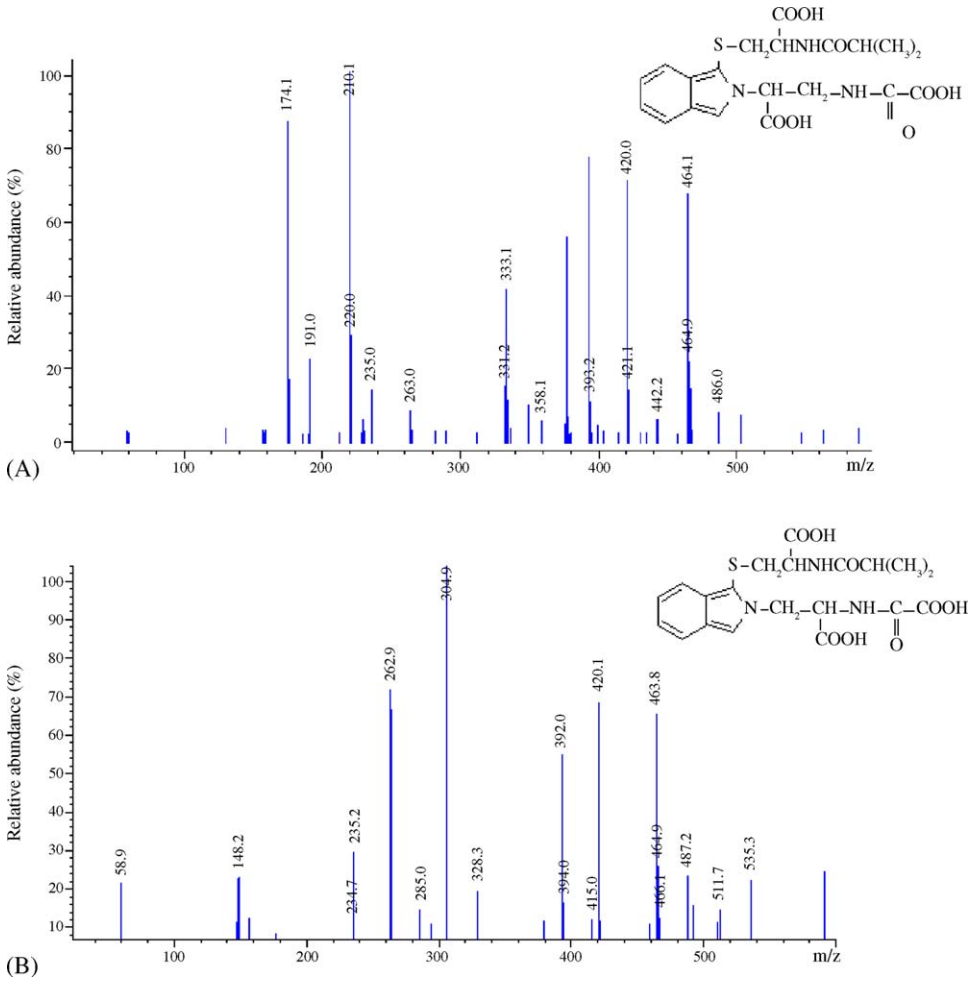

Fig. 2. Mass spectrums of OPA/NIBC derivatives of Den(β-ODAP) (A); mass spectrums of OPA/NIBC derivatives of α-ODAP (B).

voltage between the skimmer and the exit of the capillary, which may be used to vary the amount and characteristics of fragmentation observed. In the study, pure α-ODAP and D-Den should have been injected into the present system to select the suitable detection conditions; however, this was not possible due to the absence of any commercial preparation of pure α-ODAP. An alternative way was to use heated and transformed L-Den as test solution; on-column full-scan MS data were acquired for the OPA/NIBC derivatives of L-Den and α-ODAP. To investigate the difference between the mass spectra of L-Den and α-ODAP derivatives, total ion chromatograms (TIC) were recorded at different fragmentation voltages, varying from 30 to 150 V. It was observed that the absolute intensity of the 464 *m/z* ion (molecular ion) showed a maximum; other characteristic ions were absent in the spectra of L-Den and α-ODAP derivatives at fragmentation voltages between 30 and 80 V. There was no difference between the two mass spectra. As the fragmentation voltages increased in the range 90–130 V, the intensity of 464 *m/z* ion decreased and fragment ions increased. Mass spectra for the OPA/NIBC L-Den and α-ODAP at the fragmentation voltage 100 V are shown in Fig. 2. The typical differences were noted between the two mass spectra. The peaks at 174,219 *m/z* were prominent in the case of L-Den, whereas they did not occur in the mass spectra of α-ODAP.

