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Simultaneous determination of free catecholamines and epinine and estimation of total epinine and dopamine in plasma and urine by high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

Epinine (N-methyldopamine) is the pharmacologically active hydrolysis product of the prodrug ibopamine, which is currently being widely studied for the treatment of congestive heart failure. This paper reports a sensitive and reliable method for the simultaneous determination of free catecholamines and epinine in plasma and urine. The compounds are isolated from plasma or urine by a specific liquid–liquid extraction, derivatized with the selective fluorogenic agent 1,2-diphenylethylenediamine, and quantitated by high-performance liquid chromatography with gradient elution and fluorimetric detection. The limits of detection for the derivatized catecholamines and epinine are 0.3–0.6 pg of injected compound. Intra- and inter-assay coefficients of variation of all four compounds are good (1–8%), as are the accuracy and linearity. A method is also reported for the determination of total dopamine and epinine in plasma and urine based on the same principle. This method, in which deconjugation is accomplished by acid hydrolysis at 95°C, also shows good sensitivity and reproducibility.

INTRODUCTION

The prodrug ibopamine, the diisobutyryl ester of N-methyldopamine (epinine, EPI), is rapidly hydrolysed by esterases after oral administration to form the biologically active product EPI. EPI acts pharmacologically at the α and β adrenoceptors and at the dopaminergic DA1 and DA2 receptors. Owing to its vasodilator, inotropic and natriuretic effects, ibopamine is currently being widely studied for the treatment of congestive heart failure [1–3].

EPI is rapidly cleared from the circulation by conjugation with inorganic sulphates and glucuronic acid and through metabolization by monoamine oxidase and catechol-O-methyltransferase to 4-hydroxy-3-methoxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid. Consequently, free EPI has a relatively short plasma half-life (7.5 min), which is typical for catecholamines.

For studies on the clinical pharmacology of ibopamine, sensitive and reliable determinations of EPI in plasma and urine are required. The simultaneous determination of catecholamines in urine and plasma would be advantageous for two reasons: (1) EPI lowers the increased levels of plasma norepinephrine (NE) in patients with congestive heart failure, probably through the stimulation of ganglionic DA2 receptors, which inhibit the neuronal release of NE [4,5]; (2) some workers have claimed that EPI might be converted to epinephrine (E) *in vivo* [6,7]. The determination of total (free plus conjugated) EPI is important for investigating the rate and extent of conjugation of EPI.

A few methods for determining the concentra-

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tions of free and total EPI in plasma and urine have been reported, all using high-performance liquid chromatography with electrochemical detection (HPLC–ED) [4,8,9]. Deconjugation was performed either by enzymatic or by acid hydrolysis. A method for the simultaneous determination of free plasma catecholamines and EPI, also using HPLC–ED, has also been published [10].

The determination of catecholamines by HPLC with fluorimetric detection (HPLC–FD) has advantages over HPLC–ED with respect to sensitivity and selectivity, in both plasma [11] and urine [12]. This paper reports a method for the simultaneous determination of free catecholamines and EPI by HPLC–FD after liquid–liquid extraction from plasma or urine and derivatization with the fluorigenic agent 1,2-diphenylethylenediamine (DPE). A similar method is also reported for determining total EPI in plasma and urine after acid hydrolysis, in which total dopamine is also estimated.

EXPERIMENTAL

Materials

Dopamine (DA), α-methylnorepinephrine (AMN), EPI, NE, E and α-methyldopamine (AMD) were obtained from Sigma (St. Louis, MO, USA), heptane from Baker (Deventer, Netherlands) and octan-1-ol from Merck (Darmstadt, Germany). EPI was also obtained as a gift from the Zambon Group (Milan, Italy). DPE was prepared as described previously [11].

Human blood samples were collected in chilled heparinized 10-ml polystyrene tubes containing 12 mg of glutathione and were centrifuged within 15 min at 4°C (15 min, 3000 *g*). Plasma was stored at −70°C. Urine was collected in plastic containers and stored at −20°C.

