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Short Communication

Improved measurement of urinary catecholamines by liquid–liquid extraction, derivatization and high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

We report a sensitive and reliable assay for the determination of the urinary catecholamines norepinephrine, epinephrine and dopamine, based on selective extraction by a liquid–liquid extraction procedure, followed by selective derivatization with the fluorigenic agent 1,2-diphenylethylenediamine and quantification by high-performance liquid chromatography with fluorimetric detection. Comparison with a method using electrochemical detection shows that interference of an unknown compound, most probably N-methylepinephrine, which is an often-overlooked problem with methods using electrochemical detection and results in falsely high epinephrine concentrations, does not occur with the described fluorimetric method.

INTRODUCTION

Many methods have been reported for the determination of the urinary free catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA) [1]. Most of these methods are based on high-performance liquid chromatography (HPLC) either with electrochemical detection (ED) [2–7] or fluorimetric detection (FD) [8–10] after suitable derivatization. Prior to chromatography, a pre-purification step is usually performed employing alumina, solid-phase extraction or liquid–liquid extraction. Despite all the work that has been done problems are still encountered in the accurate measurement of urinary catecholamines.

Recently we reported a sensitive and reliable assay for the determination of catecholamines in plasma, based on selective extraction from plasma by a liquid–liquid extraction procedure, followed by selective derivatization with the fluorigenic agent 1,2-diphenylethylenediamine (DPE) and quantification by HPLC–FD [11]. We have found this method, with some modifications, to be eminently

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suitable also for the determination of catecholamines in urine. The most important (and essential) modification was than not more than 100 μl of urine, diluted with 1 ml of 0.01 *M* HCl, could be used in the liquid–liquid extraction procedure (instead of 1 ml of plasma). Furthermore, the extra washing step necessary in the extraction procedure for plasma can be omitted, and only 50 μl are injected into the HPLC system. The method thus modified gives excellent results with respect to sensitivity, selectivity and precision. In particular, it circumvents the interference from an unknown compound, most probably N-methylepinephrine, which is an often-overlooked problem with HPLC–ED methods resulting in falsely elevated E concentrations.

EXPERIMENTAL

Materials

NE, E, DA, α-methylnorepinephrine, isoproterenol and dihydroxybenzylamine were obtained from Sigma (St. Louis, MO, U.S.A.), and N-methylepinephrine hydrochloride was purchased from Janssen (Beerse, Belgium). DPE was prepared as described before [11].

Apparatus

The instrumentation for chromatography consisted of a Kratos SF-400 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-2000 integrator. Separations were performed on 3-μm Cp™ MicroSpher C_{18} (100 mm × 4.6 mm I.D.) columns (Chrompack, Middelburg, The Netherlands). Electrochemical measurements were done as described before [11].

HPLC–FD method

To a glass tube were added 100 μl of urine, 125 μl of internal standard solution [218.6 n*M* α-methylnorepinephrine (AMN) in 0.01 *M* HCl], 1 ml of 0.01 *M* HCl, 1 ml of a 2 *M* ammonia–ammonium chloride buffer (pH 8.6), containing 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 1-octanol (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 1-octanol (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 aspirated off. Next, 1 ml of 0.01 *M* HCl was added, and the extraction procedure described above was repeated, except that 150 μl of 0.08 *M* acetic acid were used instead of 200 μl. The resulting frozen pellet was transferred to a 4-ml polypropylene tube, and 200 μl of acetonitrile, 50 μl of bicine buffer

[1.75 *M* in bidistilled water containing 1% (w/v) EDTA; pH 7.05] and 100 μl of DPE (0.1 *M* in 0.1 *M* HCl) were added. The derivatization reaction was started with 20 μl of potassium ferricyanide (20 m*M* in bidistilled 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 50 μl into the chromatographic system. A black cloth was put around the autosampler to keep the samples in the dark. The mobile phase consisted of 0.05 *M* sodium acetate (pH 7.0)–acetonitrile–methanol (50:40:8, v/v). 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–bidistilled water (70:10:20, v/v).

A standard mixture containing NE, E and DA (29.59, 13.66 and 261.44 n*M*, respectively) in 0.01 *M* HCl was prepared freshly every day from stock solutions (0.55–0.65 μ*M*) stored at −70°C. Response factors were determined by taking 500 μl of the standard mixture (in quadruplicate) through the procedure described above, except that the extraction was performed only once. Each assay also included a blank in duplicate.

