Journal of Chromatography, 417 (1987) 319–329
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3653

DETERMINATION OF SUB-NANOGRAM AMOUNTS OF DIHYDROERGOTAMINE IN PLASMA AND URINE USING LIQUID CHROMATOGRAPHY AND FLUORIMETRIC DETECTION WITH OFF-LINE AND ON-LINE SOLID-PHASE DRUG ENRICHMENT

H. HUMBERT*, J. DENOUEL, J.P. CHERVET, D. LAVENE and J.R. KIECHEL

Sandoz Laboratories, Pharmaceutical Research Centre, B.P. 313, 92506 Rueil-Malmaison Cedex (France)

(First received November 11th, 1986; revised manuscript received February 3rd, 1987)

SUMMARY

A highly sensitive and selective high-performance liquid chromatographic method, involving sample pre-treatment, column switching and fluorimetric detection, is described for the determination of dihydroergotamine in plasma and urine samples. The pre-chromatographic sample treatment consists of extraction by means of an Extrelut® column for plasma samples, and pre-separation with enrichment steps on a Sep-Pak® column for urine samples. The samples are then injected onto a pre-separation column (Aquapore), and the fraction containing dihydroergotamine are automatically diverted onto an analytical column (ODS reversed phase). An acetonitrile–ammonium carbamate gradient is used as the mobile phase. High recovery of dihydroergotamine from both plasma (87%) and urine (100%) and a detection limit as low as 100 pg/ml were achieved, with a linear response up to 5 ng/ml. The assay demonstrated a high degree of selectivity with regard to the extensive metabolism of dihydroergotamine especially to the main metabolite 8′-hydroxydihydroergotamine. The assay was successfully applied for more than one year to the determination of plasma and urine concentrations of dihydroergotamine after parenteral administration.

INTRODUCTION

Dihydroergotamine (DHE) is a semisynthetic ergot alkaloid. Its major therapeutic applications are the prevention and treatment of migraine and the treatment of orthostatic hypotension. The drug undergoes extensive biotransformation in animals and humans [1], and its disposition is characterized by incomplete (30%) oral absorption [2–4] and by a large volume of distribution [3,5]. As a consequence, the plasma levels of the drug are in the range from pg/ml to low ng/ml. Any assay for pharmacokinetic purposes must, therefore, provide high levels of selectivity and sensitivity.

0378-4347/87/$03.50

Preliminary pharmacokinetic parameters of DHE were obtained by administration of radiolabelled drug [2], but total radioactivity monitoring, though very sensitive, lacks selectivity. Therefore, most of the pharmacokinetic and bioavailability data reported are based on radioimmunoassay (RIA) methods. RIA involving use of lysergic acid antisera for the determination of ergopeptides [6,7] was found to be non-specific since metabolism affects mainly the peptide moiety [8,9]. Greater selectivity was obtained by RIA using an antiserum elicited against conjugates of serum albumin (human or bovine) with ergopeptide linked via a nitrogen (C-1 or C-6 position) of the alkaloid [10–12].

The only reported chemical methods applicable to DHE in plasma involve a high-performance liquid chromatographic (HPLC) assay with fluorimetric detection [13,14]. The selectivity and the sensitivity of the methods do not, however, appear to fulfill the requirements for measurements following therapeutic dosages in human studies.

The purpose of this study was to develop a sensitive and specific HPLC method, with fluorimetric detection, for the determination of DHE in plasma and in urine suitable for use in human pharmacokinetic investigations.

EXPERIMENTAL

Materials

Dihydroergotamine methanesulphonate, dihydroergocornine methanesulphonate (DHEC) (internal standard) and 8′-hydroxydihydroergotamine were obtained from Sandoz (Basle, Switzerland). Stock solutions were prepared in methanol at a concentration of 1 μg/μl and stored at 4 °C. Working solutions were prepared daily in water or in mobile phase, depending on their proposed use. Water was purified on a Milli-Q system (Millipore, Molshein, France), which gives a specific resistance higher than 18 MΩ/cm.