Apparatus

The instrumentation for chromatography consisted of a Spectra-Physics SP8800 low-pressure gradient pump, a Kontron 460 autosampler equipped with a 200-μl injection loop, a Shimadzu RFR 535 spectrofluorimeter (excitation at 350 nm, emission at 480 nm) and a Merck-Hitachi D-2500 integrator. The separations were performed on 3-μm Cp MicroSpher C_{18} (100 mm × 4.6 mm I.D.) (Chrompack, Middelburg, Netherlands) or on 3-μm PhaseSep C_{18} ODS2 (100 mm × 4.6 mm I.D.) (Phase Separations, Deeside, UK) columns.

Determination of free catecholamines and EPI in plasma

To a glass tube were added 1 ml of plasma, 125 μl of internal standard solution (AMN, 2 ng/ml), 1 ml of a 2 *M* ammonia–ammonium chloride buffer (pH 8.6) containing a diphenylborate–ethanolamine complex (8.9 m*M*) and EDTA (13.4 m*M*), and 5 ml of *n*-heptane containing tetraoctylammonium bromide (4.6 m*M*) and octan-1-ol (10 ml/l). After shaking for 2 min and centrifugation (5 min, 20°C, 1000 *g*) the aqueous layer was frozen in an acetone–carbon dioxide bath. The organic phase was poured into a polypropylene tube, 2 ml of octan-1-ol (saturated with 0.08 *M* acetic acid) and 200 μl of 0.08 *M* acetic acid were added, and the tube was shaken and centrifuged (5 min, 20°C, 1000 *g*). The aqueous layer was frozen and then the organic phase was removed by aspiration. The extraction procedure was repeated once more by the addition of 1 ml of 0.01 *M* hydrochloric acid, 1 ml of the ammonia–ammonium chloride buffer and 5 ml of the *n*-heptane solution.

The tube was again shaken and centrifuged, the organic phase was separated after the aqueous layer had been frozen, and was shaken with 2 ml of 2 *M* ammonia–ammonium chloride buffer (pH 8.6) containing EDTA (13.4 m*M*) but no diphenylborate–ethanolamine complex. It was then again separated after freezing the aqueous layer. A 2-ml volume of octan-1-ol and 150 μl of 0.08 *M* acetic acid were then added, the tube was shaken and centrifuged (5 min, 20°C, 1000 *g*), and the organic phase was removed by aspiration after the aqueous layer had been frozen. The resulting frozen pellet was transferred to a 4-ml polypropylene tube, 200 μl of acetonitrile, 50 μl of bicine buffer [1.75 *M* in doubly distilled water containing 1% (w/v) EDTA; pH 6.95] and 100 μl of DPE (0.1 *M* in 0.1 *M* hydrochloric acid) were added, and the derivatization reaction was started with 20 μl of potassium ferricyanide (20 m*M* in doubly distilled water).

After incubation for 60 min in a water-bath at

37°C in the dark, 400 μl of the resulting solution were transferred to an Eppendorf vial. The autosampler injected 75 μl of this solution into the chromatographic system. A black cloth was put around the autosampler to keep the samples in the dark. Mobile phase A consisted of 0.05 *M* sodium acetate (pH 7.0)–acetonitrile–methanol in a ratio of 76:20:4 (v/v/v), whereas mobile phase B consisted of the same ingredients in a ratio of 30:60:10 (v/v/v). The elution was carried out with a mixture of 40% A and 60% B from 0.0 to 3.0 min; between 3.0 and 3.5 min the percentage of B was increased to 100% and maintained at this level for another 4.5 min. After 8 min the system was returned to its original state. The flow-rate was 1.0 ml/min. After the last sample had been chromatographed, the column was flushed with 60 ml of acetonitrile–methanol–doubly distilled water (70:10:20, v/v/v).

A standard mixture containing NE, E, DA and EPI (250, 250, 100 and 500 pg, respectively, in 250 μl of 0.01 *M* hydrochloric acid) was prepared freshly every day from stock solutions (100 ng/ml) stored at −70°C. The response factors were determined by taking 250 μl of the standard mixture and 125 μl of the internal standard through the described procedure in quadruplicate, except that the extraction was performed only once. Each assay also included a blank sample in duplicate.

