Journal of Chromatography, 494 (1989) 303–309
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4840

Note

Measurement of urinary free catecholamines using high-performance liquid chromatography with electrochemical detection

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(First received January 9th, 1989, revised manuscript received April 20th, 1989)

High-performance liquid chromatographic (HPLC) methods of measuring catecholamines (CA) and their metabolites in urine have been extensively studied Selection of a suitable method depends on the nature of the biological source Especially for urine samples, the matrix interferences vary from one sample to another and pretreatment of the samples with activated alumina is not sufficient for direct measurement of CA with fluorimetric or electrochemical detection [1] Interferences from other biogenic substances cause peak overlapping, which results in imprecise and inaccurate determinations Further, the relatively lengthy stabilization of the baseline current in electrochemical detection (ED) is another reason why many laboratories hesitate to use this technique Numerous papers have been published recently suggesting that problems in the determination of CA can be improved by using a column-switching technique, sample enrichment [2,3] or improvements in detector construction [4] However, these techniques are complicated for those who have little experience with practical aspects of instrumentation. The simplicity of the procedure described is advantageous for workers with little experience of CA analysis.

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EXPERIMENTAL

Reagents and chemicals

Norepinephrine (NE), epinephrine (E), dopamine (DA), 3,4-dihydroxybenzylamine (DHBA), Dowex 1 (chloride form, 100–200 mesh), octanesulphonic acid and acid-washed activated alumina (super 1 grade) were purchased from Sigma (St Louis, MO, U S.A) Formic acid and the other reagents and solvents used were of analytical-reagent grade and were obtained from E Merck (Darmstadt, F R G) and BDH (Poole, U K)

Apparatus

A Model 302 liquid chromatograph equipped with a Model 5SC pump head and a Model 231-401 autosampling injector was used (Gilson, Villiers-le-Bel, France) The Partisphere 5 C_{18} column used was a 110 mm$\times$4 7 mm I D replaceable cartridge protected by a guard cartridge system (Whatman, Clifton, NJ, U S A) The detection system consisted of a Model LC-4B electrochemical detector with a glassy carbon electrode and an Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, U S A) and a CR-5A integrator (Shimadzu, Kyoto, Japan) The anion-exchange columns were prepared fresh daily by using 4 mm I D glass tubes packed to a length of 10 mm with Dowex 1 (approximately 250 mg) Preconditioning was achieved by rinsing twice with 2 ml of distilled water The chromatographic conditions are given in the figure legends

Standard preparation

An internal stock standard solution was prepared by dissolving 2 5 mg of DHBA in 250 ml of 0 1 *M* formic acid A working standard solution of 50 ng/ml was prepared daily by diluting the stock solution with a 200-fold volume of 0 1 *M* acetate buffer (pH 6 1) containing 20 g/l EDTA

A stock standard solution of CA was prepared by dissolving 2 5 mg of NE and E and 5 mg of DA in 250 ml of 0 1 *M* formic acid These stock solutions are stable for several months at 4°C Working standards were prepared daily by diluting the stock solution 2000-, 400-, 200-, 100- and 50-fold to obtain concentrations of 5, 25, 50, 100 and 200 ng/ml, respectively, for NE and E and double these concentrations for DA

Urine sample collection

Urine samples were collected and preserved with 1% (v/v) of 6 *M* hydrochloric acid (pH$<$2) and stored frozen at -20°C Urinary catecholamines were reported to be stable for at least six weeks when stored frozen at pH 2 [5]

Extraction procedure

A 1-ml volume of urine was adjusted to pH 6 1±0 2 with the addition of 2 ml of 0 5 *M* Tris buffer (pH 6 3) containing 20 g/l EDTA and 1 ml of 0 1 *M* acetate buffer (pH 6 1) containing the internal standard and EDTA The sample was mixed thoroughly and allowed to percolate through the preconditioned anion-exchange column The eluate was collected in a 10-ml disposable plastic tube that contained approximately 100 mg of alumina An additional 1 ml of acetate buffer (0 1 *M*, pH 6 1) was added to the column and the effluent was collected in the same tube The pH of effluent was adjusted to 8 4±0 1 by adding 2 ml of Tris buffer (0 5 *M*, pH 11)

