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Sensitive and specific liquid chromatographic–tandem mass spectrometric assay for dihydroergotamine and its major metabolite in human plasma

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Abstract

A sensitive and specific procedure for the simultaneous determination of dihydroergotamine (DHE) and its 8′-hydroxylated metabolite (8′-OH-DHE) in human plasma was developed and validated. The analytes were extracted from plasma samples by liquid–liquid extraction, separated through a Zorbax C_{18} column (50×2.1 mm I.D.) and detected by tandem mass spectrometry with an electrospray ionization interface. Caroverine was used as the internal standard. The method has a lower limit of quantitation (LOQ) of 10.0 and 11.0 pg/ml for DHE and 8′-OH-DHE, respectively. The intra- and inter-run precision was measured to be below 9.1% for both DHE and 8′-OH-DHE. The inter-run accuracy was within 4% for the analytes. The overall extraction recoveries of DHE and 8′-OH-DHE were determined to be about 58 and 52% on average, respectively. The chromatographic run time was approximately 2.5 min. More than 120 samples could be assayed daily with this method, including sample preparation, data acquisition and processing. The method developed was successfully used to investigate plasma concentrations of DHE and 8′-OH-DHE in a pharmacokinetic study of volunteers who received DHE orally. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Dihydroergotamine; 8′-Hydroxydihydroergotamine

1. Introduction

The ergot alkaloids are a family of chemical entities that have many pharmacologic effects. Their diversity results from their interaction with multiple receptors, their variable receptor affinity and intrinsic activity, and their variable organ-specific receptor access. Ergotamine was one of the first ergot alkaloids to be isolated. Dihydroergotamine (DHE, Fig. 1) is synthesized by reducing an unsaturated bond in ergotamine. This modification results in a changed pharmacologic profile. DHE exhibits greater α-adrenergic antagonist activity and much less potent arterial vasoconstriction and emetic potential, which is used to prevent or treat vascular headaches and orthostatic hypotension in clinical treatments [1]. After oral administration, DHE undergoes extensive first-pass metabolism, resulting in very low plasma

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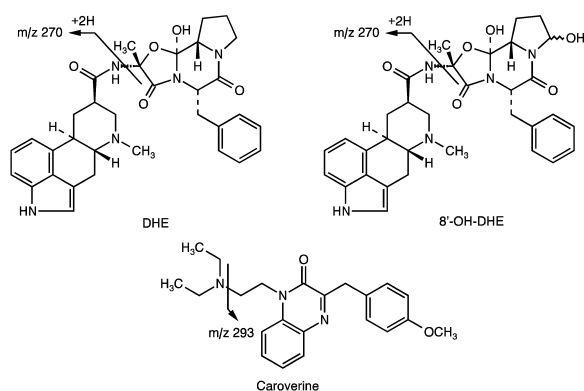

Fig. 1. Structures of DHE, 8′-OH-DHE and internal standard caroverine.

concentrations from pg/ml to low ng/ml. Several metabolites have been identified in humans and 8′-hydroxydihydroergotamine (8′-OH-DHE, Fig. 1) is expected to be the most important one [2,3]. Plasma concentrations of the metabolite after oral administration of DHE were shown to exceed those of the parent compound by several fold and its pharmacodynamic activity could be demonstrated in receptor binding studies [3].

Analytical techniques with sufficient sensitivity and specificity are needed to measure the low concentrations of DHE and its active metabolite in biological fluids. Preliminary pharmacokinetic parameters of DHE were obtained by radiolabelled drug and radioimmunoassay (RIA) methods, which were very sensitive, but lacked selectivity [4–6]. Meanwhile, some high-performance liquid chromatography (HPLC) methods with fluorescence detection were described with the limit of quantitation (LOQ) ranging from 0.1 to 0.6 ng/ml using 2.0 or 3.0 ml plasma, which did not offer a sensitivity necessary for the study of DHE pharmacokinetics following oral administration to volunteers [7–9].

The purpose of this study is to develop a highly sensitive and specific liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method for simultaneous determination of DHE and its major metabolite 8′-OH-DHE in plasma suitable for use in human pharmacokinetic investigations after oral administration of DHE.

