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DETERMINATION OF CATECHOLAMINES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: COMPARISON BETWEEN A NEW METHOD WITH FLUORESCENCE DETECTION AND AN ESTABLISHED METHOD WITH ELECTROCHEMICAL DETECTION

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SUMMARY

We report a sensitive fluorimetric method, in which catecholamines are concentrated from plasma by liquid–liquid extraction and derivatized with the selective fluorescent agent 1,2-diphenylethylenediamine prior to chromatography. Optimal conditions for extraction, derivatization and chromatography were investigated. With α-methylnorepinephrine as internal standard, the chromatographic separations are complete within 6 min. Limits of detection are 0.3 pg for norepinephrine and epinephrine and 0.5 pg for dopamine. Coefficients of variation are low (3–7%). Comparison of plasma catecholamine values determined with this method and with an established method with electrochemical detection ($n = 135$) shows good correlation ($r = 0.94$–1.00), and regression lines are close to lines of identity.

INTRODUCTION

The sensitive, but expensive and laborious, radioenzymic methods for the determination of plasma catecholamines [1,2] have in recent years increasingly been replaced by high-performance liquid chromatographic (HPLC) procedures, usually with electrochemical detection (ED) [3–6]. Although these procedures often give good results, three problems are still commonly encountered. First, the sensitivity of electrochemical detectors is barely sufficient for accurate measurements of the low physiological plasma levels of epinephrine (E) and dopamine (DA). Second, even after extensive and selective sample clean-up, interfering peaks may still occasionally occur in the chromatograms, especially with plasma obtained from patients on multiple therapy. Third, electrochemical detectors,

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despite great advances in their design, remain troublesome and need frequent attention.

Recently, a method for plasma catecholamine determination after selective precolumn derivatization of catecholamines with the fluorescent agent 1,2-diphenylethylenediamine (DPE) was reported [7]. For reasons of sensitivity, selectivity and ease of operation, such a method with fluorimetric detection would be advantageous. We describe here an assay in which catecholamines, after liquid–liquid extraction from plasma followed by derivatization with DPE, are quantitated by HPLC with fluorimetric detection. The method has been optimized with respect to conditions of extraction, derivatization and chromatography for plasma norepinephrine (NE), E and DA, and has been evaluated by comparison with an HPLC–ED method [8].

EXPERIMENTAL

Materials

NE, E, DA, α-methylnorepinephrine (AMN), isoproterenol (IP), dihydroxybenzylamine (DHBA), sodium dodecylsulphate and bicine were obtained from Sigma (St. Louis, MO, U.S.A.), ethylenediaminetetraacetic acid (EDTA), ammonium acetate, benzaldehyde, light petroleum (b.p. 60–80°C), toluene, methanol and 1-octanol from Merck (Darmstadt, F.R.G.), diphenylborate–ethanolamine complex and potassium ferricyanide (PFC) from Janssen (Beerse, Belgium), tetraoctylammonium bromide from Fluka (Buchs, Switzerland), *n*-heptane from J.T. Baker (Deventer, The Netherlands) and acetonitrile (Far UV grade) from Fisons (Loughborough, U.K.).

To remove impurities, the diphenylborate-containing buffer was stirred with activated alumina before use (45 g/l, 2 h) and 1-octanol was washed with 0.08 *M* acetic acid [9].

DPE was prepared essentially as reported previously [10], recrystallized from toluene–light petroleum (b.p. 60–80°C) (1:9, v/v) and dried overnight at 60°C.

Human blood for catecholamine determination was collected in chilled heparinized polystyrene tubes containing 12 mg of glutathione and centrifuged within 15 min at 4°C (15 min, 3000 *g*). Plasma was stored at −70°C.

Apparatus

The instrumentation for the chromatography consisted of a Kratos SF-400 pump, a Rheodyne 7125 injection valve equipped with a 100-μl loop, a Kontron SFM 23/B spectrofluorimeter (150-W xenon lamp source, 20-μl flow-cell) and a Merck-Hitachi D-2000 integrator. For ED the same instrumentation was used but equipped with an amperometric electrochemical detector as decribed by Van Valkenburg et al. [11]; both detector and column were placed in a Spark SpH99 column oven.

All separations were performed on 3 μm particle size Cp™ MicroSpher C_{18} (100 mm × 4.6 mm I.D.) columns (Chrompack, Middelburg, The Netherlands).