Determination of free catecholamines and EPI in urine

The method for the determination of catecholamines and EPI in urine was basically the same as for the determination in plasma, with the following exceptions: (1) instead of 1 ml of plasma 100 μl of urine and 1 ml of 0.01 *M* hydrochloric acid were used; (2) the internal standard was 125 μl of AMN (40 ng/ml); (3) the washing step with ammonia–ammonium buffer without the diphenylborate–ethanolamine complex was omitted; (4) 50 μl of the derivatization mixture were injected into the chromatographic system; and (5) the standard mixture consisted of 1.25 ng of NE, 0.625 ng of E, 10 ng of DA and 5 ng of EPI in 250 μl of 0.01 *M* hydrochloric acid.

Determination of total EPI in plasma and urine

To a 1.5-ml Eppendorf tube were added 200 μl of twenty-fold diluted plasma or fifty-fold diluted urine, 100 μl of 6 *M* hydrochloric acid and 50 μl of AMD (50 ng/ml) as an internal standard. The tube was closed, vortex-mixed and heated for 60 min at 95°C in an Eppendorf thermostat. After 3 min centrifugation (10 000 *g*), 100 μl of the supernatant were added to a glass tube containing 900 μl of 2 *M* disodium hydrogenphosphate. Liquid–liquid extraction was performed as described previously, but was only performed once, without the extra washing step and with back-extraction into 150 μl of 0.08 *M* acetic acid. Derivatization was carried out as described for free catecholamines and EPI in urine, except that no AMN was added as an internal standard and a bicine buffer of pH 6.5 instead of 6.95 was used.

The response factors were determined by extraction and derivatization in quadruplicate of a standard mixture of 0.357 ng each of DA and EPI and 0.714 ng of AMD in 0.01 *M* hydrochloric acid. A 50-μl aliquot was injected into the chromatographic system. Elution was carried out as described earlier.

RESULTS AND DISCUSSION

Liquid–liquid extraction procedure

The liquid–liquid extraction procedure reported previously for the nearly quantitative extraction of catecholamines from plasma or urine [11,12] works equally well for EPI and AMD: when EPI and AMD (10 ng) were injected both directly and after liquid–liquid extraction into an HPLC–ED system [13], a comparison of peak areas showed absolute recoveries of 99.5 ± 0.4 and 102.1 ± 2.6% (mean ± S.D.), respectively ($n = 8$).

Derivatization procedure

The derivatization procedure as optimized for catecholamines [11] also gives good results for EPI and AMD. Variations in the pH of the bicine buffer showed that both EPI and AMD give a rapid decrease in the fluorescence signal when the pH of the bicine buffer is higher than 7, even more so than DA, whereas maximum fluorescence for NE and E are reached at pH values slightly above 7.0. A good compromise is to keep

the pH of the buffer slightly below 7. In the method for the determination of total DA and EPI, in which NE and E are not determined simultaneously, such a compromise is not necessary and good results are obtained with the bicine buffer at pH 6.5. Variations in the derivatization time showed that for EPI and AMD plateau levels of fluorescence were reached within 60 min. The stability of the fluorescence signal in the dark at 20°C was excellent for EPI (still unchanged after 10 h), but showed a slow and gradual decline for AMD (92 and 86% of the original signal after 6 and 10 h, respectively).

Determination of free catecholamines and EPI

Chromatography. Isocratic elution with a mobile phase of 0.05 *M* sodium acetate (pH 7.0)–acetonitrile–methanol (50:40:8, v/v/v) as used in the method for the determination of catecholamines, gives a late and broad signal for EPI at 18 min. It was therefore decided to apply gradient elution. Using mobile phases A and B, consisting of the same components in ratios of 76:20:4 and a 30:60:10 (v/v/v), respectively, an initial mixture of 40% A and 60% B was used switching to 100% B at various times. As the switch in eluting solution always gave some increase in the baseline fluorescence signal, the aim was to obtain this increase in baseline after the elution of DA, and to obtain a stable baseline again when EPI eluted. This could be accomplished by switching between 3.0 and 3.5 min. At

