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Sensitive determination of ephedrine and norephedrine in human plasma samples using derivatization with 9-fluorenylmethyl chloroformate and liquid chromatography

G. Aymard, B. Labarthe, D. Warot, I. Berlin, B. Diquet*

Laboratoire de Pharmacocinétique, Service de Pharmacologie, Centre Hospitalier et Universitaire de la Pitié-Salpétrière, Assistance-Publique Hôpitaux de Paris, 47–83 Bd de l'Hôpital, 75634 Paris Cedex 13, France

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Abstract

A high-performance liquid chromatography procedure for the determination of ephedrine and norephedrine using fluorimetric detection in plasma samples is described. A double liquid–liquid extraction was performed, followed by derivatization with 9-fluorenylmethyl chloroformate. The extracts were chromatographed with a 5-μm C_{18} (150×4.6 mm I.D.) column using a mobile phase composed of acetonitrile and water (52:48; v/v). The excitation and emission wavelengths were respectively 264 nm and 313 nm. Calibration curves were linear over the range 0 to 300 ng/ml for each analyte. The specificity of the method was demonstrated with several FMOC-reacting drugs. The limits of quantification are similar to those obtained with the reference method: 2 ng/ml for ephedrine and 5 ng/ml for norephedrine. This method has been successfully applied to the determination of ephedrine and norephedrine plasma levels after administration of low doses of ephedrine to healthy subjects. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Ephedrine; Norephedrine; 9-Fluorenylmethyl chloroformate

1. Introduction

Ephedrine (E) and its active metabolite norephedrine (NE) also called phenylpropanolamine are direct and indirect sympathomimetic drugs, commonly used for flu, cold, hay fever, rhinitis. Both are stimulants of the central nervous system and as such forbidden by International Olympic Committee (IOC). E is mostly excreted unchanged in urine [1]; metabolites such as NE are also excreted in urine.

The method of reference for the quantification of E and NE is gas chromatography coupled with mass-spectrometry detection [2] which allows high specificity and sensitivity.

A radioimmunoassay has also been described [3], a method which provides an intermediate specificity with some cross-reactions, and the limit of quantification (LOQ) was 2.5 ng/ml of pseudoephedrine (PE). Both procedures require an equipment not commonly available in a laboratory. Several HPLC methods have been developed, using UV detection [4–9], and the LOQ for amphetamine-like drugs including E, NE and PE were within the range of 10 ng/ml to 2.5 μg/ml. These methods use wavelengths in the range of 205–220 nm for the detection of both E and NE. A problematic non-specificity is however,

*Corresponding author. Fax: +33-01-4216-1688.

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the rule at such wavelengths which, added to the fact that the mobile phase has a very high cut-off, decreases the sensitivity. These methods generally use urine samples without extraction procedure [5–9]; furthermore E and NE were determined in plasma samples in only three procedures [4,6,9].

A sensitive and selective HPLC method for the quantification of E and NE in plasma samples was needed to study pharmacokinetic of E in healthy subjects receiving low doses of E (5 or 10 mg by intranasal route and 50 mg after oral administration). In order to increase both sensitivity and specificity, the fluorimetric detection, using 9-fluorenylmethyl chloroformate (FMOC) as fluorescent derivatizing agent was chosen.

Al-Dirbashi et al. [10] already developed, for the analysis of urine samples, a procedure using a fluorimetric detection with fluoresceine isothiocyanate (FITC) which allows a limit of detection of 1.6 ng/ml for NE. Despite a liquid–liquid extraction, only the quantification of NE in urine samples has been validated and the method was consequently unusable for our study. FMOC as already been used by Herráez-Hernández et al. [11], with on-line extraction of E and metamphetamine in urine samples.

We present in this paper a sensitive and specific HPLC analysis method with fluorimetric detection for quantification of E and NE in human plasma after liquid–liquid extraction and derivatization.