2. Experimental

2.1. Chemicals and reagents

DHE mesylate was obtained from the United States Pharmacopoeia (Rockville, MD, USA). 8′-OH-DHE (95.7% by HPLC) was supplied by Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany), which was prepared from rat liver microsomes. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra indicated that 8′-OH-DHE was a mixture composed of two stereoisomers 8′α- and 8′β-OH-DHE in a ratio of 78:22. Caroverine (internal standard, I.S.) was obtained from Phafag (Schaanwald, Germany). Acetonitrile and methanol were of HPLC grade, and other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. LC–MS–MS instrumentation and analytical conditions

A Shimadzu LC-10AD pump (Kyoto, Japan) was used in the LC–MS–MS system. Chromatography was performed on a Zorbax SB C_{18} column (50×2.1 mm I.D., 5 μm, Hewlett-Packard, Wilmington, DE, USA) and a SecurityGuard C_{18} guard column (4×3.0 mm I.D., Phenomenex, Torrance, CA, USA) using a mobile phase of acetonitrile–water–formic acid (30:70:1.5, v/v). The liquid flow-rate was 300 μl/min. The column temperature was maintained at 25 °C.

A Finnigan TSQ (an improved type of TSQ 7000) triple quadrupole mass spectrometer (San Jose, CA, USA) interfaced with the liquid chromatograph via an electrospray ionization (ESI) source was used for mass analysis and detection. The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 4.5 kV. Nitrogen was used as the sheath gas (80 p.s.i.) and auxiliary gas (3 l/min) for nebulization (1 p.s.i.=6894.76 Pa). The heated capillary temperature was set 280 °C to assist in desolvation. The electrospray interface and mass spectrometer parameters were optimized to obtain maximum sensitivity at unit resolution. Quantitation was performed using selected reaction monitoring (SRM) of the transitions of m/z 584→m/z 269.5 for

DHE, m/z 600→m/z 269.5 for 8′-OH-DHE, and m/z 366→m/z 292.5 for the I.S., with a scan time of 0.4 s per transition. Argon was used as the collision gas at a pressure of approximately 1.4 mTorr (1 Torr=133.322 Pa). The collision energy for each ion transition was optimized to produce the highest intensity of the selected ion peak. The optimized collision energy of 42 V was used for each analyte and the I.S.

Data acquisition was performed with Xcalibur 1.1 software (Finnigan). Peak integration and calibration were performed using Finnigan LCQuan software. Calibration curves were constructed by plotting peak area ratio (each analyte to the I.S.) against plasma concentration using a $1/x^2$ weighted linear regression model. Concentrations of analytes in quality control samples (QCs) or unknown samples were subsequently interpolated from these curves.

2.3. Preparation of standard and quality control samples

Stock solutions of DHE and 8′-OH-DHE were prepared in methanol to give final concentrations of 400 and 440 μg/ml, respectively. The solutions were then serially diluted with the mobile phase to obtain the desired concentrations. All concentrations were based on the free base form. The resulted working solutions were kept refrigerated (4 °C) and were prepared freshly every week. I.S. working solution (5 ng/ml) was prepared by diluting the 400 μg/ml stock solution of caroverine with the mobile phase.

The standard solutions (100 μl) were used to spike blank plasma samples (1.0 ml), either for calibration curves of both analytes or for QCs in the prestudy validation and during the pharmacokinetic study.

2.4. Sample preparation

To a 1.0-ml aliquot of plasma sample, 100 μl of the mobile phase (to maintain the same pH values as the calibration curves), 100 μl of the I.S. and 100 μl of 1 mol/l buffer (dissolving 5.35 g NH_4Cl in 100 ml concentrated aqueous ammonia) were added. The mixed samples (ca. pH 9) were extracted with 3 ml of diethyl ether by shaking for 10 min. After centrifugation at 2000 g for 5 min, the upper organic layer was removed and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase. A 20-μl aliquot of the resulting solution was injected into the LC–MS–MS system for analysis.

2.5. Method validation

The calibration curves for the determination of DHE and 8′-OH-DHE were prepared by analyzing spiked plasma samples. The samples at three concentration levels (see Table 1) were used as QCs and analyzed by the LC–MS–MS system.