Following this, the rac-Den was analysed under the same conditions. The D-Den was equivalent to the L-form with respect to the mass peak and their intensities. To separate Den and α-ODAP, therefore, the characteristic 174,219 *m/z* fragment ions of Den were chosen for SIM.

3.2.2. MS parameters

As the monitored ions were selected, the purpose of the optimization of the mass-selective detector parameters was to find the optimal nebulization conditions of the sample solution and ionization of the analytes. Based on the maximum response (peak height), the optimized parameters were as follows: capillary voltage, 3.5 kV; nebulizer pressure, 35 psig; drying gas temperature, 350 °C; drying gas flow rate, 10 l/min; and fragmentation voltage, 110 V.

3.3. Sample preparation

Using the mass spectrometer as the detector, the complex matrix of biological samples may have an influence on the performance of it. It is well known that electrospray ionization suffers from suppression effects when polar/ionic compounds other than the analytes of interest, such as protein, originating from the biological sample are present. Matrix effects have two distinct implications for quantitative procedures: a loss of absolute sensitivity may render the analyte of interest undetectable; the accuracy and precision of the determination may well be affected. Sample preparation is, therefore, an important process in the quantitative determination of target compounds in biological matrices by MS detection. Depending on the nature of Den and its OPA/NIBC derivative isoindole, we have investigated liquid–liquid extraction (LLE) and solid-phase extraction (SPE).

Liquid–liquid extraction was first considered for sample preparation in the study. It can be seen from the structure of Den that it is a polar compound, and LLE into a water-immiscible organic medium can, therefore, be achieved only with difficulty. However, the formed isoindole after derivatization is more hydrophobic and may be extracted with organic solvents. Meanwhile, non-volatile buffers that are used in derivatization can be removed with LLE. This method was evaluated in terms of recovery of both Den enantiomers and carbocisteine (I.S.). Although Mehta et al. [20] had successfully used ethyl acetate to extract 2, 4-dinitropheylhydrazine Den derivative and determined Den in monkey tissues, the recovery of LLE was found to be remarkably low in our study. By optimizing the extraction conditions of LLE, including extracting with various organic solvents – such as ethyl acetate, acetone, dichloromethane, chloroform and toluene – extracting under different pH and increasing the volume of extraction solvent, it was observed that absolute recovery did not obviously increase. According to the experiments performed in our laboratories, such a procedure gave a 20–25% recovery for Den from rabbit plasma in the concentration range of 100–400 μg/ml. The recovery decreased strongly below 100 μg/ml and is negligible at the level of 20 μg/ml. The same result was observed when extraction was performed from H_2O instead of plasma, which indicated that the reason for the low recovery of Den from plasma was not due to the high affinity of the drug for albumins. It may be attributed to the long and polar side-chain of the amino acid and thiol moieties in the structure of the derivative isoindole, which made the solution of isoindole in organic medium limited, and resulted in the loss of analytes. The low recoveries are unacceptable for pharmacokinetic measurements when low amounts of drug are administrated or when low drug concentrations exist for a long time after administration.

As the application of the method mentioned above was unsuccessful, the SPE procedure was developed using RP C_{18} cartridges (see Section 2.5). Extraction of Den and I.S. from plasma included three procedures: precipitation, derivatization and solid-phase extraction. It should be emphasized that with and without a protein precipitation and centrifugation step yielded different results in terms of MS detection. The initial work was performed without incorporating a protein precipitation step. After direct derivatizing and loading of the plasma sample, the SPE cartridge was washed with water to eliminate salts and proteins, respectively. The analytes were eluted with alkali methanol (0.5% ammonia). The result demonstrated that some protein remained on the cartridge column and eluted with target compounds. The matrix effects originating from plasma proteins were prominent. The ionization of Den was extensively suppressed, although it had no influence on the I.S. ionization. As a result, the sensitivity for Den was low and reproducibility was bad. We solved the problem by adding a protein precipitation step. The rabbit plasma was deproteinized with a 6% $HClO_4$ solution prior to the SPE step to remove most of the proteins in the plasma. In this way, a clean final solution was obtained.

