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Fully automated high-performance liquid chromatographic assay for the analysis of free catecholamines in urine

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ABSTRACT

A totally automated and reliable high-performance liquid chromatographic method is described for the routine determination of free catecholamines (norepinephrine, epinephrine and dopamine) in urine. The catecholamines were isolated from urine samples using small alumina columns. A standard automated method for pH adjustment of urine before the extraction step has been developed. The extraction was performed on an ASPEC (Automatic Sample Preparation with Extraction Columns, Gilson). The eluate was collected in a separate tube and then automatically injected into the chromatographic column. The catecholamines were separated by reversed-phase ion-pair liquid chromatography and quantified by fluorescence detection. No manual intervention was required during the extraction and separation procedure. One sample may be run every 15 min, *ca.* 96 samples in 24 h. Analytical recoveries for all three catecholamines are 63–87%, and the detection limits are 0.01, 0.01, and 0.03 μM for norepinephrine, epinephrine and dopamine, respectively, which is highly satisfactory for urine. Day-to-day coefficients of variation were less than 10%.

INTRODUCTION

The main interest in the determination of urinary catecholamines lies in the diagnosis of pheochromocytomas. These tumours develop in chromaffin cells, which are distributed throughout the body and are characterized by their high content of catecholamines. Most of these cells (and hence pheochromocytomas) are located in the adrenal medulla. Numerous techniques for catecholamine determination have been developed. The colorimetric techniques first used [1] seem obsolete today because of the development of techniques with greater precision and sensitivity. Prior to the introduction of high-performance liquid chromatography (HPLC), the techniques most widely used were fluorimetry [2–4], radioenzymatic techniques and gas chromatography (GC) [5–7].

It is evident from the large number of papers published, that HPLC has now become the method of choice for determination of catecholamines [8,9]. It offers the advantages of reasonable simplicity, a high degree of versatility and adequate sensitivity and specificity. The results of an HPLC determination depend on factors such as detector/column performance, chromatographic conditions, biological fluid conditions, precolumn manipulations for sample clean-up. etc.

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All urinary catecholamine HPLC methods require meticulous sample purification because of the many closely related amines and catecholamines found in urine.

Until very recently, pre-treatment consisted of an off-line step by a separate batch or chromatographic alumina purification method [10,11]. Some progress has been made in simplifying the pre-treatment step, notably through the use of on-line purification techniques [12]. Many of the previously described methods require a preliminary manual step, particularly to adjust the urine to the necessary pH of extraction [11,12] and to centrifuge or adjust the total urine sample to a precise volume [13].

This paper describes a simplified native fluorescence detection method for urinary catecholamines, which includes fully automated solid-phase sample clean-up on small alumina cartridges and on-line injection. The adjustment of pH is standardized, and this makes the method completely automated. The separation is carried out by reversed-phase ion-pair chromatography. A sample may be analysed every 15 min.

EXPERIMENTAL

Reagents

Hydrochloric acid (37%), perchloric acid (65%), hydroxymethylaminomethane, sodium dihydrogenophosphate, EDTA, phosphoric acid, methanol and sodium hydroxide were analytical-grade reagents supplied by Carlo Erba (Paris La Défense, France). Sodium heptanesulphonate was obtained from Aldrich (Strasbourg, France). Ultra-pure glass-distilled water was used throughout. Standards of (−)-epinephrine (E). (±)-norepinephrine (NE), dopamine (D) and the internal standard (dihydrobenzylamide hydrobromide, DHBA) were obtained from Sigma (L'Isle d'Abeau Chesnes, France). The solid-phase extraction was performed on prepacked columns of 1 ml volume, containing 200 mg of alumina:70–230 Mesh-ASTM (Touzart et Matignon, Vitry sur Seine, France). The columns were sealed by polypropylene caps from Gilson.

Reference solutions

Individual stock standards of NE, E, D and internal standard (I.S.) containing 1 m*M* (free base) were prepared every month in perchloric acid diluted 1:200 and stored at 4°C protected from light. Working solutions of NE, E, D and I. S. containing 10 μM were prepared daily by dilution with 0.1 *M* perchloric acid. Standards prepared in urine were obtained by dilution of working solutions to obtain, in addition to their basal concentration, 0, 0.25, 0.50, 0.75 and 1 μM of each standard.