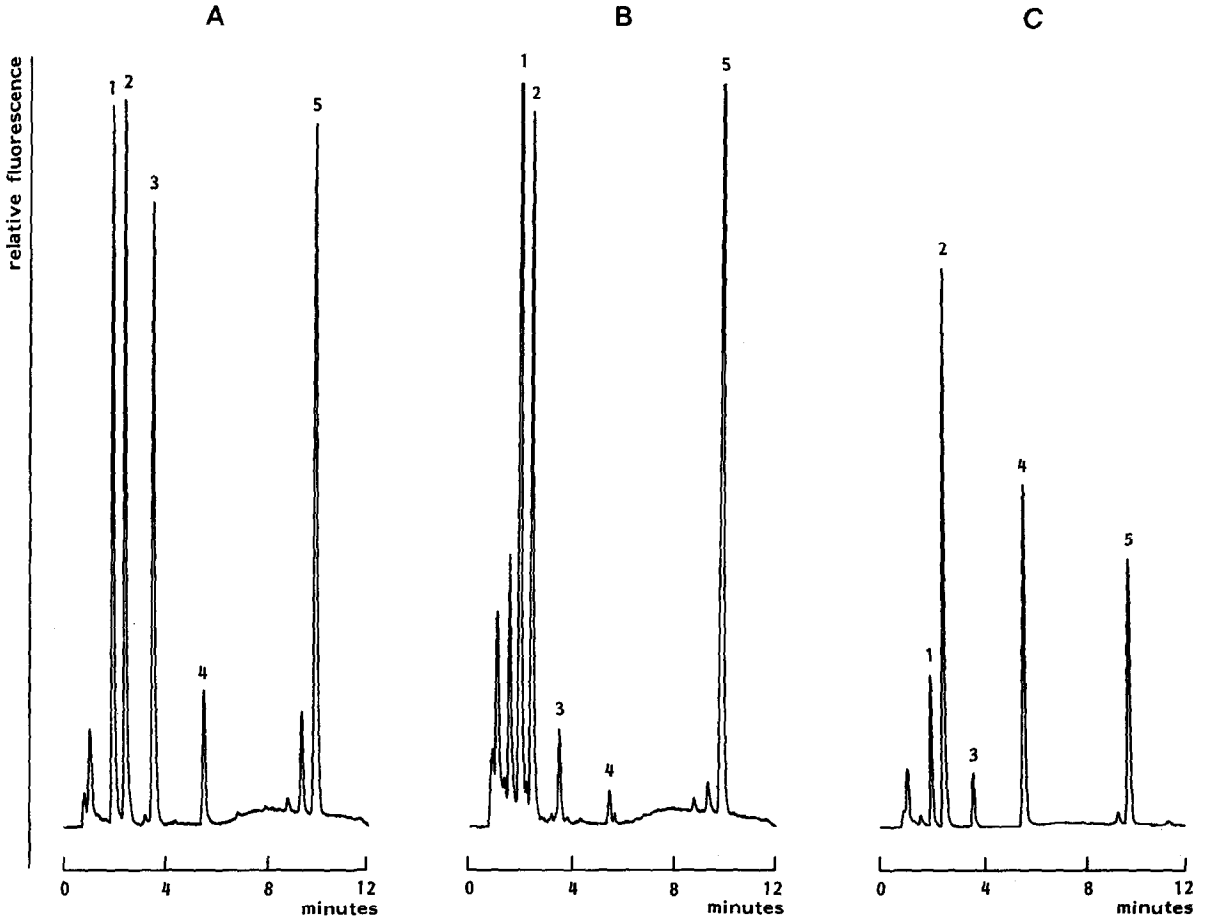

Fig. 1. Chromatograms of free catecholamines and epinine obtained by the HPLC–FD method. Peaks: 1 = norepinephrine (NE); 2 = α-methylnorepinephrine (AMN); 3 = epinephrine (E); 4 = dopamine (DA); 5 = epinine (EPI). (A) Standard mixture. Amounts injected: 33 pg of NE, AMN and E; 13 pg of DA; and 66 pg of EPI. (B) Plasma sample from a patient treated with ibopamine (NE, E, DA and EPI 414, 40, 25 and 603 pg/ml, respectively; internal standard 250 pg/ml AMN). (C) Urine sample from a patient treated with ibopamine (NE, E, DA and EPI 15.4, 6.1, 78.8 and 55.8 ng/ml, respectively; internal standard 50 ng/ml AMN).

