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MEASUREMENT OF CATECHOLAMINES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A COMPARISON OF FLUORIMETRIC WITH ELECTROCHEMICAL DETECTION

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SUMMARY

An improved method for the determination of catecholamines in biological fluids, by reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection is presented. The pH titration previously employed in the alumina extraction was abandoned in favour of the use of a molar excess of pH 8.5 Tris—HCl buffer. A novel lyophilisation step serves to concentrate the catechols and by reconstituting in mobile phase, chromatography disturbances are minimised. The addition of 2 m*M* octanesulphonic acid to a citrate—phosphate mobile phase at pH 6.0 gave optimal resolution and sensitivity.

That HPLC separation can improve the specificity of the trihydroxyindole reaction, to the extent of providing a reliable analytical method, has been demonstrated and validated by the technique of HPLC with electrochemical detection. A correlation coefficient of 0.98 was obtained between the two techniques as applied to the measurement of urinary catecholamines. The HPLC—fluorimetric method was sensitive enough to measure 0.1 ng/ml of noradrenaline or adrenaline at a signal-to-noise ratio of 2.0. Application of the method to the quantitative determination of catecholamines in human urine, plasma and rat brain homogenates is demonstrated.

INTRODUCTION

The measurement of catecholamines in biological fluids has always proved a difficult task, because of their low concentrations, susceptibility to oxidation

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and the complex methods for their analysis. Despite these problems, the investigation of adrenergic pathophysiology in man has led to the development of diverse methods for the quantitation of catechols.

The most sensitive techniques available — gas chromatography—mass spectrometry (GC—MS) [1—3] and radioenzymatic assay [4—8] — have been limited to those workers with the considerable funds and expertise necessary. This situation has led to the recent upsurge in the use of high-performance liquid chromatographic (HPLC) separation of the catecholamines, followed by ultraviolet [9,10], fluorimetric [11—15] or electrochemical detection [16—24].

One of us recently described a fluorimetric method for the estimation of urinary catecholamines [25] which required a 5-ml sample of urine to achieve a detection limit of 5 μg/l. We now report on an improved extraction procedure and chromatography in which catecholamines can be measured down to 0.1 μg/l using only 500 μl of urine or 2—4 ml of plasma. This can be achieved using fluorimetric detection after a post-column trihydroxyindole reaction or alternatively by direct electrochemical detection.

We also describe the application of the HPLC—fluorimetric technique to the measurement of the decrease in the noradrenaline content of selected areas of rat brain, after chemical sympathectomy with 6-hydroxydopamine.

EXPERIMENTAL

Apparatus

A Model RR/015 solvent delivery system (HPLC Technology, Cheshire, Great Britain) equipped with an extra pulse dampener was used for chromatography. Samples were introduced through a Rheodyne 7125 injection valve, fitted with a 100-μl loop. The column used was a 250 × 4.6 mm I.D. Hypersil ODS, 5 μm particle size (Shandon Southern, Cheshire, Great Britain) protected by a guard column of Co:Pell ODS, 50 × 2.1 mm I.D. (Whatman, Maidstone, Great Britain).

The electrochemical detection system comprises a Faraday cage enclosing a Model TL-3 thin-layer cell packed with CP-O paste and a Model LC-2A electronic controller (BioAnalytical Systems, Lafayette, IN, U.S.A.).

The post-column trihydroxyindole reaction was achieved by combining the column eluent with a mixture of sodium acetate and potassium ferricyanide followed by a mixture of sodium hydroxide and ascorbic acid, in a continuous-flow AutoAnalyser (Technicon Instruments, Basingstoke, Great Britain). The manifold details are as given previously [25] except that the concentration of ascorbic acid was increased to 0.20% and air delivery increased to 0.42 ml/min. The fluorescent derivatives were detected by means of a Locarte Mark VI fluorimeter (Locarte, London, Great Britain) fitted with a flow-through cuvette and filters providing excitation at 380 nm and emission at 510 nm. The signal from the fluorimeter was considerably amplified by the Linseis chart recorder, itself fitted with a 4-μF capacitor in series to reduce noise.