2. Experimental

2.1. Chemicals

E hydrochloride, NE base and pseudoephedrine (PE) base (internal standard) were purchased from Sigma (St. Louis, USA). Stock solutions of each compound (1 mg/ml) were prepared by dissolving 10 mg of equivalent free base in 10 ml of water. Stored at +4°C, in the dark; these solutions were stable for months. The working solutions were prepared each day. The concentrations were 2 and 0.2 μg/ml for both E and NE and 5 μg/ml for the internal standard. Three standard solutions of E and NE at the concentrations of 5, 60 and 160 ng/ml were prepared in order to determine within-day and between-days reproducibilities. Because of the photosensitivity of all three molecules, bright light has to be avoided during the preparation of the solutions.

The derivatizing agent, 9-fluorenylmethyl chloroformate (FMOC) was purchased from Sigma. FMOC solution ($C_{15}H_{11}ClO_2$, MW=258.7 g/mol) at 4 m*M* was prepared each day by adding 10.4 mg of FMOC to 10 ml of acetonitrile.

Acetonitrile (Carlo Erba Reagenti, Milan, Italy) and Ter-Butyl-Methyl-Ether (TBME) (Sigma) were HPLC grade. Isopropylic alcohol (Carlo Erba Reagenti), hydrochloride acid (Prolabo, Paris, France), sodium carbonate buffer 2 *M* (Merck, Darmstadt, Germany) and acetone (Carlo Erba Reagenti) were analytical reagent grade. Distilled water was purchased from Fresenius France Pharma (Louvier, France).

2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of a 114 M pump (Beckman, Berkeley, CA USA), solvent delivery module (pump 1), a LC-6A pump (Shimadzu, Dyson instruments, Hotton, UK) (pump 2) set at 1.5 and 2 ml/min respectively, a high pressure six-port valve (Eurosop EPS 130) and a wisp 717 Plus Autosampler injector (Waters, Milford, MA, USA) set at 150 μl for sample injection volume. The RF 551 fluorescence HPLC monitor (Shimadzu) with the excitation wavelength set at 264 nm and the emission wavelength set at 313 nm, was linked to an integrator (Beckman System Gold Analogue interface module 406) for data acquisition and treatment. The chromatography was achieved at room temperature using a Symmetry 5-μm C_{18} (150×4.6 mm I.D.) column (Waters).

The analytical mobile phase consisting of a mixture of acetonitrile and water (52:48; v/v) was degassed ultrasonically before use. At the beginning, the switching valve was in position A (Fig. 1) and pump 1 flushed the analytical mobile phase in the column. After a 150-μl sample injection, a 19-min time period is required for analysis. After this time, the switching valve was automatically turned to position B for 3 min to wash the column with a 100% acetonitrile mobile phase. To return back to initial conditions, 3 min of the first mobile phase

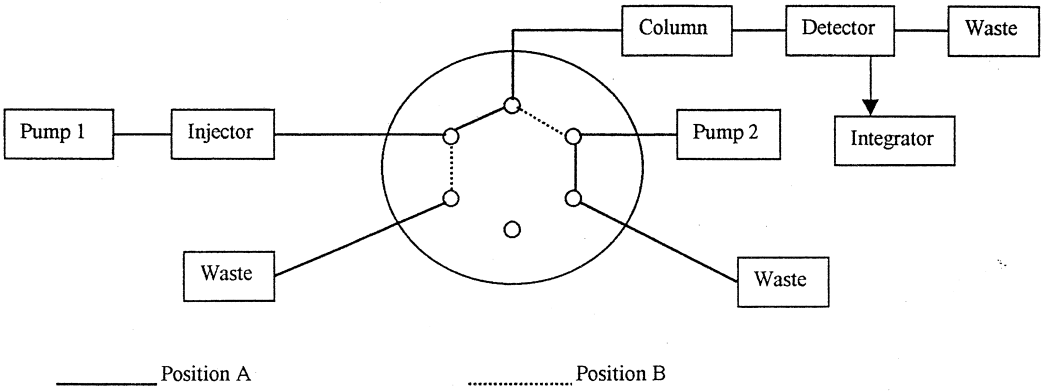

Fig. 1. Chromatographic system.

were needed (position A) before the following injection.