Fluorimetric method (HPLC-FD)

Liquid–liquid extraction. An extension of a previously reported extraction method [12,13] was used. To a glass tube were added 1 ml of plasma, 250 µl of AMN solution (1 ng/ml), 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 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. The extraction procedure was repeated once more by 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 described above. The tube was again shaken and centrifuged as described above, the organic phase was separated after the aqueous layer had been frozen, 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, and again separated after freezing of the aqueous layer. Then 2 ml of 1-octanol and 150 µl of 0.08 *M* acetic acid were added, the tube was shaken and centrifuged (5 min, 20°C, 1000 *g*), and the organic phase was aspirated off after the aqueous layer had been frozen.

Derivatization. To the concentrate resulting from the extraction procedure were added 250 µl of acetonitrile, 50 µl of 1.75 *M* bicine (pH 7.05) and 100 µl of DPE solution (0.1 *M* in 0.1 *M* hydrochloric acid). The derivatization reaction was started by the addition of 20 µl of PFC (20 m*M* in bidistilled water) and continued for 60 min in a closed water-bath at 37°C. After the reaction the tubes were kept at 20°C in the dark, and 100 µl were injected into the chromatographic system.

Chromatography. 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. The fluorescent derivatives were monitored with excitation at 350 nm and emission at 480 nm.

Electrochemical method (HPLC-ED)

Catecholamines from 1 ml of plasma were concentrated by the liquid–liquid extraction method described above. The extraction procedure was performed only once, without the extra washing step. Instead of AMN, 100 µl of a DHBA solution (12.5 ng/ml) were added as internal standard, and 100 µl of the acidic concentrate were injected into the HPLC system. The mobile phase was 0.23 *M* acetic acid, containing 0.05 *M* sodium acetate, 100 mg/l EDTA and sodium dodecylsulphate each, and 25% (v/v) of methanol; the apparent pH was 4.3. The column oven was kept at 30°C, and the electrochemical detector was operated at +600 mV vs. an Ag/AgCl reference electrode. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

Derivatization procedure

The fluorescence intensity and the stability of the diphenylquinoxaline derivatives formed by reaction of catecholamines with DPE are influenced by many factors, such as type, molarity and pH of the buffer used, type and concentration of accelerator, concentration of PFC, order of adding the reagents and reaction time. A further complication arises because a change in one particular factor may influence different catecholamines in opposing ways, so that for example an increase in the fluorescence intensity of DA can be brought about at the expense of a decrease in stability of the fluorescence intensity of NE. Not surprisingly, therefore, conditions for derivatization are different for the various assays of constituents of the catecholamine biosynthesis cascade reported by the Japanese group who developed the procedure [14–22].

We have investigated the relevant factors in order to find optimal conditions for the derivatization of catecholamines after concentration from plasma by liquid–liquid extraction. Special attention was given to E and DA because of their low physiological plasma concentrations. AMN and IP were investigated as potential internal standards. In the experiments to determine optimal conditions we initially used standard mixtures of 1 ng of NE, E, DA, IP and AMN in 200 μl of 0.02 *M* acetic acid, 100 μl of DPE solution, 50 μl of buffer, 20 μl of PFC solution and various amounts of organic accelerator; incubation was carried out for 60 min at 37°C.

Accelerator. Originally, glycine was reported to accelerate the derivatization [14], but also to result in multiple peaks for DA [15]. Later, ethanol and acetonitrile were found to be more effective accelerators [19]. We found acetonitrile to be slightly more effective than ethanol, with maximum effectiveness occurring with ca. 40% (v/v) of acetonitrile in the derivatization mixture. A further increase in the percentage of organic accelerator led to a further small increase in fluorescence peak areas, but also to peak broadening and thus a decrease in peak heights. Thus 250 μl of acetonitrile were taken as optimal (total volume of derivatization mixture 620 μl).

Potassium ferricyanide. The concentration of the PFC solution was varied in the range 0–100 m*M*. Maximum response was seen with 20 m*M*; at higher concentrations the DA response decreased slowly to 92% of the maximum at 100 m*M*. Mitsui et al. [7] started the derivatization with the addition of the DPE, but we found very rapid destruction of catecholamines, dependent on pH, when PFC was added before DPE, but not when the order of addition was reversed. Therefore, best results were obtained when 20 μl of 20 m*M* PFC were added to start the reaction.