8 min the composition of the eluting solution was returned to the starting composition of 40% A and 60% B. A new sample could be injected 5 min later. Under these conditions catecholamines and EPI gave clear, well separated peaks and a stable baseline, as can be seen in Fig. 1. Some batches of octanol used in the liquid–liquid extraction gave an unidentified peak at nearly the same retention time as EPI, which could not be clearly resolved from the EPI peak by changes in the mobile system. Exchanging the Chrompack MicroSpher C_{18} column for a PhaseSep C_{18} ODS2 column while maintaining the same elution conditions resulted in an equally good separation between the catecholamines and EPI, whereas the unknown peak eluted just prior to, and clearly separated from, EPI.

It should be noted that the timing of the changes in the mobile phase composition depends on the particular chromatographic system used and should be established separately for each system.

Characteristics of the assay. The analyses of standard mixtures with increasing concentrations of EPI showed that linear fluorescence responses were obtained up to at least 13.2 ng of EPI injected as the fluorescent derivative, resulting in linearity up to 100 ng/ml for the plasma and 1500 ng/ml for the urine method. The detection limit, at a signal-to-noise ratio of 2, was 0.6 pg injected as the fluorescent derivative, *i.e.* 5 pg/ml of plasma and 68 pg/ml of urine. The accuracy was good: the determination of EPI in plasma samples spiked with 10, 100, 1000 and 10 000 pg of EPI per ml gave concentrations of 10, 97, 984 and 9729 pg/ml, respectively ($n=2$). The reproducibility was tested with a plasma pool enriched in EPI and with a urine pool from a patient treated with ibopamine, and was found to be good (Table I). It should be noted that in plasma enriched with EPI, higher than normal DA concentrations are found because of the contamination of commercial EPI with DA (1.88%). The EPI obtained from the Zambon Group also contained DA, but less than the commercial samples (0.66%).

In the chromatogram extra peaks were only observed in a plasma sample of a patient also treated with α-methyldopa. In this instance, a peak of α-methyldopamine was observed (between DA and EPI) and a peak just before EPI, which corresponded to α-methyldopa itself, which is extracted and derivatized to a very small extent.

TABLE I

REPRODUCIBILITY OF THE ASSAYS

	n	NE		E		DA		EPI	
		Mean concentration	C.V. (%)	Mean concentration	C.V. (%)	Mean concentration	C.V. (%)	Mean concentration	C.V. (%)
Free compounds									
Plasma (pg/ml)									
Intra-assay	8	334	2.8	30	3.0	71	2.4	980	2.5
Inter-assay	5	260	2.9	34	7.6	63	5.3	912	6.6
Urine (ng/ml)									
Intra-assay	6	112.3	0.7	18.8	0.7	467.8	2.5	244.3	1.9
Inter-assay	5	120.1	8.2	18.6	3.5	513.6	9.5	260.2	7.7
Total compounds									
Plasma (ng/ml)									
Intra-assay	5	–	–	–	–	16.1	2.1	272.5	0.6
Inter-assay	5	–	–	–	–	30.5	6.7	863.9	2.9
Urine (μg/ml)									
Intra-assay	5	–	–	–	–	2.5	1.5	45.7	2.4
Inter-assay	5	–	–	–	–	2.0	5.8	36.2	8.5

Determination of total EPI and DA

Although the primary interest was in determining total EPI, determining also total DA has the advantage of providing an additional check on the performance of the deconjugation procedure. For a chromatographic method to determine only DA and EPI, AMD was found to be a good internal standard with a retention time in between those of DA and EPI under the chromatographic conditions used for free catecholamines and EPI. Sharp, well separated peaks were obtained in this method (Fig. 2).