Acetonitrile (HPLC grade, from Rathburn, Walkerburn, U.K.) was distilled before use. Methanol was of UV grade (Carlo Erba, Milan, Italy). Other reagents were of analytical grade: ammonium hydroxide 25% (Merck, Darmstadt, F.R.G.), ammonium carbamate (Merck), diethyl ether RPE (Carlo Erba). An aqueous stock solution of 0.01 *M* ammonium carbamate was prepared fortnightly and stored at 4 °C.

Drug-free heparinized plasma and drug-free urine were drawn from healthy volunteers and stored at −18 °C.

Extrelut® R^3 (3 ml) columns were obtained from Merck and Sep-Pak® C_{18} columns were purchased from Waters (Milford, MA, U.S.A.) and affixed to a Sep-Pak cartridge rack (Waters) to accelerate the passage of solvent through the sorbent bed by vacuum.

Extraction from plasma

A 3-ml plasma sample was pipetted into a conical glass tube, to which 100 μl of internal standard solution (75 pg/μl DHEC in water) and 300 μl of ammonium hydroxide (25%) were added. The tubes were vortexed for 15 s, then centrifuged at 1500 *g* for 5 min. The supernatant (3 ml) was transferred onto the dry Extrelut

column. After 15 min, DHE and DHEC were extracted from the column by elution with 15 ml of diethyl ether and collected in a pointed tube. The organic phase was evaporated under a gentle stream of nitrogen at 35°C. To the dry residue, 200 μl of ammonium carbamate–acetonitrile (50:50, v/v) solution were added. The tube was vortexed for 15 s and put in an ultrasonic bath for 30 s. After centrifugation for 5 min at 2000 *g*, 150 μl of the supernatant were transferred to a glass autosampler vial and capped with a septum. An aliquot of 100 μl was injected into the liquid chromatograph.

Extraction from urine

C_{18} cartridges (Sep-Pak) were activated by passing them successively through 5 ml of acetonitrile and 5 ml of ammonium carbamate (0.01 *M*). Then 5 ml (or less for concentrations higher than 5 ng/ml) of urine sample were pipetted into a glass conical tube, and 100 μl of internal standard solution (250 pg/μl DHEC in water) and 400 μl of ammonium hydroxide (25%) were added. The tube was vortexed for 15 s, and then centrifuged at 1500 *g* for 5 min. The supernatant (5 ml) was then poured into a syringe barrel attached to a previously conditioned Sep-Pak cartridge. A vacuum was applied to obtain a flow-rate of ca. 10 ml/min. After passage of the sample through the cartridge, the packing was rinsed with 1.5 ml of ammonium carbamate–acetonitrile (55:45, v/v).

The vacuum was maintained for a few seconds to remove most of the liquid phase. The cartridge was then slowly eluted with 2.0 ml of methanol–acetonitrile (10:90, v/v) into a pointed glass tube. The recovered fraction was evaporated at 40°C under a gentle stream of nitrogen. The procedure was then continued as described for plasma.

Liquid chromatography

A Model 1084B HPLC system (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. It consisted of two solvent containers, two pumps, an automatic sample injector, a pre-separation column I (Aquapore RP 300, 10 μm 30×2.1 mm I.D., Brownlee, Santa Clara, CA, U.S.A.), an air-activated valve (Rheodyne 7040, Cotati, CA, U.S.A.), an analytical column II (HS3 C_{18}, 3 μm, 83×4.6 mm I.D., Perkin Elmer, Norwalk, MA, U.S.A.). The two columns and the valve were kept in a thermostatted oven at 40°C. The two solvent containers were kept at the same temperature. All these parts were regulated by the HP 1084 microprocessor. The mobile phase was composed of (A) 0.01 *M* ammonium carbamate (pH 8.5) and (B) acetonitrile. These solutions were filtered through a 0.6-μm filter before use and degassed under vacuum on the HPLC system. The HPLC system connections downstream of the injector were via 0.13 mm I.D. stainless-steel tubes. Detection was performed with a Model LS4 spectrofluorimeter detector (Perkin Elmer) equipped with a xenon lamp and a 3-μl detector cell. Detection of DHE and DHEC was performed at 296 nm for excitation and 355 nm for emission (slit width 10 nm).