DPE. In agreement with a previous report [7], we found that 100 μl of a 0.1 *M* solution of DPE in 0.1 *M* hydrochloric acid (resulting pH 6.8) gave best results.

Buffer. Five types of buffer were examined. Phosphate and Tris buffers resulted in much lower fluorescence intensities of DA than acetate, hydrogencarbonate and bicine buffers. In agreement with a previous report [15], we found a rapid fall in fluorescence intensity with increasing molarity of the sodium acetate

buffer (0.05–1.75 *M*), especially for DA, despite maintaining the same pH. The hydrogencarbonate buffers (0.05–0.3 *M*) had the disadvantage of poor pH stability, which resulted in lack of reproducibility of fluorescence responses. Maximum fluorescence intensity was obtained with a 1.75 *M* bicine buffer. The dependency of the fluorescence intensity of the various catecholamine derivatives on the pH of the bicine buffer is shown in Fig. 1A. The fluorescence of DA espe-

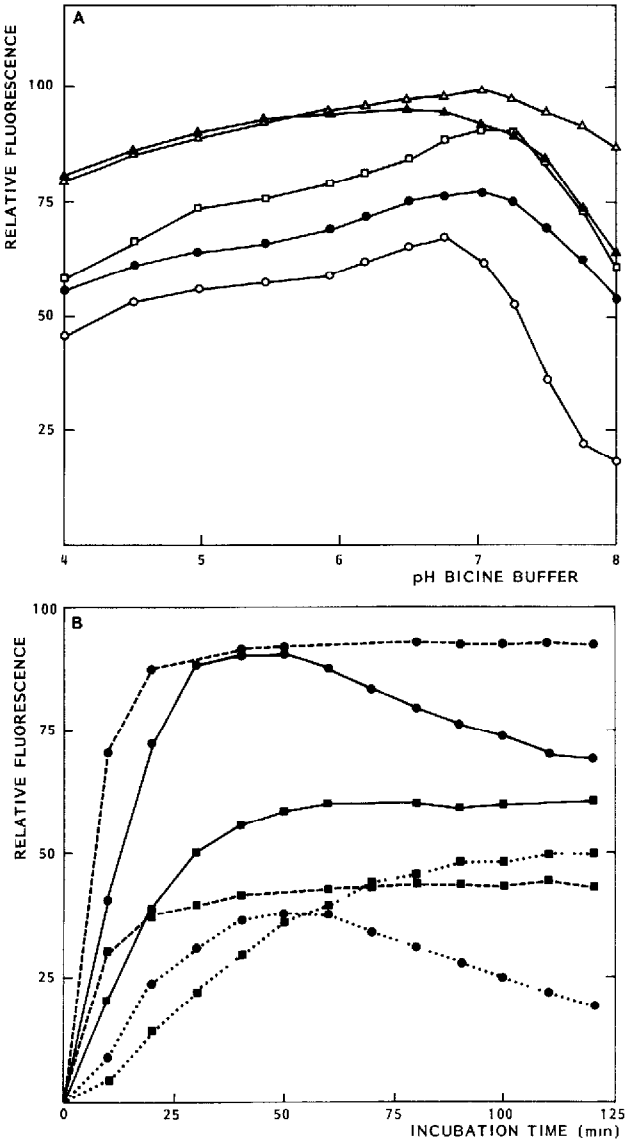

Fig. 1. (A) Effect of pH of the bicine buffer on the fluorescence intensity of the catecholamine derivatives: (□) NE; (▲) E; (○) DA; (△) AMN; (●) IP. (B) Effect of time of incubation on the fluorescence development of NE and DA at various pH values of the bicine buffer: (●) NE; (■) DA; (·····) pH 4.0; (——) pH 5.7; (– – –) pH 7.2.

