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Short communication

Simplified solid-phase extraction method for determination of dihydroergotamine in rabbit and human serum using high-performance liquid chromatography with fluorescence detection

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Abstract

A rapid, selective and sensitive method for the determination of dihydroergotamine (DHE) in serum was developed. Dihydroergocristine (DHEC) was used as an internal standard. Human and rabbit serum samples were extracted using commercial solid-phase cyano (CN) columns. Proteins were washed from these columns with pure acetonitrile, resulting in clean extracts. Extracts were subsequently separated by HPLC in an isocratic way, using a reversed-phase C_{18} analytical column. Fluorometric detection was performed at excitation and emission wavelengths of 277 and 348 nm, respectively. Calibration curves with amounts of DHE ranging from 2 to 32 ng, were linear. The limit of detection found for DHE was 0.2 ng, extracted from 0.5 ml rabbit or from 2.5 ml human serum. The limit of quantification in serum of both species was 0.7 ng. The method has been shown to be suitable for monitoring DHE in serum during pharmacokinetic studies in rabbits.

Keywords: Dihydroergotamine

1. Introduction

Dihydroergotamine (DHE), a semisynthetic ergopeptide, is widely used in the treatment of migraine. The stimulation of serotonergic receptors of the capacitance vessels [1] and effects on serotonergic neurons of the central nervous system [2] have been suggested as mechanisms of action. A number of studies have been published, demonstrating the efficacy of DHE. With an intramuscular dose of 1 mg DHE, headaches were successfully aborted in 71% of the patients [3]. Using venoconstriction as a measure of efficacy, intranasally administered doses of 1 mg DHE appeared to give a similar effect as intramuscular injections of 0.5 mg DHE [4]. In one study DHE nasal sprays were found to be significantly superior to a nasal placebo formulation [5], whereas in another study no differences were observed [6]. In a non-placebo controlled study, 77% of the patients reported good efficacy for the DHE nasal spray [7]. In a placebo controlled study on nasal dosages of 1 mg DHE in the treatment of cluster headaches, no effect on duration or frequency of the attacks was observed, but the intensity of the single attacks was significantly reduced [8]. No adverse effects apart from mild to moderate local nasal pharyngeal were reported after nasal treatment with DHE or the placebo [5–8].

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In order to measure DHE in biological fluids, radioimmunoassay (RIA) methods have been developed. However, most were found to be not specific enough, due to extensive biotransformation and interference of DHE metabolites [9–12]. Some HPLC methods with fluorescence detection of DHE in plasma after liquid or solid-phase extraction have been described, with detection limits ranging from 0.1 to 0.6 ng ml^{-1} in plasma [13–15].

None of the described methods could be performed within our laboratory, since specific DHE antibodies are not commercially available and the HPLC column switching techniques described in literature are very complex. Therefore, an improved, more simple solid-phase extraction method in combination with HPLC separation and fluorometric detection of DHE and dihydroergocristine (DHEC; used as an internal standard) in rabbit and human serum is presented in this paper. The method described is also shown to be suitable for pharmacokinetic studies of DHE.

2. Experimental

2.1. Chemicals

Dihydroergotamine methanesulfonate salt (DHE) was obtained from Sigma (St. Louis, MO, USA) and dihydroergocristine methanesulfonate salt (DHEC) was a gift of Sandoz (Basle, Switzerland). Acetonitrile (HPLC grade) and 2-propanol (analytical grade) were from Rathburn (Walkerburn, UK) and Mallinckrodt Baker (Deventer, Netherlands), respectively. $Na_2HPO_4 \cdot 2H_2O$ and citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) were of pharmaceutical grade and from Brocacef (Maarsen, Netherlands). Water used was from a Milli-Q UF plus ultrapure water (MQ) system from Millipore (Etten-Leur, Netherlands).

2.2. Calibration and quality control samples

A stock solution of 100 μg ml^{-1} DHE was prepared every week in MQ water and stored at 4°C. After dilution of the stock solution in MQ water, rabbit calibration sera were prepared by spiking 0.5 ml of sera with DHE to final concentrations of 4, 8, 16, 32 and 64 ng ml^{-1} DHE. Human calibration sera were obtained by spiking 2.5 ml with DHE, giving final concentrations of 1.6, 3.2, 6.4 and 12.8 ng ml^{-1} DHE.

