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High-performance liquid chromatography of the quinazolinocarboline alkaloid dehydroevodiamine

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the determination of dehydroevodiamine (DeHE), an active principle from *Evodia* fruit. Plasma was denatured with acetonitrile and centrifuged, the supernatant was separated and blown dry, and the residue was redissolved in water. Bile was acidified with perchloric acid and centrifuged to yield the supernatant. Aliquots were used for analysis. Elution was isocratic on a reversed-phase column with acetonitrile–water–phosphoric acid (64:35:0.8, v/v) adjusted to pH 3.5 as the mobile phase. Ultraviolet detection was at a wavelength of 367 nm. The detection limits were 2 ng/ml for plasma and 10 ng/ml for bile. The intra-day and inter-day variations were mostly below 10%.

INTRODUCTION

The dried unripe fruit from *Evodia rutaecarpa* (Juss.) Benth., popularly known in China as Wu-Chu-Yu, has been prescribed, according to traditional medical practice, for the treatment of gastrointestinal disorders, headache, abdominal pain, dysentery, postpartum haemorrhage, amenorrhea [1] or as a cardiotonic [2] and an analgesic [3]. It also possesses central stimulant [1], transient hypertensive [2] and positive inotropic and chronotropic [4] effects. However, the observed pharmacological effects may represent the combined effects of a plethora of compounds contained in the fruit of *Evodia*. Use of the fruit itself is therefore less desirable in terms of interpretation of experimental results and definitive therapeutic applications. The isolation and purification, in greater than 97% purity, of the active ingredient dehydroevodiamine (DeHE) [5] (Fig.

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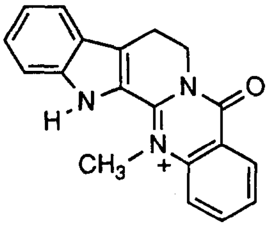

Fig. 1. Structure of dehydroevodiamine.

1) from the unripe fruit of Wu-Chu-Yu has permitted definitive studies of its pharmacological effects. Unlike the fruit itself, the demonstrated pharmacological effects of DeHE include hypotensive [6,7], negative chronotropic [7] and ion-channel depressant properties [8,9]. Because of its therapeutic potential, intensive investigations are warranted, but there is no extant method for its quantitation. This paper describes the development of a sensitive high-performance liquid chromatographic (HPLC) assay for the determination of DeHE.

EXPERIMENTAL

Chemicals

DeHE (>97% purity) was provided by the National Research Institute of Chinese Medicine (Shin-Dian, Taiwan). Berberine (internal standard) was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, orthophosphoric acid (85%) and ammonia solution (25%) were obtained from Merck (Darmstadt, Germany). Triply deionized water was used for the preparation of solutions.

Animals

Male New Zealand White rabbits, weighing 2.0–2.5 kg, were obtained from The Laboratory Animal Center at The National Yang-Ming Medical College. The animals were kept in environmentally controlled quarters at 24 + 1°C and a 12-h light–dark cycle for at least one week before being used in experiments. Standard laboratory chow and water were given *ad libitum* except for the bile experiments, when food only was withdrawn 18 h before the experiments.

Drug administration and sampling

The drug was always administered in a single bolus through the marginal vein in the left ear. For whole blood and plasma, sampling was done through the ear artery in the right ear at scheduled intervals. Both minimally restrained conscious and pentobarbital (30 mg/kg intraperitoneally) anesthetized animals were used. For bile studies, the rabbits were anesthetized with pentobarbital, and the bile duct was cannulated with polyethylene tubing (PE 160, Clay Adams). Bile collection would begin 1 h following surgery and drug administration in another hour. Bile was thereafter collected at 10 min intervals until no trace of the drug was found, which normally occurred in *ca.* 6 h.