Reagents

Noradrenaline, adrenaline, dopamine, 3,4-dihydroxybenzylamine, 6-hydroxydopamine, isoprenaline and α-methyldopa were purchased from Sigma (Poole,

Great Britain). The octanesulphonic acid was obtained from Fisons (Loughborough, Great Britain). All other chemicals including the alumina (Brockmann grade 1, neutral) were from BDH (Enfield, Great Britain). Catecholamine standards were prepared at 10 μg/ml in a mixture of 0.010 *M* hydrochloric acid and 0.00260 *M* sodium metabisulphite. Working concentrations were achieved by subdilution in 0.30 *M* acetic acid prior to use.

Sample collection

Human urine samples were collected into plastic bottles containing 15 ml of freshly prepared 0.26 *M* sodium metabisulphite solution. When completed, the volume and time were recorded and a portion of the urine frozen at −20°C.

Blood samples were obtained from sitting subjects by venipuncture and transferred to lithium heparin tubes containing 100 μl of 0.25 *M* sodium metabisulphite as preservative. The separated plasma was then stored at −20°C for up to eight weeks [26].

Rats were sacrificed by decapitation, their brains were removed and frozen on solid carbon dioxide. The cut sections were then weighed and homogenised in an ice-cold mixture of 1 *M* sodium metabisulphite (1 ml), 0.1 *M* disodium EDTA (1 ml) and 0.05 *M* perchloric acid (27 ml) in the ratio of 3 volumes per g tissue. The homogenate was then centrifuged at 11,000 *g* at 4°C for 15 min and the supernatant transferred to a ground-glass stoppered tube for storage at −20°C until required.

Chromatography

All mobile phases were citrate—phosphate buffers containing methanol as the organic modifier and were pumped at flow-rates of 1—2 ml/min. Retention of the catechols was altered by changing the concentration of the ion-pairing agent and the methanol content, and in the case of methyldopa, changing the pH.

Extraction

Prior to extraction, alumina was acid washed in 0.3 *M* acetic acid followed by several water washes and finally activation was accomplished by heating overnight at 200°C. Once activated the alumina remained potent for several months in a tightly capped dark glass container.

A 500-μl sample of urine (or aqueous standard) was added to a 12-ml plastic tube, contained in an ice-bath. Then 100 mg of activated alumina and 500 μl of 12 ng/ml dihydroxybenzylamine (internal standard) were added followed by 2 ml of distilled water and 3 ml of 1 *M* Tris buffer, pH 8.5. The tubes were stoppered and gently mixed for 10 min on a rotary mixer. The alumina was allowed to settle and the liquid aspirated at the sink, followed by three washes with 0.0050 *M* acetate buffer, pH 7.0. The wash water was removed as thoroughly as possible and then the catechols were eluted from their binding sites on the alumina, by increasing the pH with a 200-μl addition of 0.30 *M* acetic acid. The tubes were mixed for 2 min on a vortex mixer and then centrifuged at 2200 *g* at 4°C for 15 min to compact the alumina. Finally the upper acid layer was transferred into a 1.5-ml Eppendorf tube and 20—100 μl injected into the HPLC system. In the case of plasma samples 2—4 ml plasma

or standard were extracted with only 200 μl of 12 ng/ml dihydroxybenzylamine. For brain homogenate samples 1 ml homogenate or standard was extracted with 50 μl of 0.2 μg/ml α-methyldopa as internal standard, in place of dihydroxybenzylamine which does not undergo the trihydroxyindole reaction. A further concentration of the catechols was achieved by freezing the alumina extracts in a mixture of solid carbon dioxide and acetone, lyophilising and reconstituting in 50 μl of the mobile phase.