Using the SPE sample preparation protocol described in Section 2.5, we were able to achieve excellent recoveries for both Den enantiomers and I.S. The results are summarized in Table 1. The recovery values of 75% or higher indicate that isoindole

Table 1
Recoveries of Den enantiomers and carbocisteine (I.S.) from plasma by the SPE procedure ($n = 3$)

Concentration spiked (μg/ml)	L-Den		D-Den	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
0.05	79.5	7.2	76.5	6.8
1	73.1	9.7	75.7	8.5
10	83.5	6.3	86.6	9.6
50	89.6	5.1	91.2	3.6
CAR (I.S.) 32 μg/ml	91.5	4.1		

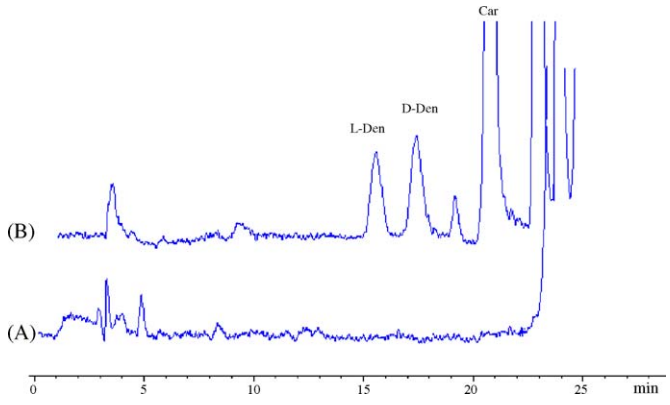

Fig. 3. Chromatograms of: (A) drug-free blank plasma; (B) plasma sample spiked with 0.08 μg/ml of per cichine enantiomers and 32 μg/ml I.S.

derivatives have a good retention on the C_{18} cartridges. This feature makes it suitable for the chiral determination of Den at low concentration, where ample pre-concentration is required for analytical procedures.

Fig. 3 presents the HPLC–MS chromatograms obtained from a drug-free blank plasma and from the rabbit plasma spiked with Den (0.08 μg/ml per enantiomer) calibrators, which contained 32 μg/ml of the internal standard.

3.4. Linearity and detection limits

Calibration curves were generated by weighted ($1/x^2$) least squares linear regression. The linear relationships between peak height ratio and drug enantiomer concentrations for Den in the range of 0.05–50 μg/ml were described by the following equations: $Y = 0.0312X + 0.0027$, $r^2 = 0.9996$ (L-Den); $Y = 0.0385X + 0.0048$, $r^2 = 0.9994$ (D-Den). The data were based on three replicates of an eight-point calibration curve.

The LLOQ is the lowest concentration of the drug in the matrix that can be determined with a high percentage of accuracy (80–120%) and precision (C.V. $\leq$ 15%). The LLOQ for Den was 50 ng/ml per enantiomer. The LLOD was defined as the concentration of the compound at which the signal versus noise ratio (S/N) was equal to 3. For each enantiomer, the LLOD was 10 ng/ml for L-Den and 8 ng/ml for D-Den.

3.5. Accuracy and precision

Accuracy and precision of the method for Den were evaluated from sextuplet analysis of each QC standard level (0.1, 2.5, 12.5 and 40 μg/ml) and repeated for 5 days.

The intra- and inter-day precision of the method were determined as the coefficient of variance (C.V. (%)). The results were

Table 2
Intra-assay validation of L-Den and D-Den

Added amout (μg/ml)	L-Den				D-Den			
	Mean (μg/ml)	SD (μg/ml)	C.V. (%)	Accuracy (%)	Mean (μg/ml)	SD (μg/ml)	C.V. (%)	Accuracy (%)
0.1	0.0972	0.00463	4.8	−3.3	0.105	0.00599	5.7	0.5
2.5	2.57	0.129	5.0	2.8	2.48	0.114	4.6	−0.8
12.5	12.6	0.268	2.1	0.8	12.7	0.347	2.7	1.6
40	39.9	0.560	1.4	−0.3	40.4	0.352	0.9	1.0

Values were obtained from six replicates.

Table 3
Inter-assay validation of L-Den and D-Den

Added amout (μg/ml)	L-Den				D-Den			
	Mean (μg/ml)	SD (μg/ml)	C.V. (%)	Accuracy (%)	Mean (μg/ml)	SD (μg/ml)	C.V. (%)	Accuracy (%)
0.1	0.102	0.00571	5.6	2.0	0.0988	0.00617	6.2	−1.2
2.5	2.54	0.0762	3.0	1.6	2.60	0.0843	3.2	4.0
12.5	12.6	0.603	4.8	0.8	12.4	0.435	3.5	−0.8
40	39.6	1.44	3.6	−1.0	40.7	1.09	2.7	1.8

Values were obtained from five replicates.