Sample preparation

Plasma. Plasma was separated following centrifugation at 10 000 *g* for 5 min. To 0.35 ml of plasma, 0.1 ml of the internal standard berberine (0.4 μg/ml) was added. After vortex-mixing, 2 ml of acetonitrile were added to precipitate the plasma protein. Following a second centrifugation at 10 000 *g* for 5 min, the supernatant was separated and blown dry with nitrogen through a multichannel manifold with the temperature kept constant at 25°C by a heating block. The residue was finally redissolved in 300 μl of water, and 200-μl aliquots were injected into the HPLC apparatus for analysis.

Whole blood. Whole blood samples of 0.6 ml were used. The blood was placed in a freezer at −20°C fracture the cells. Further fracturing was done by sonication. A 0.2-ml volume of an aqueous solution containing the internal standard berberine (0.4 μg/ml) was then added, followed by 2.0 ml of acetonitrile. The remaining steps in handling and analysis were similar to those described for plasma.

Bile. To 100 μl of bile, 100 μl of internal standard were added, followed by 100 μl of $HClO_4$. After mixing and centrifugation at 10 000 *g* for 5 min, the supernatant was separated. Aliquots of 50 μl were injected directly into the HPLC apparatus for analysis.

HPLC conditions

The system consisted of a Micromeritics 760 solvent-delivery system (Micromeritics, Norcross, GA, USA) delivering the mobile phase, which was acetonitrile–water–phosphoric acid (64:35:0.8, v/v) with the pH adjusted to 3.5 with ammonia solution, at the constant flow-rate of 1.0 ml/min to a reversed-phase column (25 cm × 0.46 cm I.D., RP-C_{18}, 5 μm particle size; Cosmosil, Nacalia Tesque, Kyoto, Japan) fitted with a guard column (5 cm × 0.46 cm I.D., RP-C_{18}, 10 μm particle size; Cosmosil, Nacalia Tesque). Detection was performed using a Sama UV–VIS/S-3702 detector (Soma Optics, Tokyo, Japan) with the detection wavelength set at 367 nm. Chromatography was carried out isocratically at the ambient temperature of 25°C. Data were processed by a Sic Chromatocorder 12 integrator (Sic, Tokyo, Japan).

Quantitation

In order to construct a standard curve, various amounts of DeHE and a constant amount of the internal standard berberine were added to aliquots of the corresponding pooled blank biological fluid, which were then carried through the entire procedure. The amounts of DeHE used covered the expected range of DeHE in the samples to be assayed. A standard curve was constructed by plotting the amounts of spiked DeHE against the response (peak-area) ratios with the constant amount of internal standard. The amount of DeHE in a sample was calculated from the slope of the standard curve.

Recovery

Recoveries from plasma and bile were determined from extracts of blank rabbit plasma and bile to which DeHE had been added. The samples were carried through the extraction procedure, and extraction recoveries were determined by comparing the peak-area ratios from the chromatograms obtained with those obtained after direct injection of the respective reference solutions in corresponding concentrations.

Precision

Intra- and inter-day variations were determined using rabbit plasma spiked with DeHE standards. Intra-day determinations were carried out five times at different times of the day, and inter-day assays were carried out on five different days.

Stability

The stability at −20°C was determined in samples that had been frozen at −20°C for three days.

RESULTS

Optimization of HPLC conditions

Detection. The UV absorption spectrum of DeHE indicated the optimal absorption maximum was at 367 nm [10]. One of the absorption maxima for the internal standard berberine was at 343 nm [11]. It was chosen because its peak was well separated from that of DeHE, it is apparently not interfered with by compounds in plasma or bile, and it elicits a good detector response. The relative detector responses (DeHE/berberine) exhibited a linear relationship to the amounts of standards assayed in the ranged tested.