RESULTS AND DISCUSSION

Reversed-phase chromatography systems suitable for the separation of catecholamines have been extensively studied by several groups [27—29]. The measurement of urinary catecholamines in alumina extracts has proved difficult with reversed-phase systems because of void volume interferences [30,31]. This has led to the development of alternative extraction techniques based on ion exchange and boric acid gels [30]. However these interferences can be eliminated by using ion-pairing agents such as alkylsulphonic acids, to selectively increase the retention of the catechols so that they are separated from the more polar void volume peaks (Fig. 1). The chromatography solvent

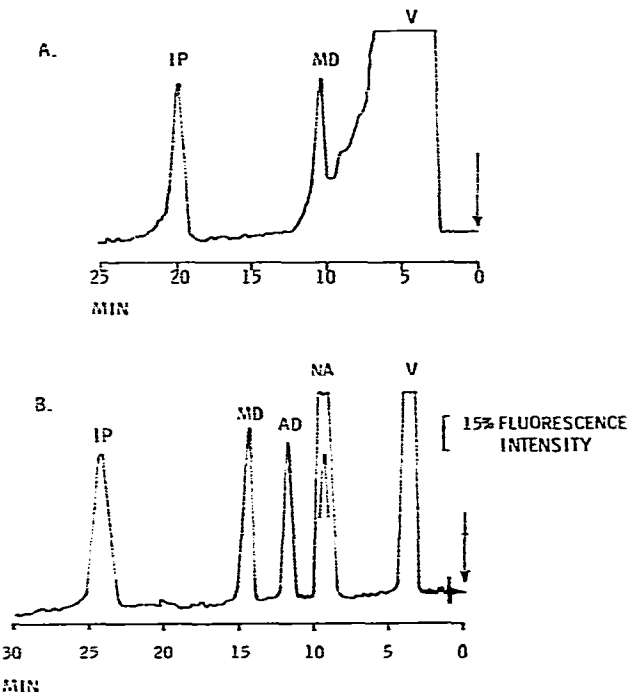

Fig. 1. Chromatograms of urinary catecholamines measured by HPLC with fluorimetric detection. (A) The void volume interference in the absence of ion-pairing agent; (B) the elimination of this interference by the ion-pairing agent 1-octanesulphonic acid at 2 m*M* concentration. Peaks: V = void volume peak, MD = methyldopa, IP = isoprenaline, NA = noradrenaline, AD = adrenaline. Mobile phase, citrate—phosphate buffer (pH 3.1) with 10% methanol, flow-rate, 1 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

system outlined in Fig. 1B is analogous to that in our previous paper [25] but the sensitivity on the column was improved to 100 pg. However, this system could not be used successfully with plasma extracts, or in combination with electrochemical detection. It appears that the void volume contaminants are electroactive and present to a greater extent in plasma than in a 24-h urine specimen. A further problem noticed with extracted standards was that α-methyldopa and dopamine had similar retention. Changing the pH of the mobile phase from 3.1 to 6.0 reduced the retention of methyldopa, with little effect on the retention of the other amines, and had the added bonus of providing more stable baselines with both detectors. To eliminate the void volume interference in plasma extracts it was necessary to increase the ion-pair concentration to 2 m*M* (Fig. 2). This gave a mobile phase composed of 300 ml 0.1 *M* citric acid, 150 ml 0.1 *M* disodium hydrogen phosphate, 100 ml methanol, 20 ml 0.1 *M* octanesulphonic acid and 20 ml 0.1 *M* disodium EDTA per litre, adjusted to pH 6.0 with 5 *M* sodium hydroxide.

With electrochemical detection all three endogenous catecholamines could be measured in urine using dihydroxybenzylamine as internal standard. The separation of standards and a typical urine extract is shown in Fig. 3.

Rat brain homogenate extracts gave exceptionally clean chromatograms (Fig. 4); HPLC-fluorimetric quantitation was achieved using α-methyldopa as internal standard, calibration curves for noradrenaline (NA) being linear over the range 0.1—200 ng/ml.