During prestudy validation, the calibration curves were defined in three runs based on triplicate assays of the spiked plasma samples, and QCs were determined in replicates ($n=6$) on the same run. Overall assay performance was assessed by calculating the accuracy and intra- and inter-run precision of QCs analyzed. During routine analysis, each ana-

Table 1
Summary of precision and accuracy from QCs of human plasma extracts ($n=3$ days, six replicates per day)

Analyte	Added concentration (pg/ml)	Found concentration (pg/ml)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
DHE	10.0	9.9	9.1	3.8	−1.0
	200.0	193.0	7.9	5.2	−3.5
	800.0	780.2	5.4	4.5	−2.5
8′-OH-DHE	11.0	11.2	7.7	4.6	2.0
	219.6	213.5	6.5	4.0	−2.8
	878.4	898.5	6.0	4.7	2.3

lytical run included a set of calibration samples, a set of QCs in duplicate and the unknowns.

2.6. Application to pharmacokinetic study

The LC–MS–MS procedure developed was used to investigate plasma profiles of DHE and its major metabolite 8′-OH-DHE after a single oral dose of 20 mg DHE mesylate to 16 healthy volunteers.

3. Results and discussion

3.1. Mass spectrometry

DHE, 8′-OH-DHE and the I.S. caroverine were at first characterized by MS and MS–MS by flow injection analysis to ascertain their precursor ions and to select product ions for use in SRM, respectively.

Fig. 2 shows the full-scan Q1 mass spectra of DHE and 8′-OH-DHE in the positive-ion mode. Both formed protonated molecular ions $[M+H]^+$ as major ion peaks. Small amounts of solvent clustered ions $[M+H+CH_3CN]^+$ were also found. The product ion spectra of $[M+H]^+$ showed several fragment ions at m/z 253, 270, 297, 322 and 538 for DHE and at m/z 253, 270, 313, 322 and 554 for 8′-OH-DHE (Fig. 3). Structurally DHE consists of a D-dihydrolysergic acid linked to a tricyclic peptide by a peptide bond (see Fig. 1). The fragment ion at m/z 270, formed by breaking the single carbon–nitrogen bond of alanine in the tricyclic peptide and present in the highest abundance, was chosen to be used in the SRM acquisition for both DHE and 8′-OH-DHE. The most suitable collision energy was determined by observing the maximum response obtained for m/z 270. Using similar procedures the precursor ion of the I.S. was determined to be the base peak $[M+H]^+$ ion at m/z 366. The SRM transition of m/z 366→293 was selected.

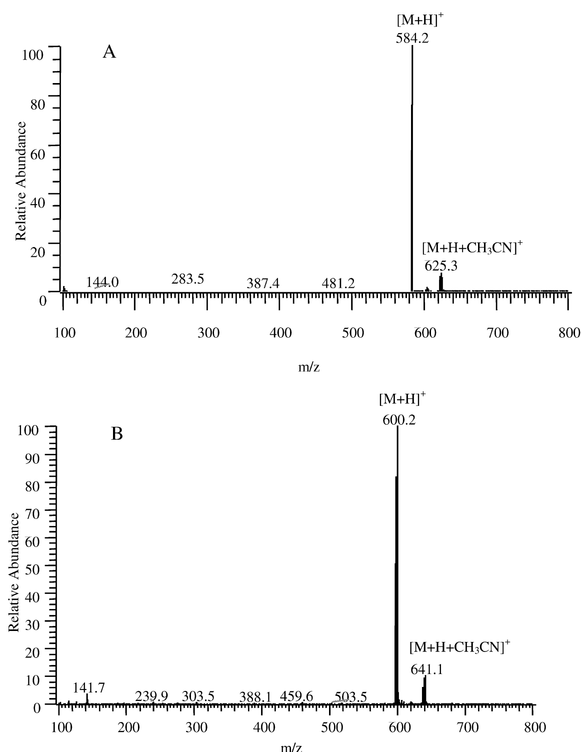

Fig. 2. Full-scan Q1 mass spectra of (A) DHE and (B) 8′-OH-DHE.

The use of atmospheric pressure ionization (APCI) for the analysis of DHE and 8′-OH-DHE was also explored. Abundant $[M+H]^+$ ions were observed for both compounds, which were identical to those obtained using ESI, but with reduced intensity (about 2.5:1). Compared to ESI, more fragment ions such as m/z 564, 380, 270, and 253 (base peak) were found in APCI product ion scan spectra of DHE, which also resulted in decreased SRM sensitivity. Therefore, APCI was not chosen for the analysis of DHE and 8′-OH-DHE.