Deconjugation of EPI sulphate and EPI glucuronide has been accomplished by enzymatic hydrolysis with sulphatase or glucuronidase [8,9]. Pocchiari *et al.* [9] found acid hydrolysis with perchloric acid to be equally good and much faster: heating for 1 h at 60°C hydrolysed the sulphate, whereas the glucuronide was deconjugated by heating for 1 h at 100°C. As the use of perchloric acid leads to problems in the subsequent extraction and derivatization procedure used here for EPI, other acids were investigated. Hydrochloric acid (6 *M*) was suitable; neutralization (necessary for the liquid–liquid extraction) could be accomplished with 2 *M* phosphate buffer and the derivatization procedure was not hampered.

The deconjugation procedure was carried out at 95°C with 6 *M* hydrochloric acid for various lengths of time with a plasma and urine pool

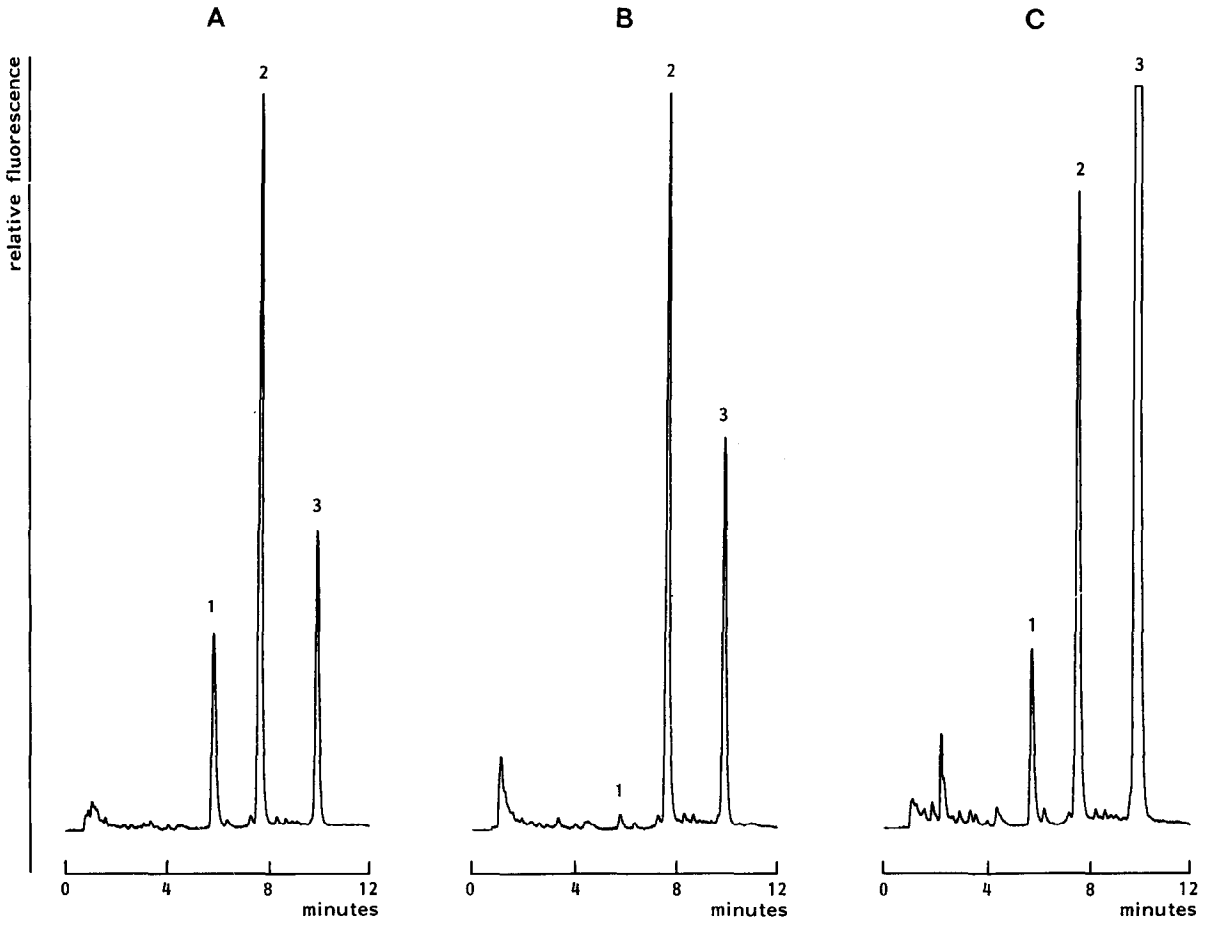

Fig. 2. Chromatograms of total dopamine and epinine obtained by the HPLC–FD method. Peaks: 1 = dopamine (DA); 2 = α-methyldopamine (AMD); 3 = epinine (EPI). (A) Standard mixture. Amounts injected: 31 pg of DA and EPI; 63 pg of AMD. (B) Plasma sample from a patient treated with ibopamine (total DA and EPI 11.2 and 161.4 ng/ml respectively; internal standard 250 ng/ml AMD). (C) Urine sample from a patient treated with ibopamine (total DA and EPI 0.3 and 6.1 μg/ml, respectively; internal standard 0.625 μg/ml AMD).