Resolution. Both DeHE and the internal standard berberine are quaternary ammonium-containing alkaloids that ordinarily do not lend themselves to good resolution by HPLC. Indeed, using the basic mobile phase components of acetonitrile and water alone, neither DeHE nor berberine was ever eluted. However, the addition of phosphoric acid and ammonia solution to the mobile phase appeared to enable these compounds to be well resolved from each other and from other potentially interfering compounds in the biological fluids. The effect of the phosphoric acid concentration was studied by varying it while the pH was held at 3.5 by adjusting with ammonia solution. The results indicated that even though the compounds could be eluted with 0.4% of phosphoric acid, the peak shapes were much better when the concentration of phosphoric acid reached 0.6%.

The effect of the pH was studied by keeping the relative proportions of acetonitrile and water relatively constant and varying the concentrations of phosphoric acid and ammonia solution. Elution patterns were studied for pH values from 2.5 to 7.5. The results indicated that the peaks appeared to be distorted at pH 5.5 and above. For pH values from 2.5 to 7.5. The results indicated that the peaks appeared to be distorted at pH 5.5 and above. For pH values from 2.5 to 4.5, the effects, with regard to selectivity and peak symmetries, were insignificant. The value of 3.5 was chosen because it was further from the lower tolerable pH limit of the reversed-phase material and it could be reached with approximately the optimal phosphoric acid concentration of 0.6%.

The final composition adopted was then 35% acetonitrile–64.4% water–0.6% phosphoric acid with the pH adjusted to 3.5. These conditions were employed for the assays of DeHE in rabbit plasma (Fig. 2), whole blood and bile (Fig. 3). DeHE and berberine were clearly separable and quantifiable under these conditions.

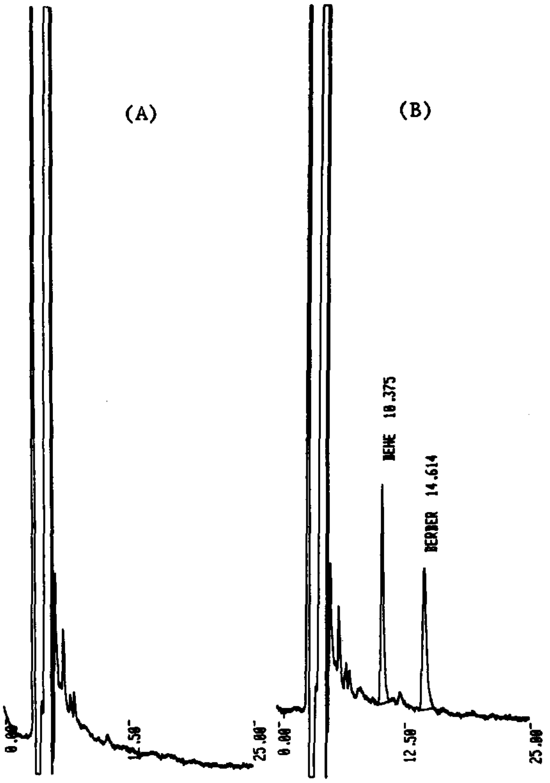

Fig. 2. Chromatograms showing the detection of DeHE in rabbit plasma. (A) Blank rabbit plasma; (B) plasma sample taken 2.5 min following intravenous administration of 0.5 mg/kg DeHE and spiked with the internal standard (berberine). The calculated DeHE concentration was 0.08 μg/ml.

Recovery

Table I shows the recoveries of DeHE in rabbit plasma and bile over the concentration ranges of 0.001–100 μg/ml for plasma and 1–50 μg/ml for bile. Recoveries ranged between 79.9 and 100.1% for plasma and 72.1 and 98.6% for bile.

Linearity, detection limit and reproducibility

In five determinations of spiked rabbit plasma (injection volume 200 μl), the response ratios for various amounts of DeHE to a fixed amount of the internal standard berberine was linear over the test range 0.005–1 μg/ml. The correlation coefficient was better than 0.999. The detection limit for DeHE in rabbit plasma was 2 ng/ml at a signal-to-noise ratio (S/N) of 4. Similarly, the standard curve for five spiked whole blood samples showed excellent linearity over the same range. Again the correlation constant was better than 0.999. The detection limit at an S/N of 4 was 2 ng/ml.