3.2. Chromatography

LC conditions were developed to optimize for sensitivity, speed and peak shape. The contents of mobile phase were optimized by flow injection analyses with mobile phases containing varying percentages of organic solvents. It was found that the low organic solvent content (about 30%) in HPLC system decreased the background noise, and provided stable MS signal throughout an analytical run, allowing an enhancement of sensitivity. It was found that using acetonitrile was more favourable to chromatographic separation of DHE and 8′-OH-DHE isomers than using methanol, while sensitivity was the same. It was also demonstrated that the addition of acidic modifiers such as formic acid to the mobile

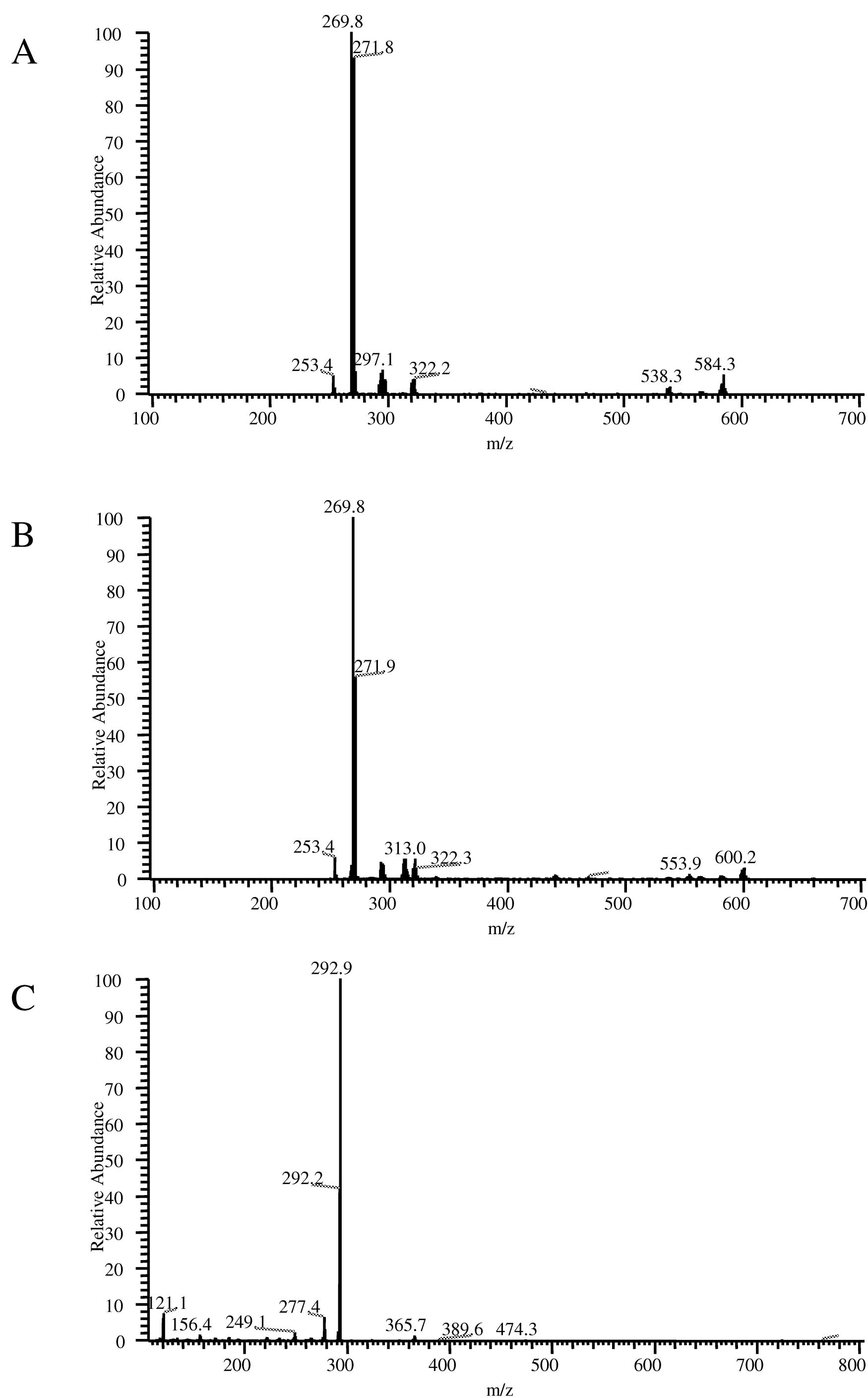

Fig. 3. Full-scan product ion spectra of $[M+H]^+$ of (A) DHE, (B) 8′-OH-DHE and (C) caroverine.

phase could improve sensitivity by promoting the ionization of the analytes.