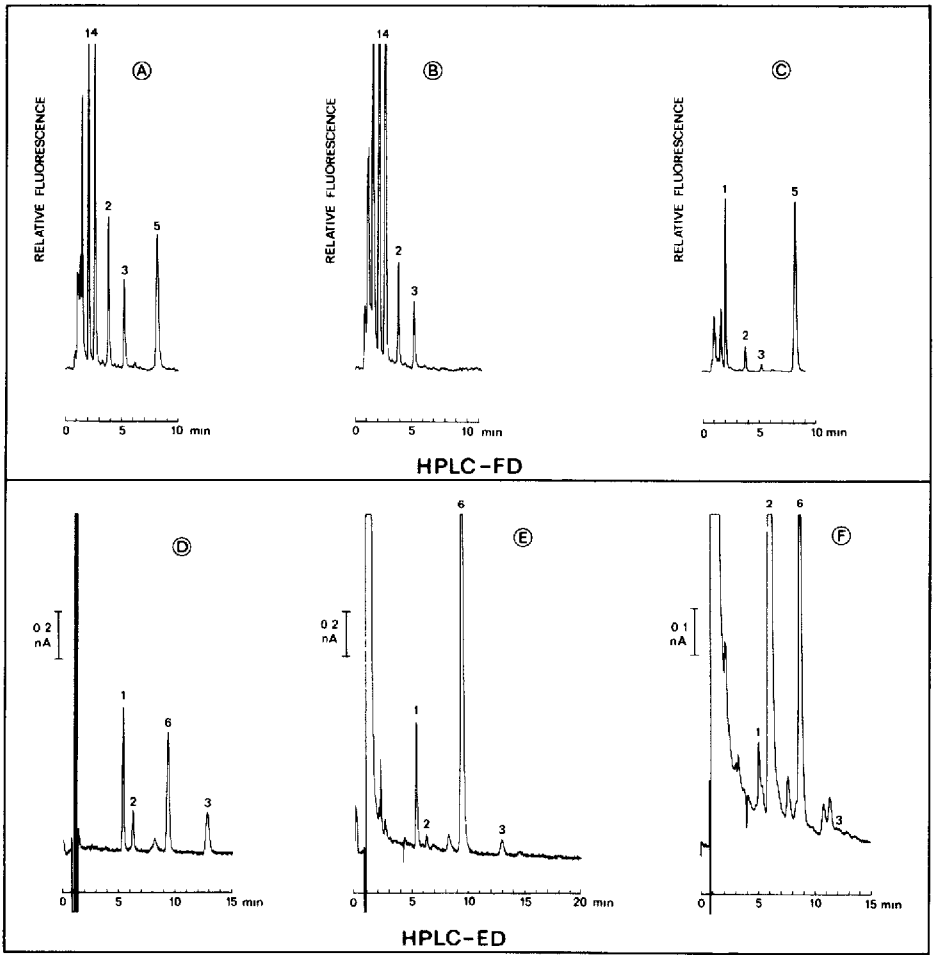

Fig. 2. Chromatograms of the HPLC-FD (upper panel, A, B and C) and HPLC-ED (lower panel, D, E and F) methods. Peaks: 1 = norepinephrine (NE); 2 = epinephrine (E); 3 = dopamine (DA); 4 = α-methylnorepinephrine (AMN); 5 = isoproterenol (IP); 6 = dihydroxybenzylamine (DHBA). (A) Standard mixture; injected amounts 16 pg of NE, AMN and IP and 8 pg of E and DA; (D) standard mixture; injected amounts 100 pg of NE and DHBA and 40 pg of E and DA; (B and E) plasma sample (NE, E and DA 244, 36 and 43 pg/ml, respectively), internal standard 250 pg/ml AMN (B) or 1250 pg/ml DHBA (E); (C and F) plasma sample (NE, E and DA 201, 29 and 19 pg/ml, respectively), internal standard 500 pg/ml IP (C) or 1250 pg/ml DHBA (F).

cially is seen to decrease sharply when the pH of the bicine buffer becomes greater than 7.

When the effect of time of incubation, at various pH values of the buffer, on the fluorescence development was investigated, a striking difference was found between NE, E, AMN and IP on the one hand and DA on the other. The maximum stable response of DA was obtained after 75 min of incubation at 37 °C at pH < 7. The other catecholamines had maximum stable response at pH > 7; at pH values below 7 the response was not stable. This point is illustrated in Fig. 1B, which shows the responses of NE and DA at various incubation times at three

TABLE I

RECOVERY AND PRECISION OF THE HPLC-FD AND HPLC-ED METHODS

Four different plasma pools were used for the determination of intra- and inter-assay precision. Values in parentheses are coefficients of variation (%).