Apparatus

An entirely automated extraction of the urinary catecholamines was performed on an ASPEC (Automatic Sample Preparation with Extraction Columns)

device from Gilson (Villiers le Bel, France). This system consists of a 401 dilutor equipped with a 10-ml syringe. The complete system is programmed and controlled from the sample processor. The ASPEC is directly connected, via a Rheodyne 7020 valve, to an HPLC system composed of a ConstaMetric III pump (LDC, Paris), a stainless-steel column (250 mm x 4.6 mm I.D.) packed with Nucleosil 100/C_{18} (particle size 5 μm) (Macherey-Nagel Touzard et Matignon), a spectrofluorimeter (Shimadzu RF 530) and an integrator (Shimadzu CR1B, Touzard et Matignon). Excitation and emission wavelengths were 280 and 310 nm, respectively, the fluorimeter being set at high sensitivity (range 2).

Sample preparation and chromatography

Urine samples were collected over a 24-h period in polyethylene containers, and 10–15 ml of 6 *M* hydrochloric acid were added as preservative. Samples were stored at 4°C and frozen at −20°C when the determination was delayed by over 72 h. Because the technical steps were automated, extraction and measurements were carried out in sequential mode, a urine sample being analysed at the same time as the extraction of the following sample. The system was washed with 2 ml of water between samples. A schematic diagram representing the entire automated process of sample preparation and injection is shown in Fig. 1.

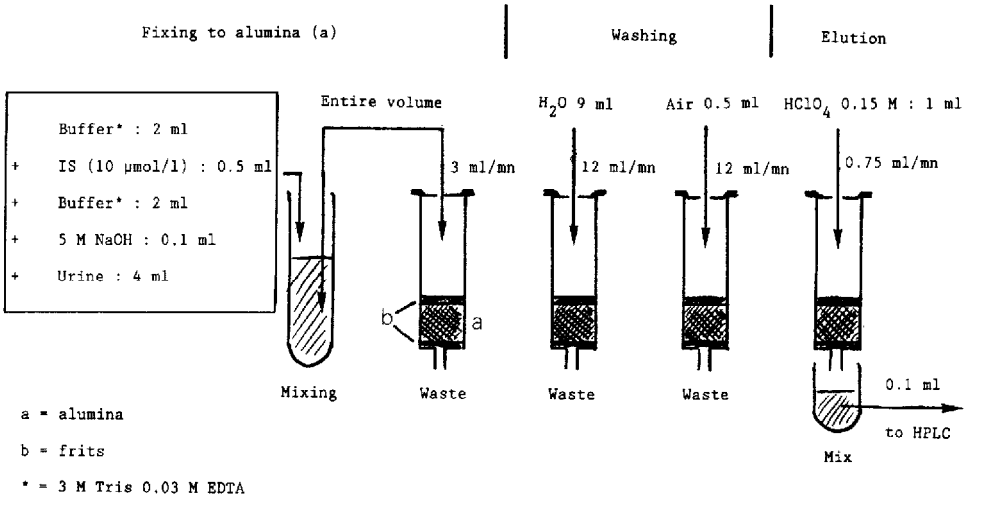

Fig. 1. Automated process of sample preparation and injection.

Chromatography

The mobile phase was methanol–buffer (18:32, v/v). The buffer consisted of 0.075 *M* $NaH_2PO_4 \cdot H_2O$, 0.00015 *M* EDTA and 0.006 *M* sodium heptanesulfonate (pH 3.96). The flow-rate was maintained at 1.25 ml/min throughout the system at all times to ensure reproducible separations.

RESULTS

Typical chromatograms of a blank reagent, a urine sample and two urine samples spiked respectively with 0.5 and 1 μM of each catecholamine are shown in Fig. 2. Under the selected HPLC conditions, retention times for NE, E, I.S. and D were 6.7, 7.6, 9.6 and 12.5 min. respectively.

The influence of the pH of the mobile phase on catecholamine retention is shown in Fig. 3. The identity of a given peak can be verified by this means, or by varying the excitation and emission wavelengths.