Because a common product ion at *m/z* 270 was monitored in SRM scan mode for 8′-OH-DHE and DHE, the chromatographic separation of both analytes has to be implemented to minimize the potential for cross-talk between channels. Therefore, a short Zorbax C_{18} column (50×2.1 mm) with a 4× 3.0 mm guard column was eventually used for the analysis. As shown in Fig. 4, excellent separation was achieved for DHE and 8′-OH-DHE within 2.5 min under the described chromatographic conditions. It has been checked that the matrix components of a sample did not interfere with the analyte signals of the next samples and cause the suppression of ionization. The total chromatography time of 2.5 min made it possible to analyse a large number of samples in a relatively short period of time.

NMR data provided by Dr. Willmar Schwabe Pharmaceuticals show that the two isomers, 8′α- and 8′β-OH-DHE, were produced by 8′-hydroxylation of DHE in rat liver microsomes. The two isomers were fully separated at retention times ca. 0.9 and 1.1 min in the experiment (see Fig. 4). They corresponded to the 8′β- and 8′α-isomers, respectively, based on NMR response ratio and HPLC–UV detection. The two isomers display the same full-scan Q1 spectra. A small difference between their product ion spectra

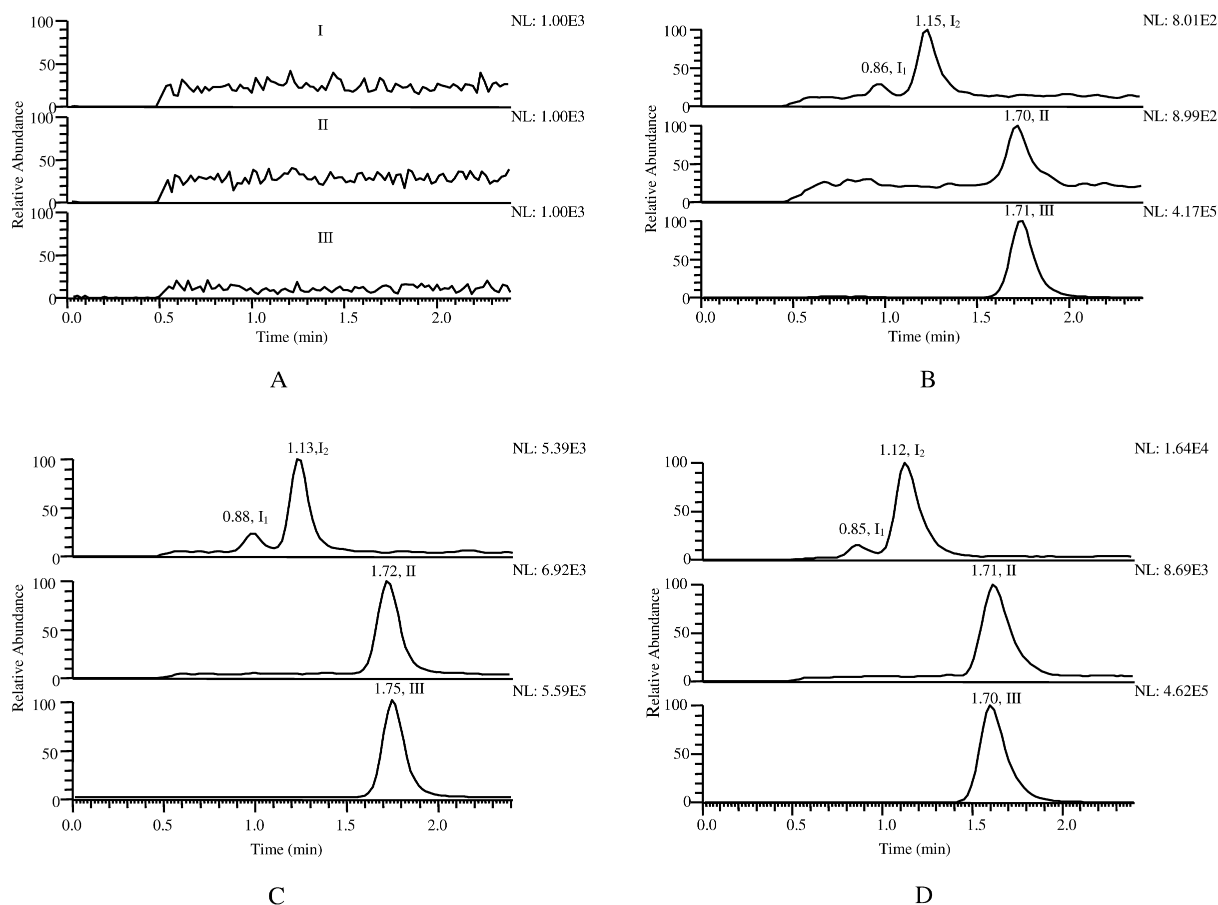

Fig. 4. Representative SRM chromatograms (A) a blank plasma sample; (B) a blank plasma sample spiked with DHE and 8′-OH-DHE at the limit of quantitation (10.0 and 11.0 pg/ml) and the internal standard caroverine (I.S., 500.0 pg/ml); (C) a blank sample spiked with DHE (200.0 pg/ml), 8′-OH-DHE (219.6 pg/ml) and I.S.; (D) plasma sample from a volunteer 0.33 h after administration of 20 mg dihydroergotamine mesylate. Peaks: I_1, 8′β-OH-DHE; I_2 8′α-OH-DHE; II, DHE; III, I.S.

were observed, in which the fragment ions of 8′β-OH-DHE at m/z 253, 296, 313 have higher response than those of 8′α-OH-DHE, while the base peak is the same at m/z 270 for the two isomers. The same peak-area ratios of 8′β- and 8′α-isomers were observed in reference (rats liver microsomes) and human plasma samples (see Fig. 4C and D). Therefore, no significant difference was shown in the stereoselectivity of 8′-hydroxylation of DHE between rats and humans. The content of 8′-OH-DHE in plasma was calculated as the sum of the two isomers.

3.3. Method validation

3.3.1. Assay specificity