HPLC–ED method

A 100-μl volume of urine was extracted, together with 50 μl of a 0.718 μ*M* solution of dihydroxybenzylamine (DHBA) in 0.01 *M* HCl as internal standard, as reported before [11]. The extraction procedure was performed only once, and 100 μl of the resulting 250 μl of acidic solution were injected into the HPLC system. Response factors were determined by taking through the same procedure a standard mixture containing 0.592, 0.546 and 0.654 μ*M* NE, E and DA, respectively.

RESULTS AND DISCUSSION

Extraction procedure

The liquid–liquid extraction procedure, which we have already used succesfully for many years for the isolation and concentration of catecholamines from plasma [11], can also be used with good results for the isolation of catecholamines from urine, provided that the amount of urine taken through the procedure does not exceed 100 μl. With larger amounts of urine recoveries of catecholamines may sometimes deviate significantly from the usual 95–100%, especially when the urine is concentrated. The amounts of catecholamines present in urine, however, are sufficient for 100-μl samples to contain more than enough for accurate determination. Absolute recoveries of NE (29.6 pmol), E (13.7 pmol) and DA (261.4 pmol) added to 100 μl of ten different urine samples were (mean ± S.D.) 93.1 ± 1.4, 93.0 ± 1.0 and 95.2 ± 1.9%, respectively. Recovery of the internal standard AMN was 95.0 ± 2.4% in 318 samples analysed.

The extraction procedure had to be performed twice in order to obtain stable and optimal fluorescence signals and to get rid of interfering signals, as reported

before [11]. The extra washing step of the heptane layer, which is necessary in the plasma catecholamine determination method, can be omitted when urine samples are extracted, probably because of the absence or low concentrations in urine samples of glutathione, which interferes in the derivatization procedure.

Derivatization procedure and chromatography

Once the extraction procedure has been performed, the derivatization of the catecholamines with DPE and the subsequent chromatography proceed as described before for plasma catecholamines [11]. Clean chromatograms are obtained with sharp, clearly separated peaks for NE, E, and DA (Fig. 1A). Although AMN and isoproterenol (ISO) are equally suitable as internal standards, we usually prefer AMN because of its shorter retention time. Interferences due to medication are very rare: we have only found additional fluorescent signals in urine samples of patients treated with α-methyldopa (at the retention time of ISO) or with labetalol (two peaks just after E). In both cases quantification of catecholamines is not hampered when AMN is used as internal standard.