For five samples of spiked rabbit bile (injection volume 50 μl), the standard curve linearity extended over the range 1–50 μg/ml. The correlation coefficient was again greater than 0.999. The detection limit was 10 ng/ml.

DeHE spiked in rabbit plasma was used to assess the intra- and inter-day variabilities. At concentrations from 0.005 to 8 μg/ml the coefficients of variation (C.V.) ranged between 2.2 and 10.7% (Table II). Over the same concentration range, the inter-day C.V. ranged between 3.36 and 12.7% (Table II). These values indicate the good reproducibility of the assay.

Stability

As shown in Table III, DeHE spiked in plasma at concentrations ranging between 0.8 and 20 μg/ml was stable for at least three days when kept frozen at −20°C.

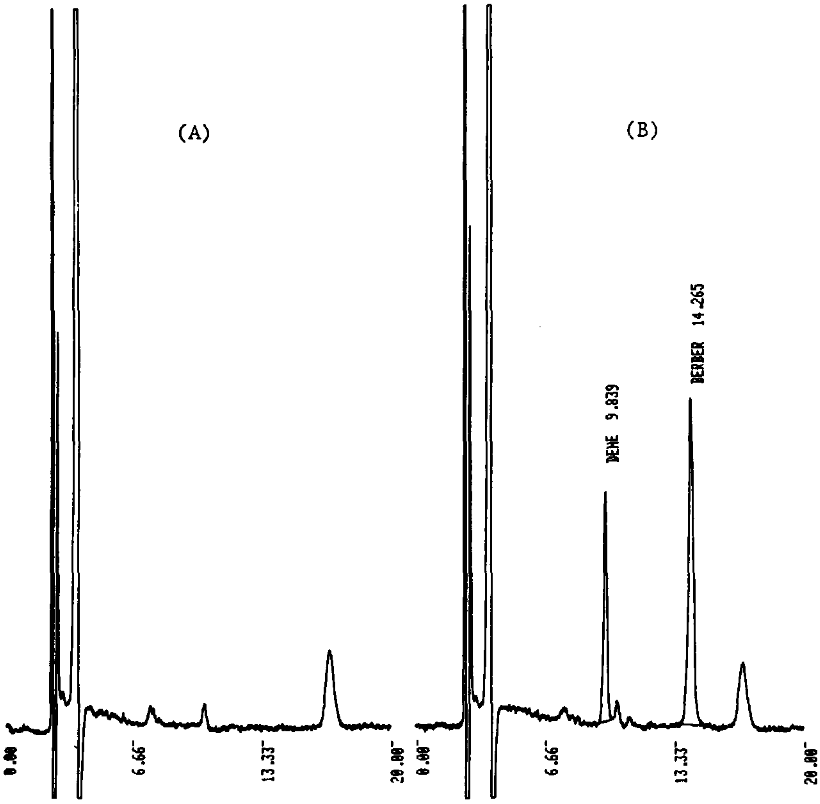

Fig. 3. Chromatograms showing the detection of DeHE in rabbit bile. (A) Blank rabbit bile; (B) bile sample taken 30 min following intravenous administration of 0.5 mg/kg DeHE and spiked with the internal standard (berberine). The calculated DeHE concentraion was 1.05 μg/ml.

TABLE I

RECOVERY OF DeHE IN RABBIT PLASMA AND BILE

Sample	Spiked concentration (μg/ml)	Recovery (mean ± S.E., n = 5) (%)
Plasma	0.001	79.7 ± 4.3
	1	90.2 ± 2.8
	10	93.2 ± 5.2
	100	100.1 ± 1.8
Bile	1	72.1 ± 1.1
	10	93.3 ± 1.0
	50	98.6 ± 0.2