In addition, quality control samples were prepared by spiking pooled sera and subsequently dividing these into small fractions just before freezing at −20°C. Calibration samples were prepared in advance and frozen in order to treat these identically to the quality control and experimental samples. For assays with rabbit sera, two fixed concentrations of 15 and 40 ng ml^{-1} DHE in serum, and for the human sera three fixed quality control samples of 2, 5 and 8 ng ml^{-1} DHE in serum were used.

2.3. Extraction procedure

Two stock buffer solutions were used, one of pH 7.2 (KH_2PO_4 2.7 g l^{-1} and $Na_2HPO_4 \cdot 2H_2O$ 8.3 g l^{-1}; 66 m*M*) and one of pH 3.6 (14.24 g l^{-1} citric acid and 11.46 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$; 66 m*M*).

Supelclean LC-CN solid-phase extraction (SPE) tubes of Supelco (Bellefonte, PA, USA) were placed on a 24-port SPE vacuum manifold (Supelco), conditioned with 1 ml acetonitrile and followed by 1 ml of the 10-times diluted buffer, pH 3.6.

For the rabbit sera, an internal standard solution was made of 16 ng ml^{-1} dihydroergocristine mesylate (DHEC) in undiluted buffer, pH 3.6. Serum samples (quality control, calibration and experimental sera) were provided with 0.5 ml internal standard solution and added to the SPE tubes. For the human sera, the internal standard solution had a concentration of 3.2 ng ml^{-1} in undiluted buffer pH 3.6 and calibration and quality control sera were provided with 2.5 ml of this solution.

The SPE tubes were subsequently washed twice with 1 ml of ten-times diluted buffer (pH 3.6) and once with 1 ml of pure acetonitrile. Because the SPE tubes were mounted with 10 cm stainless steel luer lock needles at the bottom, samples could flow through without lowering pressure. After the acetonitrile washing, the packing of the SPE tubes was dried by lowering the pressure with 127 mmHg. The DHE was extracted from the tubes with 1.5 ml of acetonitrile–buffer (pH 7.0, ten times diluted) (8:2, v/v). These extracts were collected into 10 ml disposable glass tubes and evaporated within 30 min under a

stream of nitrogen at 35°C in a Liebisch evaporator (EBMC, Kerkdriel, Netherlands). The residues were dissolved in 200 μl of a solution of acetonitrile–buffer (pH 7.0; undiluted) (1:1, v/v).

2.4. Instrumentation and chromatographic conditions

A Jasco (B&L Systems, Zoetermeer, Netherlands) PU-980 gradient pump in combination with a Jasco LG-980-02 low pressure gradient unit was used to pump an eluent of acetonitrile–2-propanol–buffer (45:18:37, v/v/v) with a flow-rate of 1 ml min^{-1} in an isocratic way. The eluent was de-aerated with helium gas. The buffer used was 10 m*M* $Na_2HPO_4 \cdot 2H_2O$ adjusted to a pH of 7.0 with concentrated phosphoric acid. An amount of 150 μl of extracted serum samples was injected with a Gilson 234 (Gilson Medical Electronics, Villiers-le-Bel, France) autosampler onto a MF plus analytical column (Alltech, Deerfield, IL, USA) of 150×4.6 mm I.D., packed with Alltima (Alltech) C_{18} 5U material. The analytical column was preceded by an Alltima C_{18} cartridge guard column of 7.5×4.6 mm and a replaceable frit was placed before the column. DHE was detected with a Jasco 821 fluorescence detector at excitation and emission wavelenghts of 277 and 348 nm, respectively. Chromatograms were integrated using EZCHROM 6.4 software (Scientific Software, San Ramon, CA, USA). Data were exported to the commercial spreadsheet program QUATTRO PRO FOR WINDOWS (Borland, Scotts Valley, CA, USA) for further calculations.

2.5. Assay validation

Standard curves were produced by plotting the DHE/DHEC peak height ratios against the DHE concentration. Regression coefficients were obtained by least-squares regression analysis for calculation of the sample concentrations. The precision and accuracy of the method were determined by analysing quality control samples with every assay.

The recoveries of both DHE and DHEC were measured for each assay by comparing the peak heights of the calibration sera after extraction with those of the same expected DHE and DHEC concentrations in a solution of acetonitrile–buffer (pH 7.0, undiluted) (1:1, v/v).