Operating conditions

A diagram of the gradient elution is shown in Fig. 1. A constant flow-rate of 1.8 ml/min was maintained during all operations. At the time of injection ($t=0$ min)

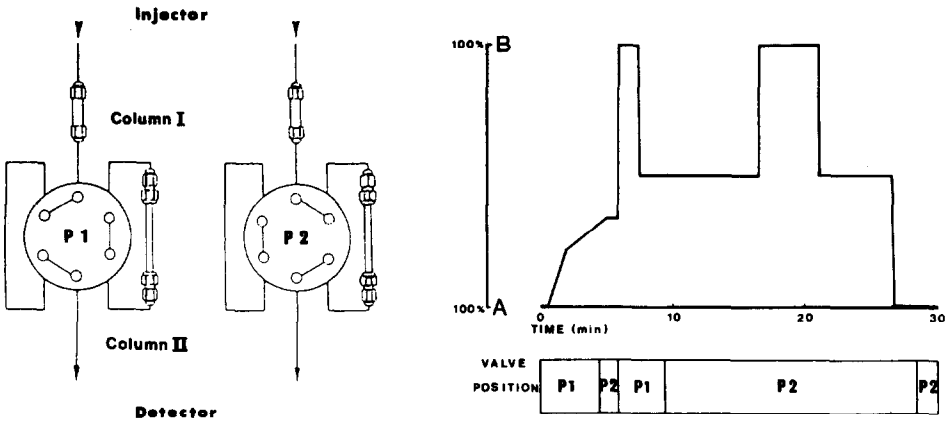

Fig. 1. Diagram of the column-switching device and the variation of the gradient (see text for details).

the mobile phase consisted of 100% A and passed only through column I (valve position P1). After 0.5 min the percentage of solvent B was linearly increased to 22% over 1.5 min, followed by a further increase to 34% over 3 min. At time $t = 4.5$ min the fraction containing DHE and DHEC was automatically diverted onto column II for 1.4 min (valve position P2). At time $t = 5.9$ min the valve was reset to position P1, and column I was washed with 100% B for 1.5 min. The mobile phase composition was then set to 40% B, and at 9.4 min the valve was again set to position P2 to elute DHE and DHEC from column II for ca. 7 min. From time $t = 16.5$ to 21.0 min columns I and II were washed with 100% B. At 21 min the eluent mixture was set to 40% B to recondition column II, and at 26.5 min the valve was reset to position P1 to recondition column I with the initial eluent, consisting of 100% A. Under these conditions the elution times for DHE and DHEC were 13.3 and 14.5 min, respectively, within a total cycle time of 30 min. Typical chromatograms of a standard solution, blank plasma, blank urine and spiked standard samples are shown in Fig. 2.

Quantification

The detector signal was fed parallel into the microprocessor of the HP 1084 system and to a 3357 LAS system (Hewlett-Packard). Concentrations of DHE were calculated by relating the peak-area ratios of DHE and DHEC to a standard calibration curve, obtained by least-squares linear regression over the range 0.250–2.500 ng/ml DHE in plasma (DHEC = 2.5 ng/ml) or 0.100–5.00 ng/ml DHE in urine (DHEC = 5 ng/ml). The percentage recovery of DHE in plasma and urine was calculated from the peak-area ratio for spiked standard samples and directly injected standard solutions.

RESULTS

Pre-chromatographic sample treatment

Sample pre-chromatographic isolation steps were considered to be helpful or even necessary, although direct sample injection into column-switching systems