2.3. Extraction procedure

A liquid–liquid with back extraction was performed. In a 15-ml silicone tube (Venoject, Terumo, Belgium), 500 μl of plasma were alkalinized with 250 μl of 2 *M* sodium carbonate. Fifty μl of the working solution of the internal standard and 500 μl of isopropylic alcohol were added and then extracted by 5 ml of TMBE. The tube was capped, shaken horizontally for 20 min, then centrifuged for 10 min at 3000 *g* at a temperature of +4°C. The tube was then placed in a dry ice–acetone bath, the lower aqueous layer was frozen and the entire upper organic layer was transferred to a clean tube. A 100-μl volume of HCl 0.1 N was added to the tube containing the organic layer for back extraction. Following shaking for 10 min and centrifugation (as described above) The aqueous layer was frozen again in the same condition. The organic layer was discarded and the remaining TMBE was evaporated under a stream of nitrogen for 7 min without heating the samples.

2.4. Derivatization

After the back extraction, the aqueous layer was alkalinized by 20 μl of Na_2CO_3 2 *M* and then shaken for 3 min. The mixture was shaken on a Büchler vortex for 10 min after adding 120 μl of the 4 m*M* solution of FMOC.

2.5. Preparation of the calibration curve

A calibration curve based on peak-height ratio was constructed for each assay by adding known amounts of each drugs to drug-free human plasma. Concentrations equivalent to 5, 10, 20, 40, 60, 120, 160 and 200 ng/ml in both E and NE were assayed. The quantification was performed by an unweighted linear calibration curve. Each spiked plasma sample was processed as described above.

2.6. Subjects samples

Sixteen healthy subjects were enrolled in a PK-PD double blind double placebo cross-over study. Three different formulations of E hydrochloride were administered, two intranasal sprays of 5 and 10 mg per dose and a 50-mg oral capsule of E respectively separated by a one week wash-out period.

The subjects received the drug at 8:30 in the morning. Blood samples were collected in lithium heparinized tubes at 0; 0.25; 0.5; 1; 2; 3; 4; 6 and 8 h following each dose. The samples were centrifuged and plasma was separated and stored at −20°C until analysis.

3. Results and discussion

3.1. Selectivity and specificity

Fig. 2 shows at T_0 and $T_{0.25}$ chromatograms of a subject receiving 5 mg intranasal E and a chromato-