3. Results and discussion

3.1. Chromatography

An example of overlaid chromatograms, obtained after extraction of rabbit sera previously spiked with amounts of DHE (ranging from 4–64 ng/ml), is given in Fig. 1. A blank chromatogram of rabbit serum is also included in this figure. The system showed a small memory effect for DHEC (also visible in the blank chromatogram in Fig. 1), which was about 7% of the spiked amount of DHEC. This memory effect did not increase during repetitive injections, so the contribution in the calculations of all samples is consistent. The pH of the eluent buffer was set to 7.0. When lower pH values were used in either eluent or samples, retention times were shortened and peaks were broadened. This is probably due to protonation of DHE and DHEC, resulting in reduced interaction with the C_{18} material of the column. The isopropanol in the eluent was used to shorten the retention time of the internal standard peak (DHEC). Retention times for DHE and DHEC were 3 and 4 min, respectively. By biotransformation, possible DHE metabolites will be more water-soluble and therefore will have shorter retention

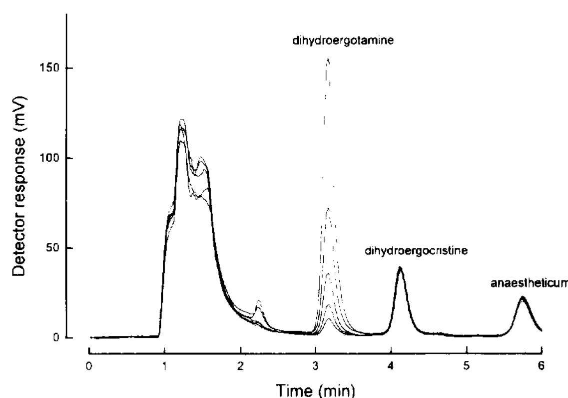

Fig. 1. Overlaid chromatograms of 0.5 ml extracted blank rabbit serum (interrupted line) and rabbit serum spiked with standard amounts of DHE. DHE peaks from top to bottom; spiked amounts of 32, 16, 8, 4 and 2 ng DHE, respectively

times. Moreover, the peak found at 3 min, after extraction of serum from an intravenously administered rabbit, was confirmed to be intact DHE by mass spectrometry. The last peak in the chromatogram was caused by the anaesthetic which was present in the rabbit sera. The guard column was replaced after analyzing experimental sera of 6 rabbits (108 in total). The analytical column did not deteriorate during the analysis of at least 2000 serum samples.

3.2. Extraction recovery

The pK_a values for DHE and DHEC are 6.75 and 6.74, respectively [16]. The interactions with the CN column are the strongest at pH values <4.7. When passing pure acetonitrile over the column to wash off the majority of proteins, DHE and DHEC were still completely retained, provided that no buffer of pH 3.6 (10-times diluted) from the former wash solution was left above the SPE packing. Washing the columns with 10-times diluted buffer (pH=3.6) was necessary to lower the buffer concentration on the CN columns before extracting with acetonitrile–buffer (pH 7.0 ten times diluted) (8:2, v/v). The pH increase resulting from this last solution was sufficient to reduce the binding interactions and to desorb both DHE and DHEC from the column. The use of higher pH values resulted in more contaminated extracts, probably due to protein extraction/denaturation and/or dissolution of the silica from the column. The extraction recoveries of DHE and DHEC from rabbit serum ranged from 73–88% and from 83–91%, respectively, whereas the recoveries of both compounds from human serum exceeded 90% (Table 1). The tendency towards higher recoveries with human serum compared to rabbit serum is probably caused by the larger volumes of human serum used. These high recoveries were found with the CN SPE Supelco columns. The same extraction procedure with CN columns of another provider resulted in recoveries of only 30–40%. The extraction recovery of DHE from rabbit sera had a tendency to increase with increasing concentrations of DHE, but this difference was not statistically significant. For the human sera such a tendency was not found.