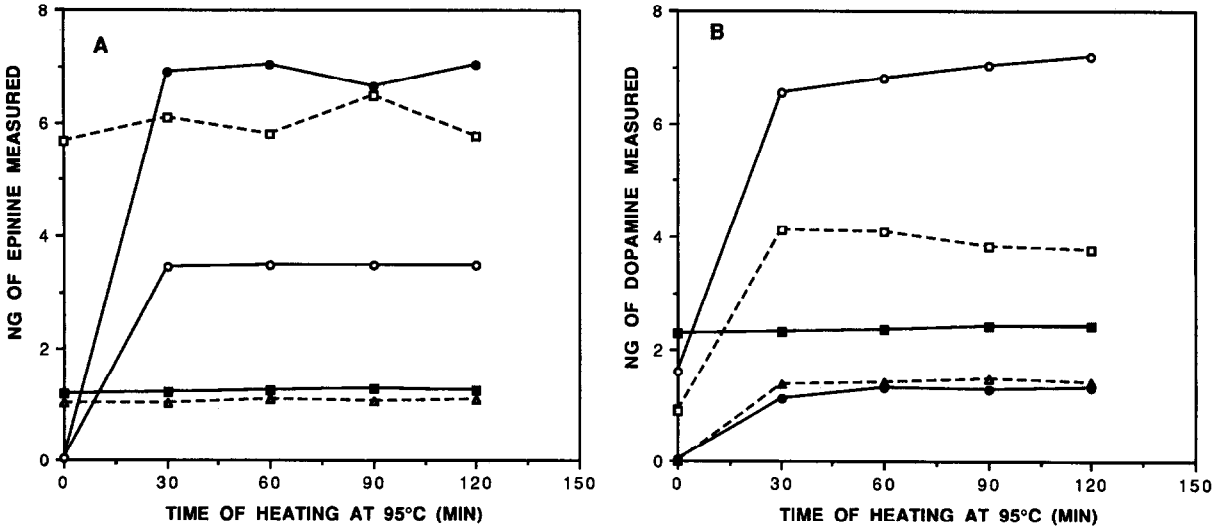

Fig. 3. Amounts of (A) epinine and (B) dopamine measured after various times of heating at 95°C. (■) DA and EPI added to doubly distilled water containing bovine serum albumin (40 g/l) and glutathione (10 g/l); (▲) EPI added to plasma pool from normal subjects; (□) EPI added to urine pool from normal subjects; (●) plasma pool of EPI-treated patients; and (○) urine pool of EPI-treated patients.

spiked with free EPI, a plasma and urine pool of patients treated with ibopamine, and a solution of DA and EPI in doubly distilled water containing 40 g/l bovine serum albumin and 10 g/l glutathione. Fig. 3 shows that the deconjugation of DA and EPI proceeds rapidly and that maximum concentrations of DA and EPI are reached within 60 min which remain stable during continued heating. Similar experiments performed at 65°C showed a much slower increase in DA and EPI, and no plateau level was reached within 2 h. Fig. 3 also shows that EPI is stable during the heating procedure in plasma and urine, and both EPI and DA in doubly distilled water containing albumin and glutathione.

TABLE II

RECOVERY OF ADDED DA, AMD AND EPI TAKEN THROUGH THE METHOD FOR THE DETERMINATION OF TOTAL DA AND EPI

Data are mean ± S.D. ($n = 6$).