The specificity of the method was demonstrated by comparing the chromatograms of six independent plasma samples from volunteers—each as a blank sample and a spiked sample. Fig. 4 demonstrates that there is no interference from endogenous substances with the analytes and I.S.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of analytes in the samples spiked post extraction with that of each analyte injected into the systems in the mobile phase. No co-eluting ‘‘unseen’’ endogenous species interfered with the ionization of the analytes and internal standard.

3.3.2. Linearity of calibration curves and lower limit of quantitation

Linear calibration curves were obtained over the concentration range of 10–1000 pg/ml for DHE and 11–1100 pg/ml for 8′-OH-DHE in human plasma. Typical equations of calibration curves were as follows:

DHE $y = 6.733 \times 10^{-4} + 7.478 \cdot 10^{-5}x$
$r = 0.9963$

8′-OH-DHE $y = 9.774 \cdot 10^{-4} + 4.658 \cdot 10^{-5}x$
$r = 0.9974$

Because the plasma concentrations of the analytes are very low, such linearity values were already satisfied. The lower limit of quantitation was 10.0 pg/ml for DHE and 11.0 pg/ml for 8′-OH-DHE, respectively. These limits are sufficient for clinical pharmacokinetic studies following oral administration.

3.3.3. Assay precision and accuracy

Intra- and inter-run precision was assessed from the results of QCs. The mean values and RSD for QCs at three concentration levels were calculated over three validation runs. Six replicates of each QC level were determined in each run. These data were then used to calculate the intra- and inter-run precision (RSD) 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).

Table 1 summarizes the intra- and inter-run precision and accuracy for DHE and 8′-OH-DHE from the QC samples. The intra-run precision was less than 9.1% for each QC level of DHE and less than 7.7% for each QC level of 8′-OH-DHE. The inter-run precision was less than 5.2% for DHE and 8′-OH-DHE. The accuracy derived from QC samples was within 4% for each QC level of DHE and 8′-OH-DHE.

3.3.4. Extraction recovery

The extraction recoveries of DHE, 8′-OH-DHE and I.S. were determined by comparing peak areas from extracted plasma samples with those from standard solutions at the same concentration. It may be more suitable to determine the recovery by comparing peak areas of analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. But the difference between both methods could not be significant, because the effect of the endogenous substances on ionization of the analytes was not observed.