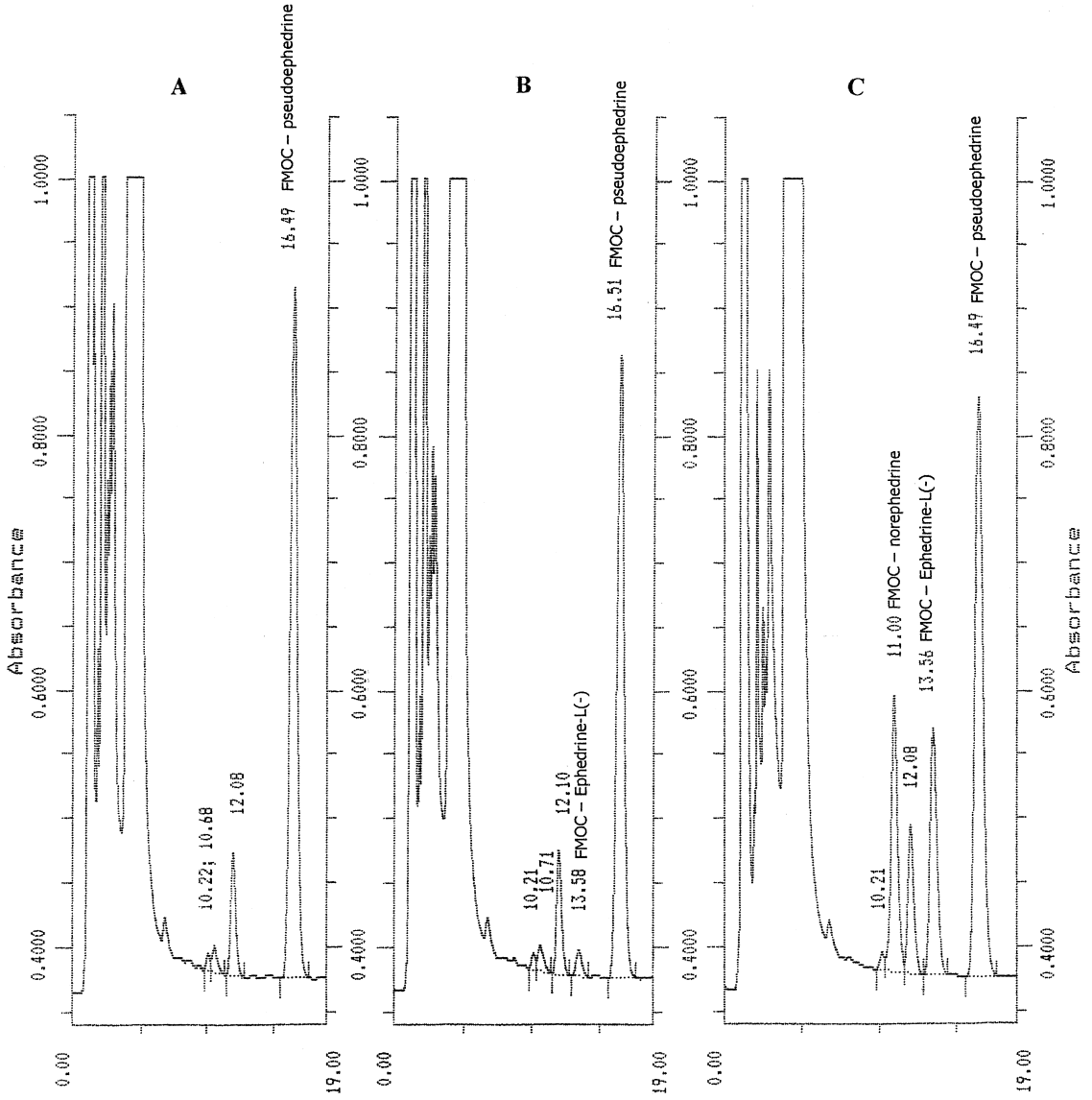

Fig. 2. Chromatograms of: (A) subject sample treated intranasally with 5 mg of E at T_0, (B) at $T_{0.25}$ containing 6 ng/ml of ephedrine, (C) standard plasma spiked with 60 ng/ml of E and NE.

gram of a spiked plasma sample containing 60 ng/ml of E and NE. Peaks of derivatized E, NE and PE were well separated; under the described conditions, the retention time was 13.6; 11.0 and 16.5 min, respectively. Blank plasma (T_0) showed no interfering peak at the retention time of the molecules studied. Other drugs containing primary or secondary amines were tested: acebutolol, amineptine, amphetamine, atenolol, clenbuterol, furosemide, lidocaine, propranolol, salbutamol, triamterene, fluvoxamine, fluoxetine, venlafaxine and viloxazine. No interference was detected with any of these derivatized compounds at the retention time of derivatized E, NE or PE. The derivatization process using FITC [10] did not show interference either with drugs commonly used for flu or allergy, but no precision about the

structure and the possibility for these drugs to effectively react with FITC was given. Amphetamines that are structurally close to E are eluted by the washing mobile phase. A large peak was detected at 4.5 min, corresponding to the non-reacted FMOC.