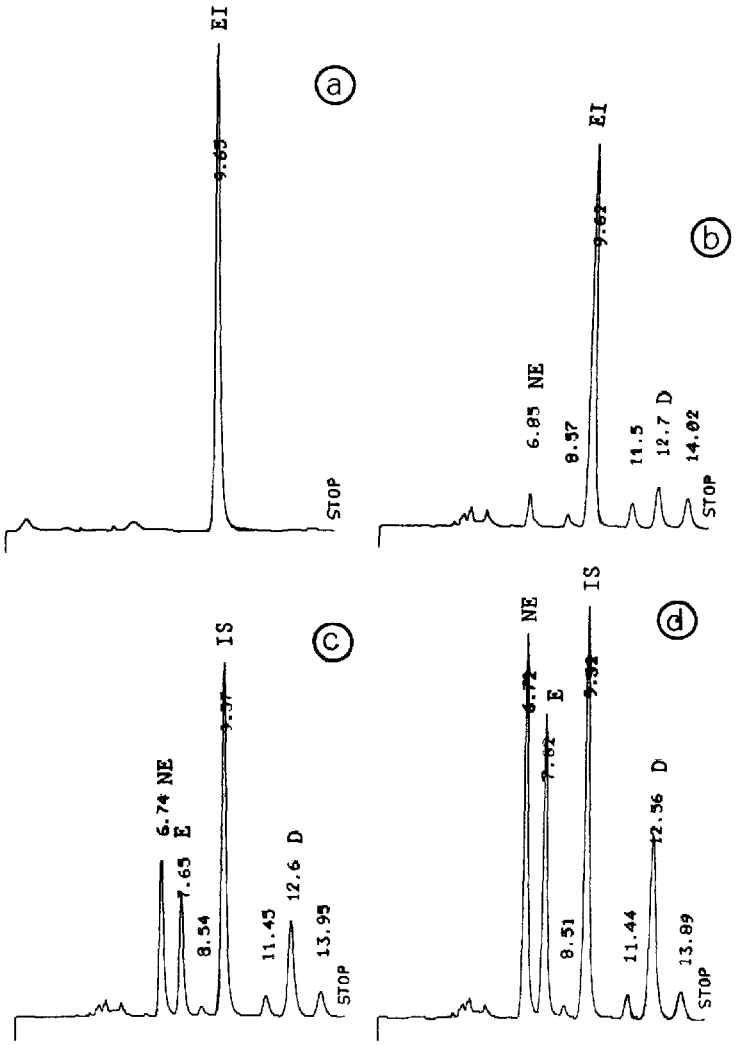

Fig. 2. Typical chromatograms showing separation of E, NE and D: (a) blank reagent; (b) catecholamine-free urinary pool; (c) catecholamine-free urinary pool spiked with 0.5 μM E, NE and D; (d) catecholamine-free urinary pool spiked with 1.0 μM E, NE and D.

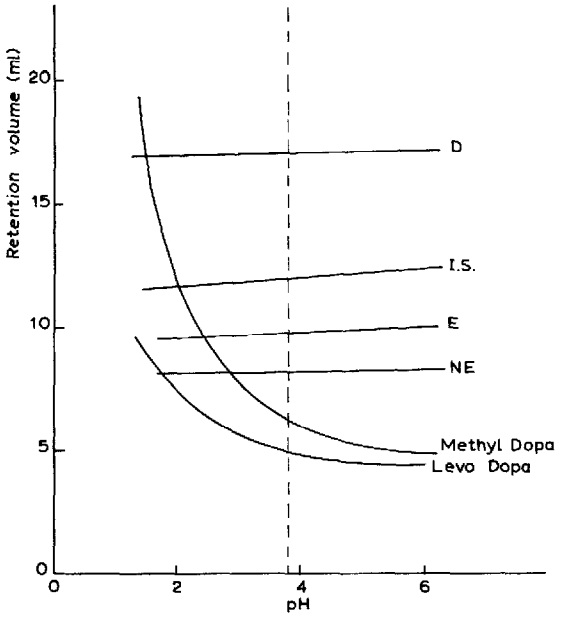

Fig. 3. Elution volume of each catecholamine as a function of mobile phase pH.

Linearity and detection studies

The detection limits were 0.01 μM for both NE and E, and 0.03 μM for D at a signal-to-noise ratio of 2.

A calibration curve was obtained for each catecholamine by plotting the peak-height ratios of NE, E and D to I.S. *versus* known concentrations. The peak-height ratios of NE, E and D to I.S. were found to be linear throughout the range 0.01–5 μM (0.03–10 μM for D). The typical equations were: NE, $y = 0.93x$; E, $y = 0.79x$; D, $y = 0.40x$. Correlation coefficients (r) in the isolation and assay procedures were: NE, 0.9999; E, 0.9997; D, 0.9995.

Optimization of the extraction procedure

The extraction efficiency was studied as a function of the pH of the urine loaded on the alumina column. The optimal pH range is 7.75–8.50, within which the ratio of each catecholamine measured to the I.S. remains constant (Fig. 4).

The buffer volume of 4 ml is justified because it results in a final pH in the optimal range independent of the initial pH or buffering capacity of the urine.