3.3. Assay validation

The precision and accuracy of the method are summarized in Table 2. The assay to assay coefficient of variation (CV) for measuring DHE in both rabbit and human serum was 2.2–5%. The accuracy of the concentrations measured for the rabbit quality control samples of 15 and 40 ng DHE ml^{-1} were about 10% too high. For the human quality control samples the accuracy was found to be better than for the rabbit samples; −1.8, 3.4 and 2.1% deviation for the three quality control sera with 2, 5 and 8 ng DHE ml^{-1}, respectively (Table 2). The linearity found for the calibration lines with human sera as well as with rabbit, expressed as r^2, was 0.999±0.001. The x coefficient was 0.075±0.011 and 0.076±0.022 for the human and rabbit calibration lines, respectively. The detection limit for DHE, measured as the signal-to-noise ratio of 3:1, was 0.2 ng. The limit of quantification with a signal-to-noise ratio of 10:1 was 0.7 ng.

Table 1
Recovery of DHE and DHEC after extraction from rabbit and human serum

Amount DHE added (ng)	Rabbit serum (0.5 ml) (mean±S.D.) (%)		Human serum (2.5 ml) (mean±S.D.) (%)	
	DHE	DHEC	DHE	DHEC
2	73±12	83±5	–	–
4	76±12	89±7	92±10	93±8
8	76±5	86±7	92±10	95±7
16	83±9	86±5	97±7	104±9
32	88±5	91±6	94±7	99±12

The values given are the means±S.D. of 10 serum samples.

Table 2
Inter-assay precision and accuracy for measuring DHE in rabbit and human serum

Species	Amount spiked (ng)	Amount of serum (ml)	Concentration spiked (ng ml^{-1})	Concentration found (ng ml^{-1})	C.V. (%)	Accuracy (%)
Rabbit	7.5	0.5	15	16.8±0.7	4.2	12
	20	0.5	40	43.2±2.2	5.1	8
Human	5	2.5	2	2.0±0.1	4.8	−1.8
	12.5	2.5	5	5.2±0.1	2.2	3.4
	20	2.5	8	8.2±0.3	3.7	2.1

The values given are the means±S.D. of ten samples.
C.V.=coefficient of variation (S.D./mean×100%); accuracy=((concentration found−concentration spiked)/concentration spiked)×100%.

3.4. Pharmacokinetic application

The presented bioanalytical method for DHE is now being used for pharmacokinetic studies with DHE in rabbits and man. In collected blood samples of rabbits, DHE appeared to be stable for at least 2 h at room temperature. The mean serum concentration–time curves, obtained after an intravenous bolus injection of 0.03 mg kg^{-1} of DHE in rabbits, is shown in Fig. 2. DHE was far above the limit of detection (0.4 ng ml^{-1}) during the 2 h of measurement. The serum disappearance curves of DHE showed a bi-exponential decline with half-lives of 2.0±0.5 (mean±S.D.) and 71.6±19.1 min for the initial distribution phase and the final elimination phase, respectively.

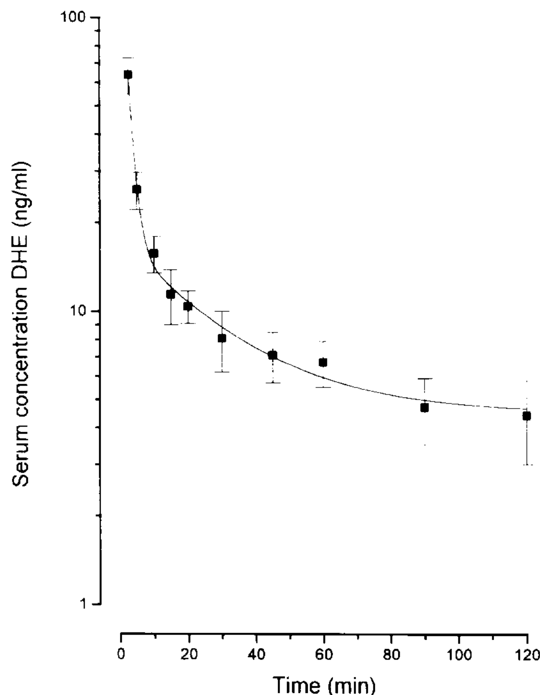

Fig. 2. DHE serum concentration versus time curve after an intravenous bolus injection of DHE (0.03 mg kg^{-1}) in rabbits. Data represent the mean±S.D. of six animals.

In conclusion, the solid-phase extraction–HPLC method for measuring DHE in rabbit and human serum, as described in the present study, is a rapid, selective and sensitive method to monitor DHE in serum during pharmacokinetic investigations with DHE.

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