Most of the published methods used UV detection with wavelengths in the range of 205 to 220 nm, but at these wavelengths, the specificity of the UV detection is cautious [4–9]. The specificity of the fluorimetric detection with the FMOC depends on two factors. First, the emission wavelength of the product FMOC-E is specific and no endogenous interference has been detected with drug-free plasmas. Second, the FMOC reacts specifically and rapidly with primary and secondary amines such as E, PE or NE (Fig. 3). Mass spectrometry as reference method provides a similar specificity but the fluorimetric detection has the advantage of being easily implementable in a routine laboratory.

3.2. Stability

Under our conditions, E, NE and PE fully react with FMOC: after derivatization with FMOC, E, NE and PE, were undetectable even at high concentrations by the HPLC method with UV detection at 220 nm. The derivatized products were found to be stable for at least 24 h in the dark at room temperature. The stability of the final mixture highly depends on the acetonitrile proportion of this mixture, but if more than 50% of acetonitrile is added, peak broadening occurs which decreases both sensitivity and selectivity of the assay. Herráez-Hernández et al. [11] have already mentioned a 22% reduction in peak areas 8 h after preparation of samples when a solution of FMOC in acetonitrile–water (10:90; v/v) was used. To avoid this degradation, on-line derivatization directly in the trapping column was carried out, but this procedure demands a sophisticated injector. FITC derivatization of NE leads to products which are stable for 24 h at room temperature [10]. In our study, derivatized products had to be stable long enough to allow analysis of the 50 samples of one subject during a single run. Loss of stability of derivatized products was observed when a single liquid–liquid extraction instead of double extraction was performed. This problem might be related to impurities that were not extracted during the first step of the extraction.

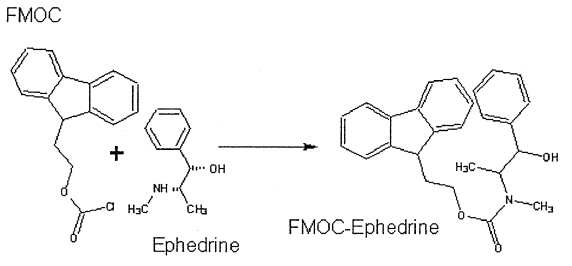

Fig. 3. Derivatization reaction of FMOC with E.

3.3. Linearity, reproducibility, limit of quantification

Calibration curves were linear in the range of 0 to 300 ng/ml for both E and NE, r was higher than 0.998 ($n=3$) in this range of concentrations. Quality controls spiked with E and NE at a final concentration of 250 ng/ml and 300 ng/ml were assayed ($n=6$) with calibration curves (0 to 300 ng/ml) for the linearity of the method. For the 250 ng/ml theory concentration, calculated concentrations (±C.V.%) were 259±6.2 (NE) and 257±5.6 (E). For the 300 ng/ml theory concentration, calculated concentrations were 319±5.9 (NE) and 316±5.2 (E). Beyond the 300 ng/ml concentrations, the signal reached the highest detection capacity of the fluorescence monitor. Consequently, in our conditions, the limit of the linearity is 300 ng/ml.

Within-day and between-day reproducibility data are reported in Table 1. Coefficient correlation of calibration curves used for between-day reproducibility were higher than 0.998.

The limit of quantification (LOQ) estimated under described conditions with a coefficient of variation below 20% and at a signal-to-noise ratio up to 3/1 were 2 ng/ml for E and 5 ng/ml for NE, respectively. The previously published methods, using UV detection, for urine samples obtain a LOQ of 2 μg/ml [7] and limit of detection (LOD) of 0.2 μg/ml [7] for E and NE respectively. The LOQ for plasma samples were 10 to 25 ng/ml for PE. All these methods are at least ten-fold less sensitive than ours. Fluorimetric detection avoids the relatively poor sensitivity of UV detection and allows a LOQ as low as 25 ng/ml to be reached for E and NE and

Table 1
Accuracy, within-day and between-day [mean (coefficient of variation, %)] precision for the analysis of NE and E in human plasma samples ($n=10$)