Compound	Absolute recovery ($n=15$) (%)	Relative recovery ($n=15$) (%)	Mean concentration found (pg/ml)	
			Intra-assay precision ($n=6$)	Inter-assay precision ($n=15$)
HPLC-FD				
NE	94.6 (6.2)	100.5 (3.8)	318 (2.6)	280 (2.7)
E	93.6 (3.6)	98.5 (2.7)	42 (5.8)	52 (6.6)
DA	94.6 (3.8)	98.9 (3.7)	89 (3.9)	44 (7.3)
AMN	95.5 (2.1)	–		
HPLC-ED				
NE	96.0 (4.5)	101.4 (5.5)	261 (0.7)	257 (3.8)
E	99.2 (7.2)	104.2 (4.6)	44 (5.3)	41 (10.2)
DA	92.1 (3.7)	97.8 (2.5)	39 (7.2)	42 (11 5)
DHBA	94.0 (2.6)	–		

different pH values of the buffer. Thus, the maximum response for DA occurs when the pH of the buffer is ca. 6.5 and the time of incubation 75 min, but at this pH and time of incubation the responses of the other catecholamines are not stable. As a compromise for obtaining stable and optimal fluorescence responses of all catecholamines, we chose a buffer pH of 7.05 and an incubation time of 60 min.

Derivatization of plasma extracts

When the derivatization procedure, optimized for standard mixtures, was used with plasma extracts, some problems arose. Although experiments in which plasma samples were spiked showed good absolute recoveries for E, IP and AMN (more than 90%), calculated recoveries for NE and DA were too low. Measurements with HPLC–ED clearly indicated that this was due not to incomplete recovery from the extraction procedure, but to inhibition of the fluorescence development. Repeating the extraction procedure once more eliminated this inhibition for NE, but not for DA. Finally, we could also eliminate the inhibition for DA by including in the liquid extraction procedure an extra washing step with ammonium buffer of the heptane layer containing the phenylboron–catecholamine complex (see Experimental). This washing step appeared effectively to remove a substance that inhibited the fluorescence development of DA. This substance may be identical with the inhibitory substance reported to be present in erythrocytes and platelets, which could be rendered inactive by the addition of N-ethylmaleimide [18]. In our experiments the addition of N-ethylmaleimide had the same effect as the washing step. We prefer the washing step, however, as the addition of N-ethylmaleimide results in a large front peak as well as in an extra, slow-eluting peak that greatly increases the analysis time.

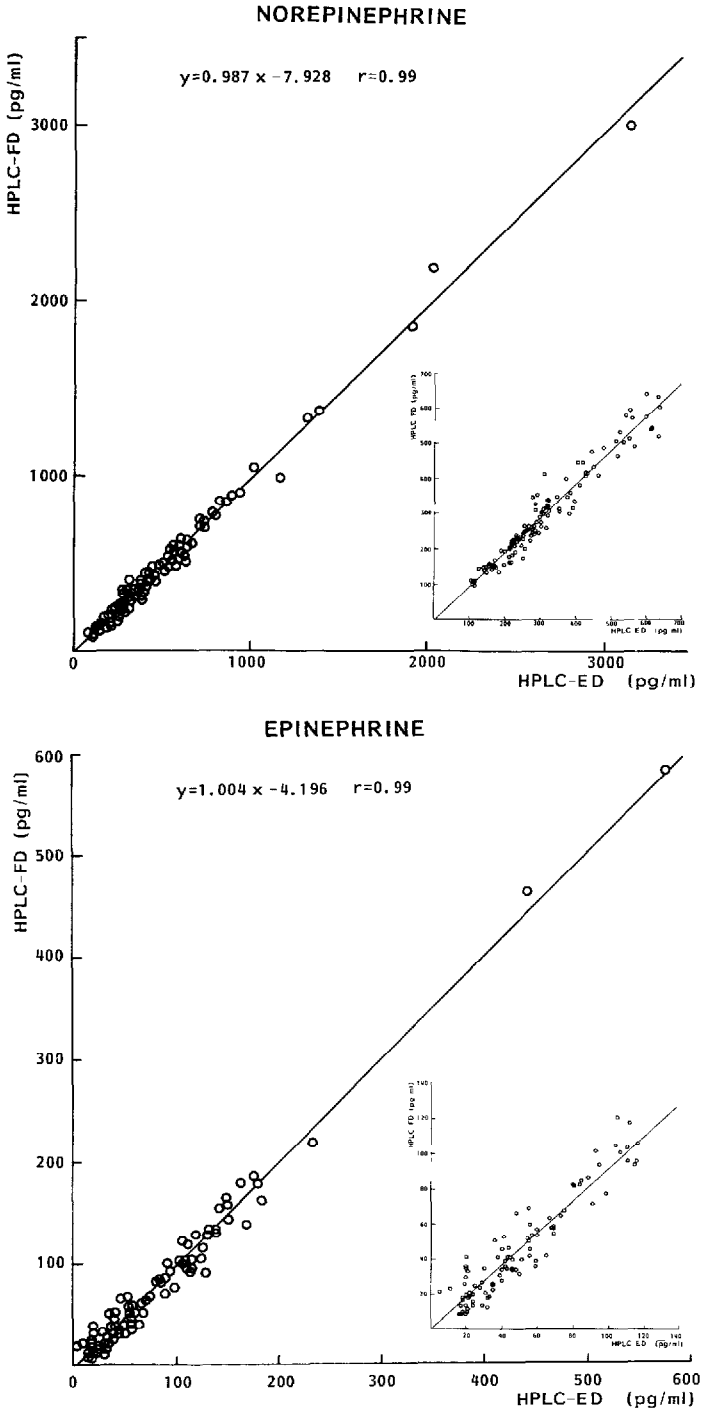
NOREPINEPHRINE
y=0.987 x -7.928 r=0.99
HPLC-FD (pg/ml)
3000
2000
1000
0
1000
2000
3000
HPLC-ED (pg/ml)
EPINEPHRINE
y=1.004 x -4.196 r=0.99
HPLC-FD (pg/ml)
600
500
400
300
200
100
0
100
200
300
400
500
600
HPLC-ED (pg/ml)