≤7.0% for L-Den and D-Den. The data for L- and D-Den are shown in Tables 2 and 3. The results of the validation studies demonstrate that the method has acceptable levels of accuracy and precision.

3.6. Specificity

The specificity of the method was examined by analysis of blank rabbit plasma and a mixture of L-Den and α-isomer. As shown in Fig. 3A, no interference from endogenous amino acids was detected in the corresponding retention times of L-, D-Den and I.S., respectively.

Fig. 4A and B present the chromatograms of a mixture of L-Den and α-ODAP obtained using ultraviolet light (338 nm) and MS (SIM, 174,219 *m/z*) as detector, respectively. It can be seen that, although the chromatographic peak of α-ODAP arose in the retention time of D-Den obtained using ultraviolet light, there was no interference at the corresponding position in the mass chromatogram. This result demonstrated that the interference that comes from α-ODAP can be eliminated under the described conditions using MS detection.

3.7. Stability

The concentrations of L-Den and D-Den in spiked samples after storage at −20 °C for 7, 14, 21 and 28 days were compared with those analysed immediately following preparation. The results indicated that both L-Den and D-Den were stable for at least 28 days.

The Standard solution of L-Den was used to assess the stability of Den during the preparation procedure. The concentrations of L-Den were determined immediately following preparation of plasma samples for analysis, and were compared with the added amount. Meanwhile, the chromatographic peaks of α-ODAP and D-Den were monitored with UVD (338 nm) and MSD (SIM, 174,219 *m/z*), respectively, to investigate the arrangement and mutarotation during the preparation. The result demonstrated that decomposition and/or interconversion of analytes during the preparation of samples accounted for less than 2%.

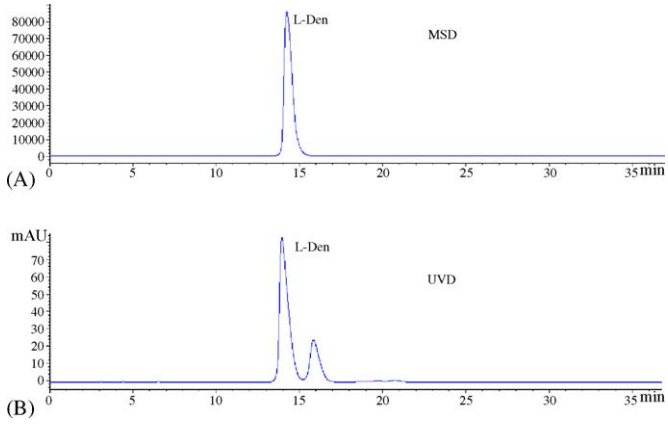

Fig. 4. Chromatograms of a mixture of L-Den and α-ODAP using MS (SIM, *m/z* 174,219) (A) and UV (338 nm) (B) detector.

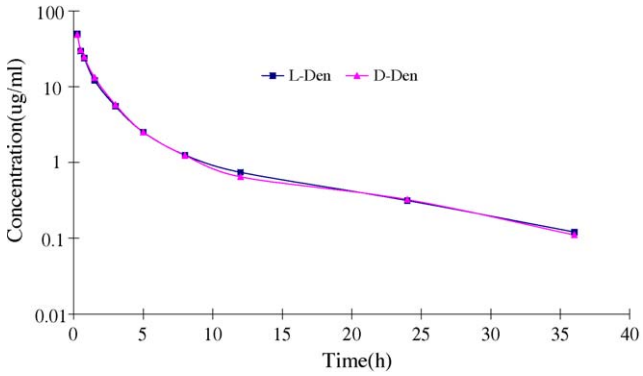

Fig. 5. Mean concentration-time profile of dencichine enantiomers after an i.v. administration of racemic dencichine at a dose of 30 mg/kg to six rabbits.

3.8. Application of the analytical method in pharmacokinetic studies

After a single i.v. administration of racemic Den at a dose of 30 mg/kg to six rabbits, L- and D-Den were determined. Fig. 5 shows the mean plasma concentration time curves of Den enantiomers after administration ($n = 6$).

4. Conclusion

This paper reports, for the first time, the study of enantiomeric separation and enantioselective analysis of dencichine in plasma by LC–MS. The indirect HPLC method described here is simple, rapid and provides separation of individual dencichine enantiomers. The technique of SIM of MS provides the high specificity and sensitivity that is required for the determination of target compounds in complex plasma samples. The proposed method was successfully validated and dencichine enantiomers could be measured in plasma with acceptable levels of accuracy and precision. The method proved to be adequate for enantioselective pharmacokinetic studies of rac-dencichine.

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