In order to demonstrate the ability of the HPLC—fluorimetric assay to detect concentration changes after pharmacological manipulations, we administered 6-hydroxydopamine intravenously at a dose of 300 μg per 30 μl of saline (containing 0.2 mg/ml ascorbic acid as antioxidant). Controls were injected with the same volume of vehicle. The results (Table I) show the expected fall in NA after 6-hydroxydopamine, but the degree of adrenergic neurotransmitter depletion varied with the region of the brain. The midbrain was most sensitive to 6-hydroxydopamine and the medulla-pons the least sensitive. In the control animals NA levels were about five times higher in the

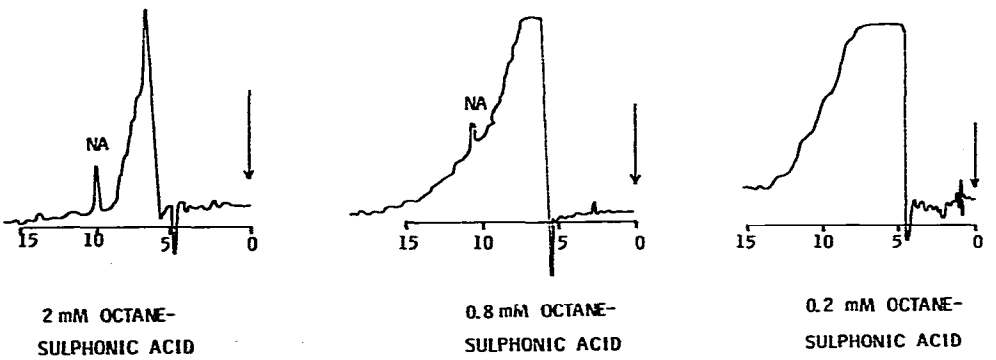

Fig. 2. Chromatograms of plasma catecholamines measured by HPLC with fluorimetric detection. The effect of 1-octanesulphonic acid is shown. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol and 2 m*M* disodium EDTA; flow-rate, 1 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