Fig. 3.

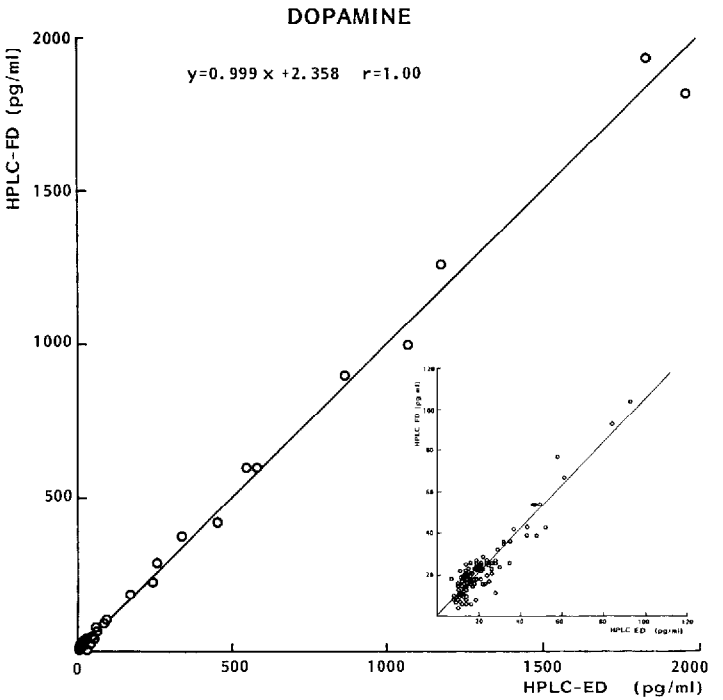

Fig. 3. Comparison between norepinephrine, epinephrine and dopamine values as determined with the HPLC-FD and the HPLC-ED methods in 135 plasma samples. Regression lines and correlation coefficients are indicated. The insets show the same comparison for samples within the normal physiological range: NE < 650 pg/ml, $n=117$, $y=0.970x-4.251$ ($r=0.99$); E < 120 pg/ml, $n=113$, $y=0.912x+0.420$ ($r=0.94$); DA < 120 pg/ml, $n=122$, $y=1.047x+0.244$ ($r=0.94$).

Stability of the catecholamine derivatives

Contrary to previous reports [14,22], we found a gradual decrease in the fluorescence intensity of the catecholamine derivatives when kept in daylight at 20 °C. The decrease amounted to 15–20% after 6 h. Storage at 0 °C gave better, but quite variable, results. Storage in daylight also led to the appearance of an interfering peak in the chromatograms, increasing in height with increasing time of exposure to daylight. Storing samples in the dark prevented the formation of the unidentified substance responsible for this peak and resulted in unchanged fluorescence responses, even after 6 h at 20 °C.