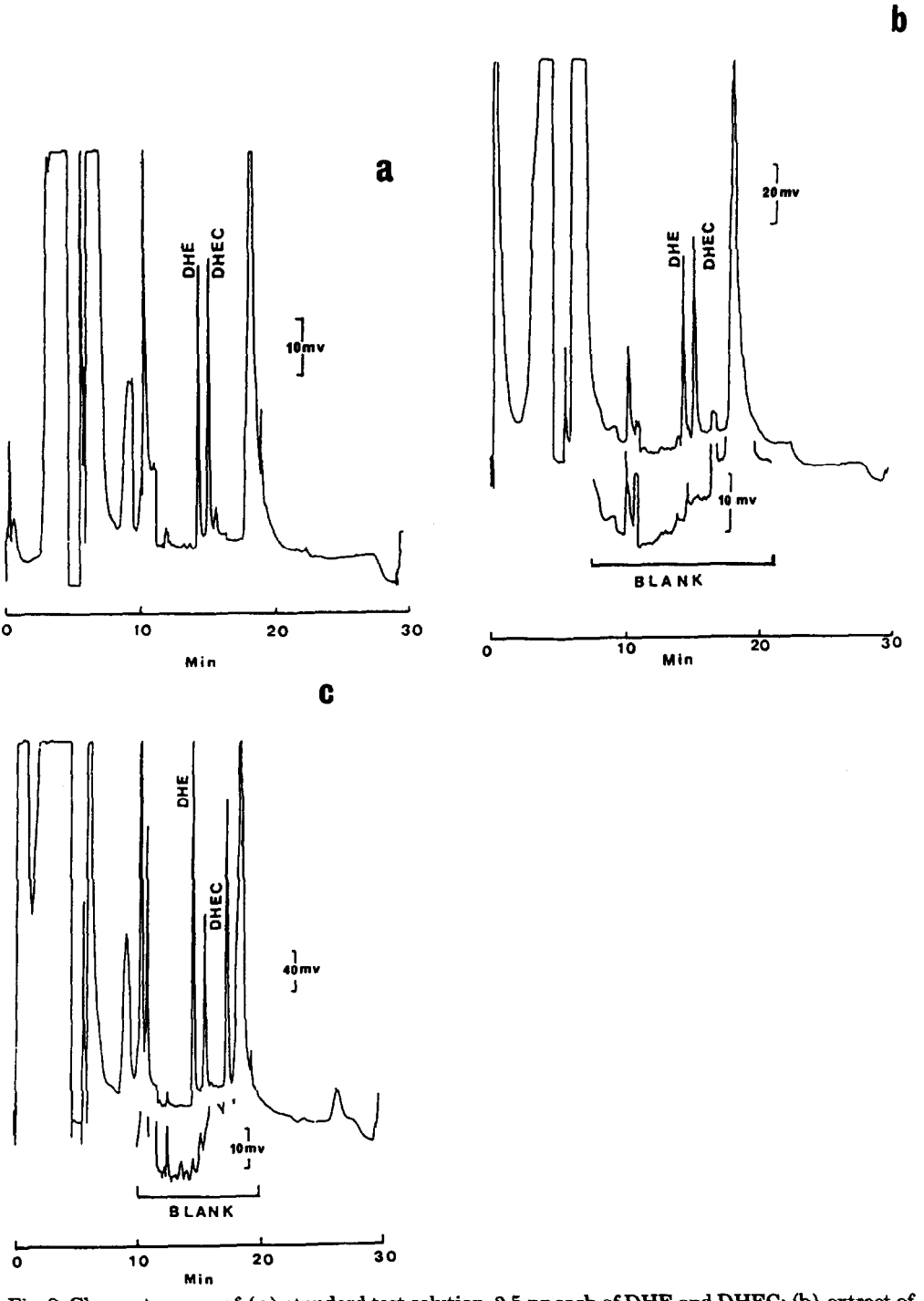

Fig. 2. Chromatograms of (a) standard test solution, 2.5 ng each of DHE and DHEC; (b) extract of human blank plasma (3 ml) with or without 2 ng/ml DHE and 2.5 ng/ml DHEC; (c) extract of human blank urine (5 ml) with or without 5 ng/ml DHE and DHEC. Note that in b and c the blank is recorded on a different scale.

TABLE I

EXTRACTION YIELDS OF DHE AND DHEC FROM SPIKED SAMPLES ($n=5$)

Compound	Spiked value (ng/ml)	Extraction yield (%)	C.V. (%)
Plasma			
DHE	0.25	87.4	22
DHE	0.50	89.1	26
DHE	2.00	85.2	20
DHEC	2.50	84.0	14
Urine			
DHE	0.10	113.2	5.7
DHE	5.00	94.6	1.7
DHEC	5.00	96.0	3.0

is common [15]. The main reasons for this decision were (i) to prevent clogging of column I, especially with the 3-ml plasma samples, and (ii) because the need for very high sensitivity necessitated a selective pre-chromatographic clean-up step.