TABLE II

INTRA-DAY AND INTER-DAY PRECISION TESTS FOR DeHE IN RABBIT PLASMA

Spiked concentration (μg/ml)	Measured concentration (mean ± S.E., n = 5) (μg/ml)	C.V. (%)
Intra-day		
0.005	0.0056 ± 0.0003	10.7
0.08	0.084 ± 0.001	3.88
0.8	0.763 ± 0.004	3.50
8	8.250 ± 0.08	2.20
Inter-day		
0.005	0.0053 ± 0.0003	12.7
0.08	0.079 ± 0.001	4.38
0.8	0.788 ± 0.009	2.61
8	8.965 ± 0.08	3.36

TABLE III

STABILITY OF DeHE IN PLASMA FROZEN AT −20°C AT DIFFERENT CONCENTRATIONS

Values in parentheses are coefficients of variation.

Spiked concentration (μg/ml)	Concentration (mean ± S.E., n = 5) (μg/ml)			
	Day 0	Day 1	Day 2	Day 3
0.8	0.85 (106.2%) ± 0.004	0.83 (103.7%) ± 0.015	0.82 (102.5%) ± 0.01	0.79 (98.8%) ± 0.003
4	3.85 (96.3%) ± 0.03	4.13 (103.3%) ± 0.015	3.88 (97.0%) ± 0.1	4.03 (100.7%) ± 0.05
20	19.69 (98.5%) ± 0.07	19.62 (98.1%) ± 0.04	18.57 (92.9%) ± 0.17	18.54 (92.7%) ± 0.14

DISCUSSION

In order to study the pharmacokinetics and disposition of drugs in the body, accurate and sensitive assays applicable to the identification and quantitation of these compounds in biological fluids are required. No previous attempt at the determination of DeHE has been made. Because DeHE has potential therapeutic properties, sensitive and reliable assays for future applications, such as quality control, bioavailability and pharmacokinetic studies, as well as clinical monitoring are necessary.

Our analytical method consists of isocratic, reversed-phase HPLC coupled to UV detection, which permits the separation and quantitative determination of DeHE. The composition and pH of the mobile phase were optimized. The incorporation of an internal standard, separable from DeHE and quantifiable under the same conditions, corrects for variability in recovery. Sample preparation is relatively simple.

The method has been successfully applied to DeHE determinations in biological fluids, including plasma, whole blood and bile, of different composition, viscosity and pH. Its applicability to the *in vivo* monitoring of plasma levels has been demonstrated. The method is therefore suitable for bioavailability and pharmacokinetic studies as well as clinical monitoring. Bile was studied because biliary excretion is a common drug elimination route, and enterohepatic circulation often affects the pharmacokinetics, although in the case of DeHE no previous information regarding biliary excretion had been reported. Indeed, comparison of the chromatograms of freshly processed bile samples and those that had been left at 4°C for days indicated a diminution of the DeHE peak and the appearance of a large peak much closer to the solvent front (retention time 7.6 min). The peak was also seen in bile samples taken 30 min after the administration of DeHE. No such changes were observed in plasma samples. A plausible explanation is that the unknown peak might represent a degradative product occurring only in the bile. The fact that the suspected degradative product was probably more polar than the drug would be compatible with the general trend of drug metabolism. No further efforts were directed at positively identifying this compound in the present study, as computer analysis ("Chem", Chem-Lab, Taipei, Taiwan) indicated that it was a simple peak. No other metabolites or other compounds appeared to interfere with the determination of DeHE in plasma, bile or whole blood.

On the other hand, although urinary excretion is another common route, attempts to analyse urinary levels of DeHE in the rabbit failed because of interfering compounds found in the rabbit urine. More elaborate sample preparation would be required. Nonetheless, the method could be applied to determinations in other biological fluids, such as cerebrospinal fluid and saliva. Another possible application, supported by preliminary studies, is the quantitative determination of DeHE in the parent plant, as concerns are often raised about the variation in concentration in different parts of the plant and among different batches. Conceivably, the method could also be adapted for the analyses of similar compounds.

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