The addition of sodium sulphite at the end of the derivatization procedure in order to decompose unchanged PFC was recently reported to be useful for increasing the stability of the derivative formed from 3,4-dihydroxyphenylalanine [22]. We found no such effect of sodium sulphite on the stability of the derivatives formed from catecholamines.

Chromatography

Good separation of all catecholamine derivatives was achieved with the mobile phase system described in Experimental. Substituting Tris buffer for the sodium acetate buffer resulted in tailing DA peaks. With acetonitrile as the sole organic

modifier resolution was poor, and with methanol as the sole modifier retention times were too long. The introduction of the second extraction and of the extra washing step, apart from improving the recovery of NE and DA, also led to the disappearance of small interfering peaks that sometimes were visible in the chromatograms.

Both IP and AMN can be used as internal standards in the HPLC–FD method. At first we used IP, but when, after analysing many plasma samples, no interfering peak had ever been observed at the same retention time as AMN, we switched over to AMN as internal standard. This change nearly halved the analysis time.

Fig. 2A shows the chromatogram of a standard mixture, while the chromatograms of two different plasma samples are depicted in Fig. 2B and 2C. For comparison the chromatograms of a standard mixture and of the same two plasma samples, as obtained with HPLC–ED, are also shown in Fig. 2D, E and F (note that here DHBA is used as internal standard). The chromatograms clearly show the much higher sensitivity of the HPLC–FD method.

HPLC–FD assay

Each assay included four standard mixtures, two blanks and one plasma pool, the latter both with and without added NE, E and DA (250, 250 and 500 pg/ml, respectively) to check recovery. Results of studies to determine the absolute recovery of NE, E, DA and AMN, the recovery relative to the internal standard and both the intra- and inter-assay precision of the assay are shown in Table I. Recovery and precision are seen to be excellent. For comparison, the corresponding data for the HPLC–ED method are also listed in Table I.

The response of the fluorimeter was found to be linear up to 7500 ng injected for all catecholamines. The limit of detection, at a signal-to-noise ratio of 3, was 0.3 pg injected for NE and E and 0.5 pg for DA, which, under the standard conditions employed, amounts to 2 pg/ml for NE and E and 3 pg/ml for DA.

Comparison between the HPLC–FD and HPLC–ED assays

In 135 plasma samples NE, E and DA were measured both with the new HPLC–FD method and with the hitherto used HPLC–ED method [8]. The data for NE, E and DA are presented in Fig. 3, both for the whole measured range and for the normal physiological range (inset). The correlations are excellent and lines of regression approach lines of identity.

With the HPLC–ED method chromatograms of plasma samples of patients sometimes showed large unidentified peaks, which either made the reliable measurement of NE and/or E and/or DA impossible, or were slow-eluting and thus interfered in subsequent analyses. In all these cases the fluorimetric method showed no or much less interference, and measurement of all catecholamines was always possible. An example is shown in Fig. 2, where the HPLC–ED method shows a large peak at the retention time of E and several other smaller peaks (Fig. 2F), which are absent in the HPLC–FD method (Fig. 2C). The higher selectivity of the HPLC–FD method is not due to the more elaborate work-up procedure from plasma, as the same work-up procedure was shown not to improve the HPLC–ED chromatogram.

CONCLUSION

The fluorimetric method described is a reliable, sensitive, selective and reproducible method for determining plasma catecholamines and compares favourably with the established HPLC–ED method. The higher sensitivity allows better measurements of low physiological plasma levels of E and DA and/or the use of smaller amounts of plasma. The higher selectivity allows for the measurement of catecholamines in plasmas containing substances that interfere with the electrochemical method. The fluorimetric response remains constant for many months without any attention, while the electrochemical response can decrease within one operating day, and the electrochemical detector needs frequent attention and cleaning. The analytical column remains in good condition for a much longer time, probably because of the much higher percentage of organic modifier in the mobile phase. The more elaborate work-up procedure is more than offset by the shorter analysis time, the lack of slow-eluting compounds, and the greater ease of introducing an autosampler because of the greater end-volume; 25 plasma samples can be assayed in a normal working day, or even twice that number when an autosampler is available.

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