For plasma, the selective pre-chromatographic clean-up step was achieved completely with the easy-to-use disposable Extrelut columns. However, when the same procedure was applied to urine, endogenous compounds interfered with DHE and DHEC. For urine, therefore, a Sep-Pak clean-up step, including a selective washing step and selective desorption, was necessary. Compared with the Extrelut procedure, the Sep-Pak treatment was more time-consuming and required more skill, especially because of the speed at which the solutions were passed through the cartridges. As can be seen in Table I, the Extrelut column gave recovery levels between 84 and 89% whereas the Sep-Pak gave recovery levels between 96 and 113%. The relatively high variability of recovery from the Extrelut cartridge was compensated in the assay by using an internal standard method, since both DHE and DHEC indicated very similar behaviour patterns during sample clean-up and chromatography.

Response curve

The linearity and precision of the chromatographic system were evaluated with standard solutions over the range 0.5–20 ng DHE per 100-μl injection with a constant amount of 10 ng of DHEC. The least-squares regression line (area ratio $= a \cdot$concentration $+ b$) demonstrated excellent linearity with a coefficient of correlation (r) of 0.999. Precision was also excellent, with a mean coefficient of variation (C.V.) of 2.5% (range 1.7–4.5%).

Analysis scheme

Because the workload and the chromatographic cycle time were rather high, standard curves were not performed every day. A standard curve was obtained at the beginning of an analysis period lasting several weeks. On each analysis day, quality-control (QC) samples obtained with spiked standard plasma or urine

TABLE II

REPRODUCIBILITY AND ACCURACY OF THE HPLC FLUORIMETRIC ASSAY OF DHE IN PLASMA AND URINE (FROM SPIKED CONTROL SAMPLES ANALYSED IN PARALLEL WITH UNKNOWNS)

Sample	Expected value (ng/ml)	n	Assay value (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
Within-day					
Plasma	0.25	5	0.23	8.8	−8.0
	0.50	5	0.51	4.0	+2.0
	1.00	5	1.03	8.9	+3.0
	2.00	5	1.99	6.2	−0.5
	Mean			7.0	−0.9
Urine	0.10	5	0.11	4.1	+10.0
	0.20	5	0.19	8.1	−5.0
	1.00	5	1.00	4.0	0.0
	5.00	5	5.00	2.4	0.0
	Mean			4.7	+1.3
Day-to-day					
Plasma	0.60	11	0.62	12.7	+3.3
	2.00	12	2.05	6.0	+2.5
	Mean			9.2	+2.9
Urine	1.00	13	1.13	9.5	+13.0
	2.00	11	1.92	16.0	−4.0
	10.00	15	11.00	11.9	+10.0
	25.00	18	24.50	11.4	−2.0
	Mean			12.0	+4.3

samples, were distributed amongst the daily series of unknowns (one QC sample for six unknowns).

Calibration curve for plasma and urine samples

The calibration curve was evaluated with replicate ($n=4$) spiked standard samples over the DHE concentration ranges 0–2.0 ng/ml in plasma and 0–5 ng/ml in urine. The least-squares regression fit showed good linearity for both plasma ($a=0.8239$, $b=0.0358$, $r=0.994$) and urine ($a=0.8969$, $b=0.0235$, $r=0.998$).

Reproducibility and accuracy

Reproducibility and accuracy were determined from the spiked control samples used in the daily analysis scheme (Table II). The within-day C.V. was 6.2–8.9% for plasma and 2.4–8.1% for urine. The mean day-to-day C.V. was 9.2% (one-month period, $n=23$) for plasma and 12.0% (four-month period, $n=57$) for urine. The accuracy of the method, expressed as the mean deviation of all concentrations from the expected value, ranged from −8.0% to +3.3% in plasma and from −5.0% to +13.0% in urine.