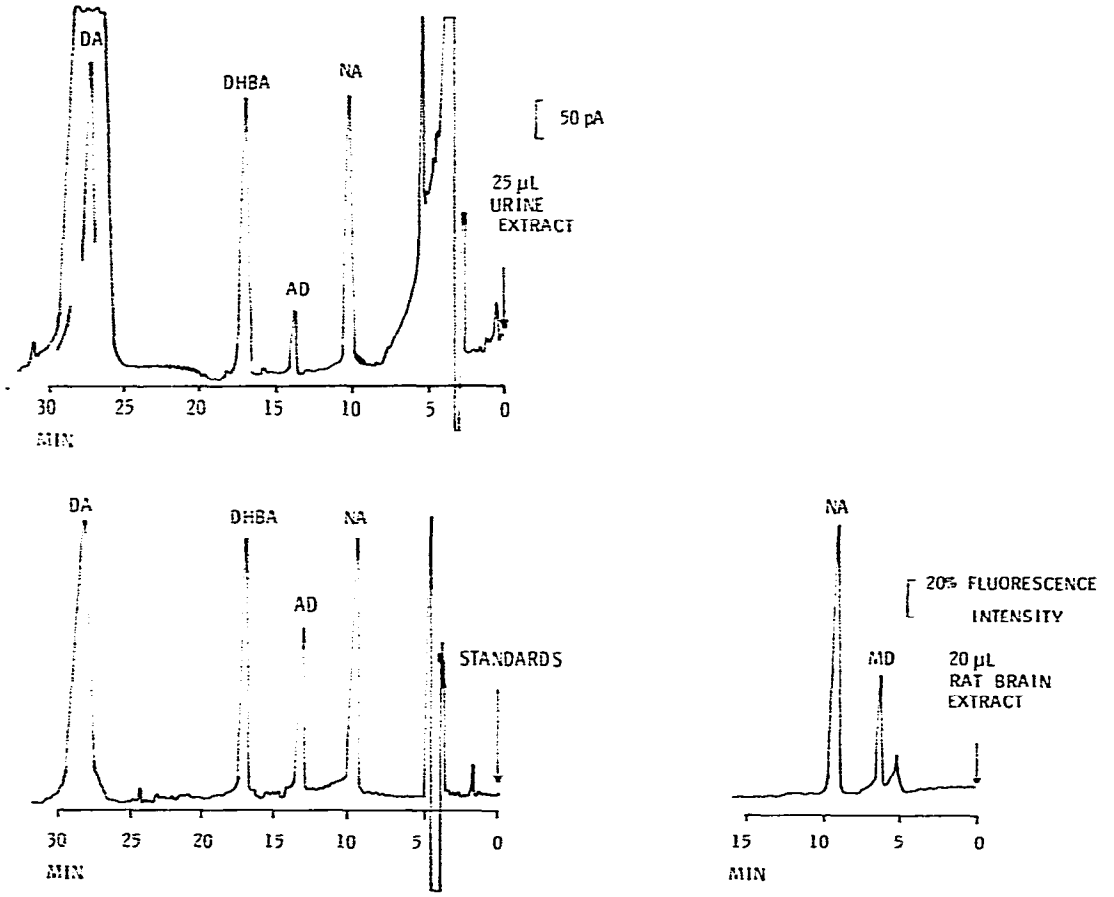

Fig. 3. Chromatograms of urinary catecholamines and extracted standards measured by HPLC with electrochemical detection. Peaks: DHBA = 3,4-dihydroxybenzylamine (internal standard), DA = dopamine. Urinary catecholamine concentrations NA = 39 µg/24 h, AD = 9 µg/24 h, DA = 190 µg/24 h. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol, 2 m*M* 1-octanesulphonic acid and 2 m*M* disodium EDTA; flow-rate, 1 ml/min; temperature, ambient; detector, BioAnalytical Systems LC-2A held at +0.55 V vs. Ag/AgCl.

Fig. 4. Chromatogram of rat brain homogenate noradrenaline measured by HPLC with fluorimetric detection. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol, 2 m*M* 1-octanesulphonic acid and 2 m*M* disodium EDTA; flow-rate, 1.5 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

midbrain and medulla-pons than in the cerebellum and cortex. These results using HPLC—fluorimetry are in general agreement with those obtained by the alternative technique of radioenzymatic assay [32] and GC—MS [33].

The combination of improved extraction and chromatography meant that urine catecholamines could be measured by HPLC—fluorimetry without the need for internal standards, thus reducing the analysis time. The percentage recoveries of 1.0 µg of NA and adrenaline (AD) added to urine samples were 70% and 68%, respectively (n=12). Extracted aqueous standards were linear over the range 0.05–250 ng/ml and the coefficients of variation for the

TABLE I

THE EFFECTS OF 6-HYDROXYDOPAMINE ON RAT BRAIN NORADRENALINE CONCENTRATION, AS DETERMINED BY HPLC WITH FLUORIMETRIC DETECTION

All values are mean wet weight ± S.E.M., $n = 3$ for each determination.

Brain region	Control (ng/g)	6-Hydroxydopamine (ng/g)
Cortex	206 ± 19.6	53 ± 17.1
Cerebellum	132 ± 22.8	26 ± 3.5
Midbrain	445 ± 73.4	28 ± 2.2
Medulla-pons	546 ± 9.7	472 ± 25.1

catecholamines determined in a pooled urine were NA 8.3% (n=9) and AD 10.5% (n=9). The method was sensitive enough to measure 0.1 ng/ml of both amines at a signal-to-noise ratio of 2.0 and so well able to cope with typical urines, which were found to have levels of NA = 43.4 ± 7.03 μg per 24 h (mean ± S.E.M., $n = 10$) and levels of AD = 15.15 ± 3.592 μg per 24 h (mean ± S.E.M., $n = 10$) by this method.

The introduction of a lyophilisation step in the extraction has enabled the measurement of NA in 2 ml of human plasma (or serum) by HPLC—fluorimetry; and although the method is still not sensitive enough to accurately measure AD in normal resting subjects, it can be determined in patients with phaeochromocytoma where levels are frequently several times the upper limit of normal [30] and in subjects undergoing maximal exercise.