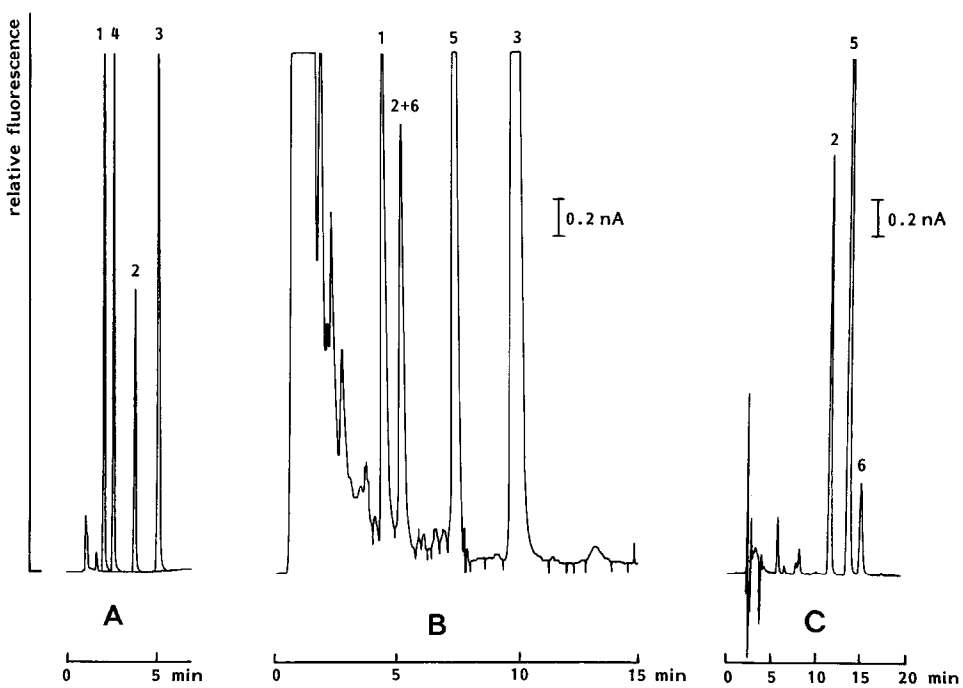

Fig. 1. Chromatograms of a urine sample assayed with the HPLC–FD (A) and HPLC–ED (B) methods and of the epinephrine peak from (B) after rechromatography (C). Peaks: 1 = norepinephrine (NE); 2 = epinephrine (E); 3 = dopamine (DA); 4 = α-methylnorepinephrine; 5 = dihydroxybenzylamine; 6 = N-methylepinephrine. Concentrations: NE, 0.036 μM; E, 0.017 μM; DA, 0.302 μM. See text for chromatographic conditions.

TABLE I

PRECISION OF THE ASSAY

Compound	Intra-assay precision (n = 10)		Inter-assay precision (n = 9)		Recovery (mean ± S.D.) (%)	
	Mean concentration (μM)	C.V. (%)	Mean concentration (μM)	C.V. (%)	Absolute	Relative
NE	0.220	3.5	0.220	6.2	94.8 ± 5.4	102.7 ± 1.3
E	0.091	3.3	0.089	6.1	95.9 ± 3.5	104.0 ± 3.7
DA	1.180	3.2	1.137	4.3	98.2 ± 6.3	106.2 ± 6.8
AMN					93.9 ± 4.4	

Characteristics of the assay

The linearity of the assay was tested by measuring a pooled urine to which incremental amounts of NE, E and DA had been added. Linearity was found to be excellent at least up to concentrations of 0.592 μM for NE, 0.164 μM for E and 4.9 μM for DA. Lowest concentrations measured (still clearly above detection limits) were 5.3 nM for NE, 1.6 nM for E and 24.8 nM for DA.

A pooled urine was used to investigate intra-assay precision (n = 10) as well as inter-assay precision (on nine consecutive days). In the latter experiment, the same pool spiked with NE, E and DA (0.30, 0.14 and 2.61 μM, respectively) was also included. Results are presented in Table I.

Comparison with an ED method

ED has been widely employed for the quantification of catecholamines in urine. Using a single extraction step and ED, reasonably clean chromatograms can be obtained (Fig. 1B), but often with more interfering peaks than with the FD method. These interferences did not disappear after a second extraction step. For comparison, we determined the catecholamine concentration in fifteen urine samples by both methods. The correlation was good for NE and DA, with regression lines and correlation coefficients $y(\text{ED}) = 0.966\ x(\text{FD}) + 0.240$ (r = 0.988) and $y(\text{ED}) = 0.960\ x(\text{FD}) + 0.166$ (r = 0.952), respectively. The ED method, however, gave significantly higher values for E: $y(\text{ED}) = 1.211\ x(\text{FD}) + 0.06$ (r = 0.852). These results suggested that in the ED method the signal for E, although a sharp peak, contained some component other than E. Indeed, re-chromatography at 40°C of the E fraction (collected with the electrochemical detector decoupled and injected after liquid–liquid extraction) on a Spherisorb C_{18} ODS-2 column (5 μm, 250 mm × 4.6 mm I.D., Chrompack) with a mobile phase consisting of 0.1 M ammonium phosphate in 0.05 M phosphoric acid containing 0.6 mM heptanesulphonic acid and 100 mg/l EDTA showed, apart from the E peak, a second, unknown peak at longer retention time (Fig. 1C).

No attempt was made to identify the unknown compound conclusively, but most probably it was N-methylepinephrine (NME) in view of the following facts: (1) the properties of this unknown peak appeared similar to those of an unknown compound reported to be present in urine and subsequently identified as NME by gas chromatography–mass spectrometry [12]; (2) authentic samples of NME showed the same chromatographic behaviour under various circumstances as the unknown peak; (3) recovery of NME after liquid–liquid extraction (which is specific for catecholamines) or alumina adsorption and its electrochemical activity are similar to that of the unknown compound and of E; (4) with the HPLC–ED method, variations in pH, type of column and mobile phase did not result in clear separation between E and the unknown peak (or NME); only when no organic modifier was added to the mobile phase could separation be achieved, but the unknown compound and NME still co-eluted at the same retention time; (5) although NME can thus interfere in the determination of E with the electrochemical method, NME cannot be derivatized with DPE (being a tertiary amine) and therefore does not interfere in the fluorimetric method.