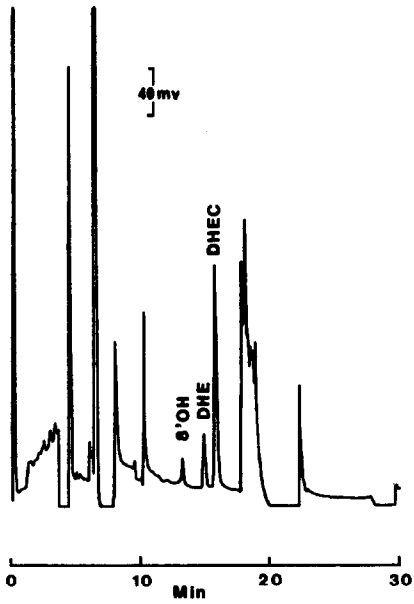

Fig. 3. Chromatogram of 100 μl of standard test solution with 2.0 ng of 8'-hydroxy-DHE (8'OH), 2.5 ng of DHE and 10 ng of DHEC.

Detection limit

The detection limit was evaluated by comparing the chromatogram of spiked standard samples of very low concentations, e.g. 100 pg/ml, with that of the corresponding drug-free sample containing only the internal standard. Typical values (expressed as the sample concentration giving a signal-to-blank ratio of 3) were 80 pg/ml for plasma and 100 pg/ml for urine. Since the detection limit depends mainly on the performance of the HPLC system and on the specimen analysed, the actual value varied somewhat from day to day.

Selectivity of the assay

The high selectivity of the assay was demonstrated by the interference-free chromatograms usually obtained within the elution window of DHE and DHEC (internal standard) for drug-free plasma and urine samples. The mean false positive value obtained from drug-free control plasma from twelve healthy volunteers was only 60 ± 60 pg/ml (range 0–280 pg/ml).

In addition, the selectivity was assessed for urine by the evaluation of the urinary excretion of DHE in volunteers ($n=4$) who were given placebo. The mean false excreted amount in 0–72 h urine (ten fractions) was less than 0.5% of the DHE excreted within the same collection period following intramuscular administration of 1 mg of DHE.

The assay also exhibited high selectivity with regard to the extensive metabolism of the drug [8]. This could be deduced from the fact that the main human metabolite in plasma and urine, 8'-hydroxy-DHE [9], was well separated from DHE and DHEC in the chromatographic system (Fig. 3). This metabolite has