Medium	Absolute recovery (%)			Relative recovery (%)	
	DA	AMD	EPI	DA	EPI
Bid–BSA–Glut[a]					
Heating	84.7 ± 2.5	87.5 ± 1.2	88.2 ± 2.2	96.7 ± 1.6	100.7 ± 2.7
No heating	83.0 ± 3.0	84.0 ± 2.0	81.3 ± 3.4	98.8 ± 1.6	96.7 ± 2.4
Plasma (undiluted)					
Heating	93.2 ± 3.4	97.6 ± 3.2	104.4 ± 1.9	95.5 ± 0.9	107.1 ± 2.4
No heating	84.8 ± 2.1	88.2 ± 1.6	93.9 ± 1.3	96.1 ± 1.3	106.5 ± 1.1
Plasma (20 × diluted)					
Heating	97.1 ± 1.2	102.2 ± 2.1	101.0 ± 2.7	95.3 ± 1.6	99.3 ± 4.4
No heating	82.7 ± 2.1	83.5 ± 1.6	84.6 ± 2.2	99.0 ± 1.1	101.4 ± 1.0
Urine (50 × diluted)					
Heating	110.9 ± 3.7	108.2 ± 2.1	107.4 ± 1.6	102.5 ± 2.9	99.4 ± 1.8
No heating	104.4 ± 2.5	103.3 ± 1.5	102.7 ± 1.7	101.1 ± 2.2	99.4 ± 1.3

[a] Bid–BSA–Glut = doubly distilled water containing 40 g/l bovine serum albumin and 10 g/l glutathione.

When dissolved in 0.01 *M* hydrochloric acid, the stability of DA, EPI and AMD on heating at 95°C is fairly erratic, and the compounds are often completely destroyed. All three compounds are stable in doubly distilled water containing bovine serum albumin (40 g/l) and glutathione (10 g/l), and absolute recoveries are similar (Table II) with or without heating. Absolute and relative recoveries were also investigated with an undiluted plasma and a fifty-fold diluted urine pool enriched with 5 ng of DA, AMD and EPI, after correction for the separately determined endogenous DA content. In the undiluted plasma pool the absolute recovery of EPI was consistently higher than that of AMD, which was in turn higher than that of DA (Table II). In freshly obtained plasma the recoveries of added DA and AMD were even lower. When plasma was first diluted twenty-fold, no such variations in recoveries were found, and the absolute recoveries were good and similar for all compounds.

It was found that performing the liquid–liquid extraction only once, and without the extra washing step, is sufficient to obtain the optimum fluorescence signals, probably because the small amounts of urine and plasma actually used in the extraction procedure greatly reduce the amounts of the substances which hamper the development of fluorescence.

As the relative recoveries of DA and EPI are the same with or without heating at 95°C, the heating step could be omitted when preparing standard mixtures for the determination of response factors.

Characteristics. With the linearity and limit of detection of the EPI fluorescence signal established, the method is valid for total EPI concentrations between 6 and 130 000 ng/ml in plasma and between 0.015 and 330 μg/ml in urine. The reproducibility of the method was good (Table I). Interference can only be expected to occur in samples from patients also treated with α-methyldopa, in which instance an apparent recovery of $>100\%$ of the internal standard AMD is seen.

As pure conjugated DA and EPI were not available, it was not possible to rigorously establish the completeness of this acid hydrolysis procedure. The stability of the compounds during heating and the plateau concentrations reached make it most likely that almost complete hydrolysis is obtained. Also, the total DA concentrations found in plasma and urine from six normal subjects (3–17 ng/ml and 0.5–3.0 μg/ml, respectively) are in agreement with previously published results [14–17].

CONCLUSIONS

It is concluded that the HPLC–FD methods described here are sensitive and reliable for the simultaneous determination of free catecholamines and EPI in plasma and urine and for the determination of total EPI and DA after acid hydrolysis. The reproducibility is good, interferences have only been seen in plasma from patients treated with α-methyldopa, and a large number of samples (40) can be processed in one day by one technician. The method can also be used to determine only free catecholamines in patients treated with EPI, as the late-eluting EPI would otherwise interfere in subsequent chromatograms.

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