Added concentrations (ng/ml)	Calculated concentrations: mean (C.V., %)			
	Between-day reproducibility		Within-day reproducibility	
	Ephedrine	Norephedrine	Ephedrine	Norephedrine
5	4.8 (12.6)	5.6 (19.6)	5.0 (7.8)	5.0 (18.4)
60	60.6 (3.9)	63.7 (7.0)	60.3 (5.3)	61.8 (6.3)
160	157.1 (8.8)	158 (3.8)	161.0 (7.8)	150.6 (5.9)

10 ng/ml for PE [11]. With FITC derivatization procedure Al-Dirbashi et al. [10] obtained an excellent LOD of 1.6 ng/ml for NE and were able to quantify as low as 2.5 ng/ml in urine samples. This method is very sensitive for the determination of NE but failed to quantify E which is actually the most active drug. No precise quantification of E by GC–MS has been found but Tatsuno et al. [2] recently determined various illicit drugs including E, a range of 2 to 40 ng/ml was given for all these drugs. Consequently, the sensitivity of our method compares adequately with the GC–MS method.

3.4. Recoveries

The recovery of the extraction was calculated by comparing peak height ratios of all the derivatized extracts from the three standard solutions to the ratio obtained after direct injection of the same amounts of derivatized E, NE and PE. The mean recoveries were, respectively, 100% for E (C.V.=6.4%), 99.6% for NE (C.V.=5.4%) and 95% for PE (C.V.=6.4%). If isopropylic alcohol was omitted, recoveries fell from 100% to 67.5% for E and from 99.6% to 76% for NE. Recoveries were of the same magnitude when a single extraction step was used including evaporation to dryness of organic solvent under a stream of nitrogen in a cold water bath. This procedure was however time-consuming and a degradation of E and NE was observed when a hot water bath was used to accelerate evaporation. Furthermore, stability of the derivatized solutions was dramatically reduced. Previous published methods using plasma samples involve back extraction [9], solid–liquid extraction [6] or in-line extraction with a trapping column [4], but these methods only quantify PE. The recoveries for PE for the last two methods are similar to ours. Al-Dirbashi et al. [10] described a double liquid–liquid extraction for urine samples and obtain comparable recoveries in the range 92 to 104.5%. But the method includes hydrolysis of the conjugated metabolites, which is time-consuming and leads to the quantification of inactive compounds.

3.5. Subjects samples

Over 450 plasma samples from 16 subjects have been assayed with this method for pharmacokinetic purpose. For all subjects and doses, E was always detected with concentrations above the LOQ, while NE was below the LOQ (5 ng/ml). Fig. 4 shows the pharmacokinetic profile of E in one subject.

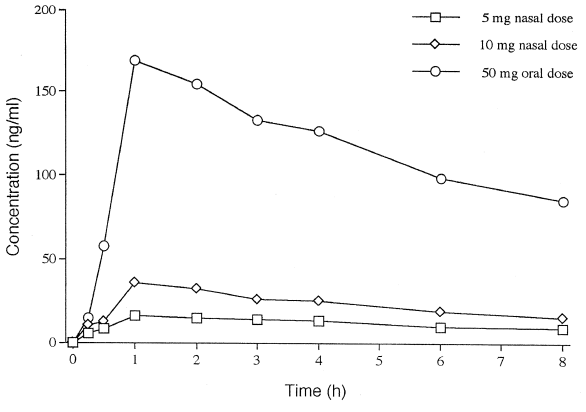

Fig. 4. Pharmacokinetic curves of E between 0 and 8 h in one subject after administration of 5 mg intranasal, 10 mg intranasal and 50 mg oral dose of E.

4. Conclusions

The method described in this paper allows a specific and sensitive quantification of E and NE with simple equipment commonly available in laboratories. This method is one of the first involving a derivatization procedure and fluorimetric detection applied to the assay of plasma samples. Chromatographic conditions can be easily adjusted to quantify amphetamines in plasma samples.

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