Recovery

Recovery in the isolation step was determined by addition of known amounts of each catecholamine (1 μM) to a pool of urine samples and comparison of the peak areas obtained with and without extraction. The recoveries ($n = 10$) were:

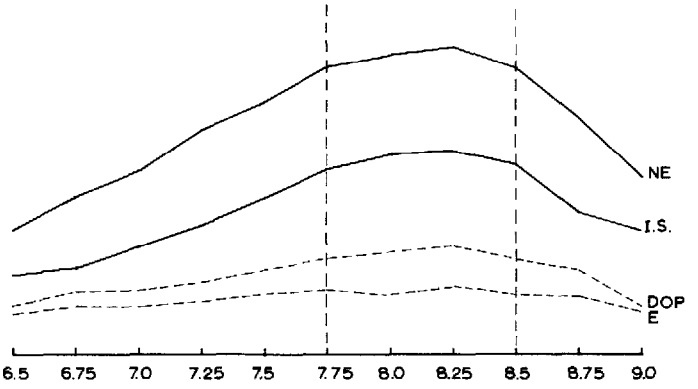

Fig. 4. Variation of the peak-height ratios (catecholamines/I.S.) as a function of the pH of the loaded urine.

NE, 75%; E, 63%; D, 87%. These values are constant from one extraction to another for a given compound.

Precision

The intra- and inter-assay coefficients of variation (C.V.) obtained are summarized in Table I. Intra-assay C.V. were determined for each catecholamine at 0.5 μM in a series of 29 samples. Inter-assay C.V. were determined at 0.2–1.2 μM in routine practice over two months, using pooled urine samples stored in aliquots at $-70°C$ and thawed just before assay.

Interference studies

We studied two compounds (L-DOPA, α-methyldopa), frequently prescribed as medications in humans, whose structures are close enough to the catecholamines assayed to be retained by the column and interfere in the determination.

TABLE I

PRECISION OF THE ASSAY

Compound	Concentration (mean ± S.D.) (μM)	C.V. (%)
Intra-assay (n = 29)		
NE	0.761 ± 0.0477	6.27
E	0.413 ± 0.0374	9.03
D	1.031 ± 0.0499	4.85
Inter-assay (n = 48)		
NE	0.581 ± 0.0477	5.58
E	0.183 ± 0.0176	9.61
D	1.236 ± 0.0942	7.62

Under the selected conditions, retention times obtained for L-DOPA and α-methyldopa were 4 and 4.1 min respectively. Although these times were dependent on the mobile phase pH (increasing when pH decreased), they are clearly shorter than the retention time of NE (6 min 15 s).

DISCUSSION

Many methods have been described for the determination of free urinary catecholamines by HPLC. These methods are differentiated by sample purification techniques (alumina [14–16], cation exchange [17], formation of complexes with boronic acid derivatives [18], reversed-phase chromatography [16,19] or ion exchange [20]) and also by methods of detection (fluorimetry [21,22] or electrochemistry [16,19]). A preliminary purification step (usually long and complex) is common to all these techniques and constitutes the most difficult and time-consuming part of the process. It presents the main obstacle to routine practice of this assay on a large scale. Hence we became interested in the development of a technique that could be entirely automated from the reception of the urine sample (of any pH or buffering capacity) to the end of chromatography, while retaining satisfactory sensitivity and specificity.

We chose alumina extraction as the separation method. This offers the advantage of good selectivity and allows a beneficial pre-concentration, which improves the sensitivity [12,23]. Alumina extraction may be carried out in sequential or batch mode on the ASPEC. We used activated alumina cartridges, which, in addition, were easier to use and less expensive than boronate derivative cartridges.

The main problem with this extraction method is obtaining urine samples with a pH of 8.0, the pH at which catecholamines bind to alumina. We have developed a standard method by systematic addition of base and a strong molar buffer. Thus, whatever the nature of the urine and its buffering capacity, a suitable pH for analysis can always be achieved.

Alumina columns allow a large volume of urine (4 ml) to be used without risk of losing the catecholamines [12] (the eluate and first washings are catecholamine-free). The extraction of the catecholamines is then carried out with 1 ml of perchloric acid, so that a satisfactory result is obtained whatever the volume extracted. Furthermore, automation allows immediate and systematic extraction from the alumina, which improves the conservation of catecholamines (known to be unstable in the pH 8.0 region [12]). The eluate is not injected on-line, which allows efficient extraction and avoids the problem of peak-broadening. The chromatogram thus obtained shows the effectiveness of the clean-up function of the cartridges, since only a few minor interfering peaks were observed and these did not affect the molecules under study.

CONCLUSION

Full automation allows excellent precision to be achieved. The use of native fluorescence as a means of detection avoids baseline instability problems and allows satisfactory sensitivity for routine clinical use. The fact that a second sample may be extracted during chromatography of the preceding sample allows a determination to be carried out every 15 min.

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