A further comparison was made by determining the catecholamine concentrations in ten urine samples (normal controls and unmedicated patients with borderline hypertension) with the HPLC–FD and the HPLC–ED methods, while NE

TABLE II

COMPARISON BETWEEN THE CATECHOLAMINE CONCENTRATIONS IN TEN URINE SAMPLES DETERMINED WITH THE FLUORIMETRIC METHOD (HPLC–FD), THE ELECTROCHEMICAL METHOD (HPLC–ED), AND THE RECHROMATOGRAPHY METHOD (HPLC–FRACTION ED)

Method	Concentration (mean ± S.D.) (μM)	Regression line[a]	Correlation (r)
Norepinephrine			
HPLC–FD	0.280 ± 0.129	–	–
HPLC–ED	0.289 ± 0.135	$y = 1.038x - 0.001$	0.998
HPLC–fractionED	0.297 ± 0.138	$y = 1.059x + 0.001$	0.996
Epinephrine			
HPLC–FD	0.177 ± 0.116	–	–
HPLC–ED	0.226 ± 0.113	$y = 0.926x + 0.063$	0.951
HPLC–fractionED	0.184 ± 0.116	$y = 0.992x + 0.009$	0.997
Dopamine			
HPLC–FD	1.899 ± 0.835	–	–
HPLC–ED	1.887 ± 0.886	$y = 1.03x - 0.07$	0.994

[a] x = HPLC–FD.

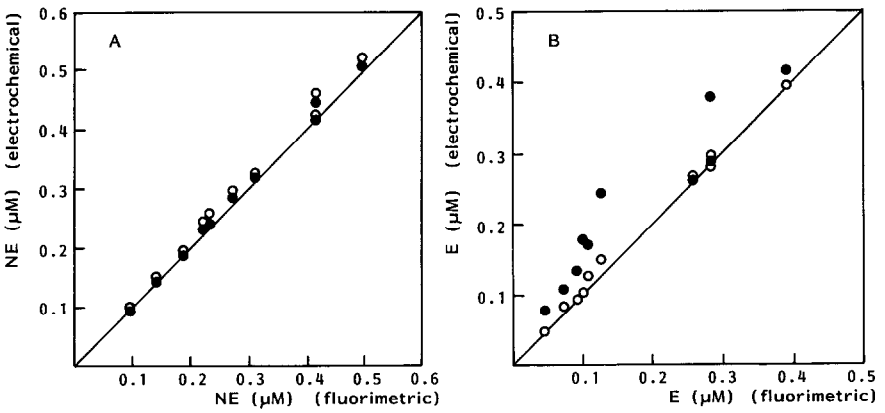

Fig. 2. Comparison of norepinephrine (A) and epinephrine (B) concentrations measured in ten urine samples by the HPLC–FD method with the concentrations found with the HPLC–ED (●) and HPLC–fractionED (○) methods.

and E were also determined by rechromatography on the Spherisorb column of four 0.5-ml fractions collected from 4 to 6 min after injection on the HPLC–ED column (HPLC–fractionED). In the latter case, the fractions were collected with the detector decoupled and were subjected to liquid–liquid extraction before rechromatography. Results are given in Table II and clearly show the good agreement between the HPLC–FD and the HPLC–ED results for NE and DA. E levels again are higher with the HPLC–ED method, but are in good agreement once the NME signal hidden in the E peak is removed by rechromatography. The similarity of NE levels between the HPLC–ED and the HPLC–fractionED methods indicates the reliability of the rechromatography procedure (Fig. 2A and B).

CONCLUSION

The combination of selective liquid–liquid extraction, selective derivatization and HPLC–FD was found to be a reliable and sensitive method for the determination of catecholamines in urine. At least 40 samples can be assayed by one technician in a working day. The often unrecognized problem of co-elution of NME at the same retention time as E in methods employing ED, leading to falsely high E concentrations, is avoided because NME cannot form a fluorescent derivative with DPE.

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