The extraction recoveries of DHE were 57.9±1.9, 58.6±2.4 and 57.1±3.7% at concentrations of 25.0, 200.0, and 800.0 pg/ml, respectively; and those of 8′-OH-DHE were 54.3±1.0, 50.1±2.0 and 52.6±4.0% at concentrations of 27.4, 219.6, and 878.4 pg/ml, respectively. The extraction recovery of I.S. was 77.6±3.8%.

3.3.5. Analyte stability

The stability of DHE and 8′-OH-DHE in human plasma was investigated under a variety of storage and process conditions. The analytes were found to be stable (>88%) in human plasma after three cycles of freeze (−20 °C)–thaw (room temperature) and for 4 h at room temperature. The analytes in the plasma were also shown to be stable for at least 24 days under −20 °C freezer conditions. Processed samples which had been analyzed by LC–MS–MS were left in the tube at room temperature for 12 and 24 h, then re-analyzed on the next day. The analytes were found degraded about 20% after 24 h, but no signs of degradation were found up to 12 h under the storage conditions. These data are summarized in Table 2.

It was reported that DHE is an air-sensitive drug [9]. In our experiment, it was also observed that the response decreased about 30% when processed plasma sample was evaporated under a gentle stream of air instead of nitrogen. The storage stability of DHE and 8′-OH-DHE in working solutions was evaluated by direct injection of the solutions every day. The working solutions were found degraded more than 10% after a week stored at 4 °C. Therefore, working solutions of the analytes should be freshly prepared every week.

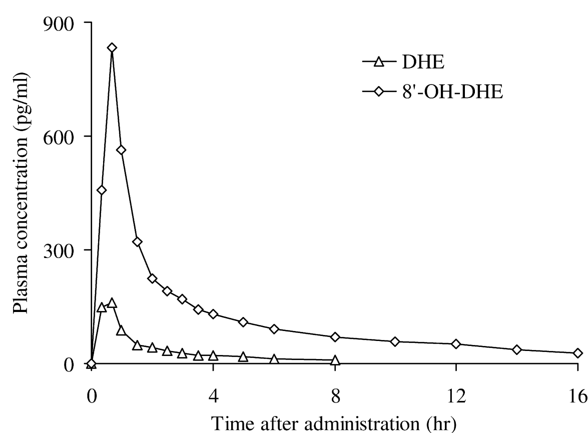

Fig. 5. Mean plasma concentration–time profile of DHE and its major metabolite 8′-OH-DHE after an oral administration of 20 mg DHE mesylate to 16 healthy volunteers.

3.4. Application of the method to pharmacokinetic study

After a single oral administration of 20 mg DHE mesylate to healthy volunteers, plasma concentrations of DHE and its metabolite 8′-OH-DHE were determined simultaneously by the described LC–MS–MS method. Fig. 5 shows mean plasma concentration–time curves of DHE and 8′-OH-DHE after administration ($n=16$).

Table 2
Stability of DHE and 8′-OH-DHE in human plasma ($n=6$)

	DHE (pg/ml)			8′-OH-DHE (pg/ml)		
	25.0	200.0	800.0	27.4	219.6	878.4
Freeze–thaw stability (relative error, %)						
0 cycles	−4.7	–	−10.5	1.1	–	−1.3
3 cycles	−0.7	–	3.1	1.4	–	0.4
Storage stability (≤−20 °C, relative error, %)						
0 day	–	−5.8	–	–	−3.8	–
24 days	–	1.9	–	–	7.0	–
Processed plasma samples at room temperature (relative error, %)						
0 h	–	−2.8	–	–	−4.6	–
12 h	–	−2.7	–	–	−2.6	–
24 h	–	−20.9	–	–	−18.9	–
Unprocessed plasma samples at room temperature (relative error, %)						
0 h	–	−5.8	–	–	−2.3	–
4 h	–	−11.3	–	–	1.4	–

4. Conclusion

An LC–MS–MS method was developed and validated for the simultaneous determination of DHE and 8′-OH-DHE in human plasma. The method is very sensitive, selective and reliable with an LOQ of 10.0 pg/ml for DHE and 11.0 pg/ml for 8′-OH-DHE. It was proved superior in sensitivity and selectivity than the reported HPLC assay with fluorescence detection, or the RIA methods. The method was used successfully to evaluate the pharmacokinetics of DHE and 8′-OH-DHE after an oral administration. More than 120 samples could be assayed daily, including sample preparation, data acquisition and processing. The method provided an example for bio-analysis of ergot alkaloids.

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