Using the HPLC—fluorimetric assay we examined the effect of treadmill exercise to exhaustion on the plasma catecholamine levels of ten male subjects, mean age 35 years. Post-exercise values were NA = 1.74 ± 0.993 ng/ml, AD = 0.23 ± 0.10 ng/ml (mean ± S.E.M.) and compared with the basal values of NA = 0.365 ± 0.041 ng/ml, AD < 0.1 ng/ml (mean ± S.E.M.).

In order to measure NA and AD in plasma from resting subjects it was necessary to utilise the increased sensitivity afforded by the oxidation of the catecholamines (at a fixed potential difference) in a thin-layer graphite electrochemical cell [23]. The routine use of the electrochemical detector to measure plasma catechols requires very careful attention to experimental details, particularly as one has to work near to the limit of the instrument's sensitivity, i.e., 0.5 nA full scale. The elimination of noise by use of a Faraday cage, electrical earthing of metal parts, inclusion of disodium EDTA in the mobile phase to chelate metal ions, passivation of the pump and column when new (using acid), use of highly polished electrode surface [34], and maintaining the system on a continuous rather than daily basis, are all important factors in ensuring operation at high sensitivity. For urine work the more reliable glassy carbon electrode (TL 4/5) is adequate and can be used in place of the paste electrode. In contrast to the trihydroxyindole reaction the electrochemical detector is very sensitive to dopamine and since urinary dopamine is present in the order of 100—600 μg per 24 h, it gives a very large peak in the chromatogram which has to be off-scaled on the chart recorder to enable its accurate quantitation. This presence of a dopamine peak also means that sample throughput is

reduced for urines since dopamine has a longer retention time than the other catecholamines.

In order to compare the two methods, the concentrations of NA and AD in twelve urine samples were determined by HPLC—fluorimetry and HPLC with electrochemical detection. The HPLC—fluorimetric method gave values of NA = 56.5 ± 12.9 μg per 24 h and AD = 27.9 ± 14.2 μg per 24 h (mean ± S.E.M.), while the HPLC—electrochemical method gave corresponding values of NA = 59.25 ± 14.2 μg per 24 h and AD = 29.6 ± 14.8 μg per 24 h (mean ± S.E.M.). No significant difference between the two techniques was found ($P > 0.05$, paired t-test) and they were shown to be well correlated ($r = 0.98$).

The improved sensitivity of the HPLC—fluorimetric method presented, derives largely from the introduction of a novel lyophilisation step, a citrate—phosphate ion-pairing HPLC system and buffering of the sample to pH 8.5 with a high-molarity buffer. The lyophilisation procedure serves to concentrate the catechols and because reconstitution is in mobile phase, injection creates the minimum of disturbance to the chromatography. The use of a molar excess of pH 8.5 buffer overcomes the need for an indicator in the extraction. This is desirable since the alkaline end-point of thymol blue is difficult to judge, being a pale blue colour, and the use of 0.5 *M* sodium carbonate is also not ideal as it could easily create local hot spots of high pH and hence degrade the catechols.

The classical manual or automated fluorimetric methods employing the trihydroxyindole reaction have fallen into disrepute mainly because of their notorious lack of specificity. That HPLC separation can improve the specificity of the trihydroxyindole reaction, to the extent of providing a reliable analytical method, has been demonstrated and validated by the widely accepted technique of HPLC with electrochemical detection.

In our hands the improved HPLC—fluorimetric method was not as sensitive as the electrochemical approach, but it was less problematic in setting up and running on a routine basis and proved quite satisfactory for the measurement of urinary catecholamines and plasma NA. Since most of the difficulties associated with electrochemical detection derive from the thin-layer cell, it is likely that improved design such as the rotating disc electrode, which is reported to have increased sensitivity and performance, because it is insensitive to the condition of the electrode surface [35] will favour the technique in future.

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