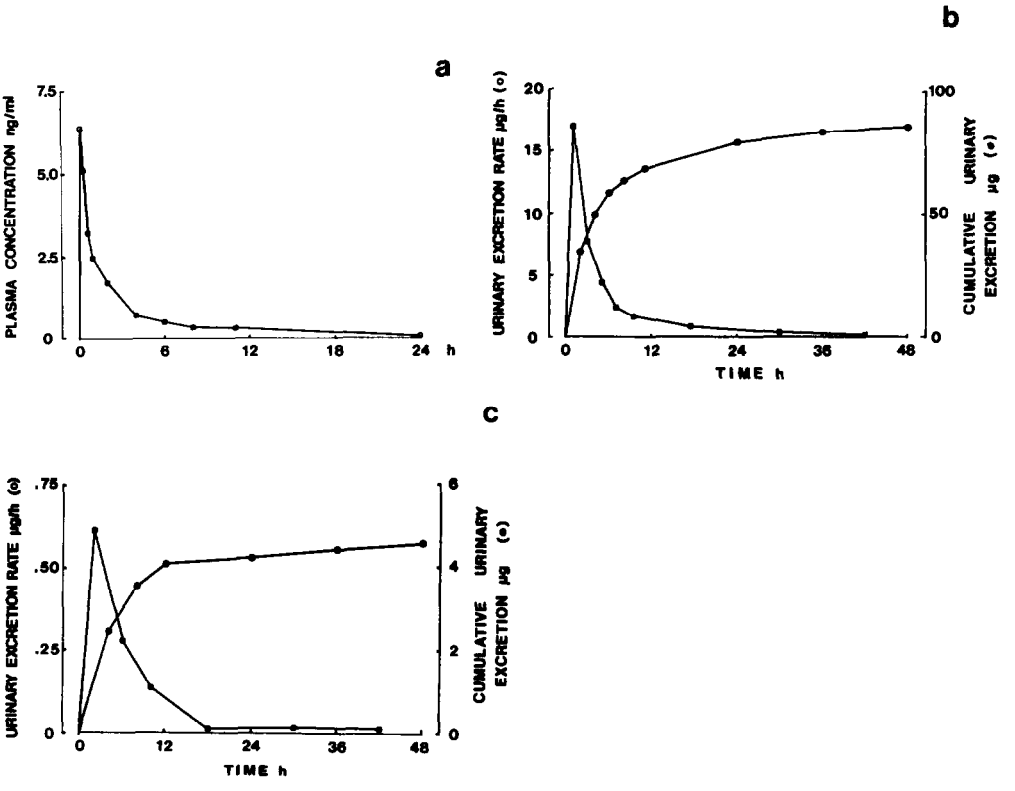

Fig. 4. Examples of plasma concentration and urinary elimination of DHE, found after administration of the drug to healthy volunteers. (a) Plasma concentration after single intramuscular administration of 1 mg; (b) rate and cumulative urinary excretion after single intramuscular administration of 1 mg; (c) rate and urinary excretion after single oral administration of 9 mg.

been found to be closest to DHE in the radiolabelled metabolite profile run on a very similar separation system [9] and, moreover, to be present at concentrations five to seven times greater than the parent drug.

Application of the method

This method has been in use for more than a year for the analysis of plasma and urine samples in human pharmacokinetic studies. Fig. 4 shows typical kinetic profiles obtained in plasma or in urine following administration of therapeutic doses of DHE to healthy volunteers.

The results of this HPLC method have been compared, for biological samples, with those of two RIA methods [7,12]. The detailed comparison will be published later. Initial findings are quite satisfacotry. Fig. 5 shows the plasma levels and urinary excretion in a healthy volunteer after intramuscular administration of 1 mg of DHE. The data collected with the RIA using an antiserum elicited against the peptide part of the molecule [12] are similar to those obtained by HPLC. However, the values obtained with RIA using an antiserum elicited against the lysergic part of the molecule [7] were higher than those found by HPLC. This

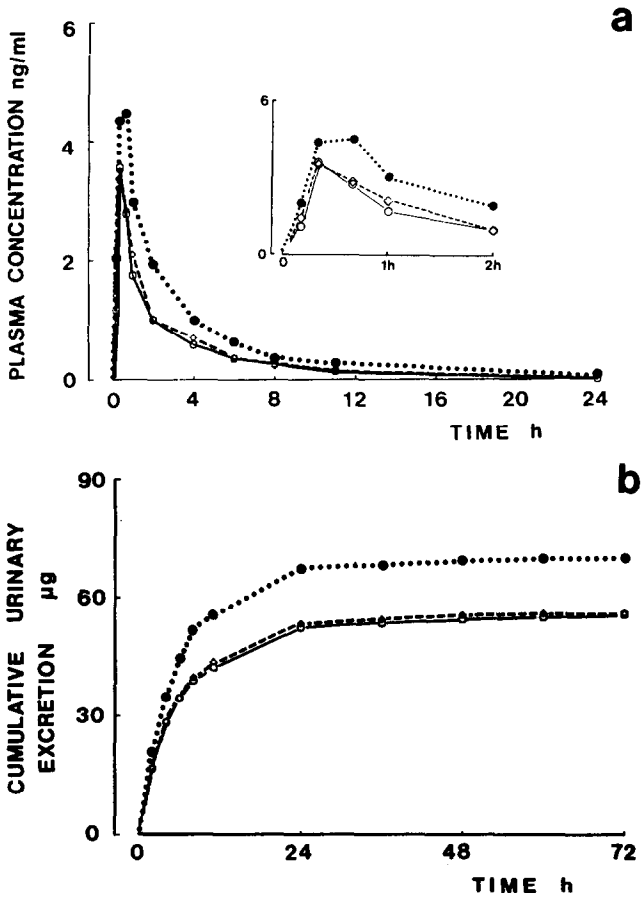

Fig. 5. Preliminary results for the comparison between three bioanalytical methodologies for DHE. HPLC with fluorimetric detection (○) and RIA with antibody reactive toward the peptide moiety of the molecule (◇) or toward the dihydrolysergic acid amide moiety (●). The data show (a) plasma concentration and (b) urinary excretion after a single intramuscular administration of 1 mg of DHE to one healthy volunteer.

preliminary result confirms that, for the RIA method, the antiserum elicited against the peptide moiety of the molecule has higher selectivity, and also gives an indirect indication of the well known metabolic pathway of DHE.

DISCUSSION

The HPLC method described is more sensitive and certainly more selective and specific than those previously described [13,14]. These results are due to a combination of three different factors: (i) sophisticated extraction and enrichment procedures; (ii) application of column switching; (iii) use of a very sensitive spectrofluorimeter. In addition, a reduction of the analysis time by ca. 10 min could be obtained by the use of two valves instead of one.

Operating conditions during both sample preparation and chromatography require careful management. The temperature of the plasma samples, before

passing them through the Extrelut column, is an important factor, as is that of urine samples before Sep-Pak treatment. In both cases, samples must be maintained at room temperature.

In chromatography for the detection of concentrations in the 50–200 pg/ml range, very efficient system performances are required. To obtain this, analytical column II is changed every 150 injections. The pre-separation column I is changed only every 300 injections. After each column change, the details of the chromatography operating conditions must be noted again.

Under these conditions, this HPLC assay is able to monitor plasma kinetics and urinary excretion after parenteral administration of therapeutic doses of DHE in humans. Unfortunately, it is not sufficiently sensitive to follow the plasma kinetics after a therapeutic oral dose, when the peak plasma levels in the range 100–200 pg/ml are close to the detection limit of the HPLC assay. Nevertheless, after a single oral dose of DHE to humans, this HPLC method, can be used to monitor the urinary elimination of parent drug and the 8′-hydroxy metabolite.

ACKNOWLEDGEMENTS

The authors thank Dr. H.P. Keller (Sandoz Basle) for critical advice about the manuscript, Dr. Maurer (Sandoz Basle) for supplying the 8′-OH-DHE and Dr. Rosenthaler (Sandoz Basle) for supplying the reagents for radioimmunoassay.

REFERENCES

1 H. Eckert, J.R. Kiechel, J. Rosenthaler, R. Schmidt and E. Schreier, in B. Berde and H.O. Schild (Editors), Handbook of Experimental Pharmacology, Vol. 49, Springer Verlag, 1978, pp. 719–802.
2 W.H. Aellig and E. Nüesch, Int. J. Clin. Pharmacol., 15 (1977) 106.
3 P.J. Little, G.L. Jennings, H. Skews and A. Bobik, Br. J. Clin. Pharmacol., 13 (1982) 785.
4 A. Bobik, G. Jennings, H. Skews, M. Esler and A. Mclean, Clin. Pharmacol. Ther., 30 (1981) 673.
5 H.F. Schran and F.L.S. Tse, Int. J. Clin. Pharmacol., 23 (1985) 1.
6 T. Kleimola, Br. J. Clin. Pharmacol., 6 (1978) 255.
7 F. Luccioni and J.R. Kiechel, Thérapie, 34 (1979) 625.
8 J.R. Kiechel, J. Pharmacol., 10 (1979) 533.
9 G. Maurer and W. Frick, Eur. J. Clin. Pharmacol., 26 (1984) 463.
10 J. Rosenthaler and H. Munzer, Experientia, 32 (1976) 234.
11 H.F. Schran, H.J. Schwarz, K.C. Talbot and L.J. Loeffler, Clin. Chem., 25 (1979) 1928.
12 J. Rosenthaler, H. Munzer, R. Voges, H. Andres, P. Gull and G. Bollinger, Int. J. Nucl. Med. Biol., 11 (1984) 85.
13 L. Zecca, L. Bonini and S.R. Bareggi, J. Chromatogr., 272 (1983) 401.
14 M. Zorz, J. Culig, Z. Kopitar, D. Milivojevic, A. Marusic and M. Bano, Hum. Toxicol., 4 (1985) 601.
15 F. Erni, H.P. Keller, C. Morin and M. Schmitt, J. Chromatogr., 204 (1981) 65.