Samples were gently mixed for 20 min on a rotary mixer The alumina was allowed to settle and the liquid aspirated The alumina was then washed twice with 1 ml of 5 m*M* acetate buffer (pH 7) After the buffer layer had been thoroughly removed, 1 ml of formic acid (0 2 *M*) was added to the alumina and mixed for 2 min on a vortex mixer Finally, the acid layer was transferred into a Costar centrifuge filter unit and centrifuged at 10 000 *g* for 30 s and 20 μl of the filtrate were injected into the HPLC system

RESULTS AND DISCUSSION

Mobile phase for HPLC with electrochemical detection system

It is usually recommended that the HPLC system be flushed to remove all traces of salt solution when not in use, especially with acetate, citric and phosphate buffers [6–10] However, it is very inconvenient to carry out this process for routine ion-pairing techniques and when an ED system is used as they require long periods of equilibration and stabilization. In this investigation, 0 1 *M* formic acid containing 2% (v/v) methanol, 0 2% (v/v) diethylamine, 2 m*M* EDTA and 1 m*M* octanesulphonic acid, with the pH adjusted to 3 6 with 5 *M* sodium hydroxide, was used as the mobile phase at a flow-rate of 1 0 ml/min This system was used continuously for up to four weeks and no significant changes in column efficiency were noted In order to maintain a high sensitivity of detection, the glassy carbonelectrode should be polished once every fourteen days

Reversed-phase chromatography and matrix interference

Measurement of urinary CA in alumina extracts has been shown to be difficult with reversed-phase systems because of void volume interference [11,12] According to Causon and Carruthers [13], these interferences can be eliminated by using the ion-pairing technique Pretreatment of urine samples with alumina alone is not satisfactory Fig 1a shows that although the concentration of the ion-pairing reagent used was 2 m*M*, there were still many interferences and peak overlapping. An increased amount of ion-pairing reagent may result in a higher resolution but it also increases the retention time Although

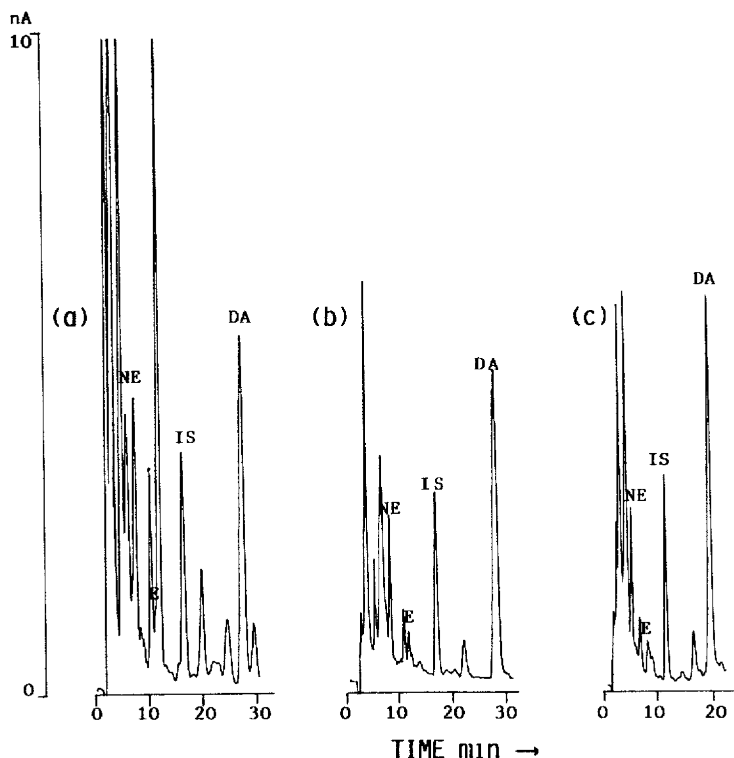

Fig 1 Chromatograms of urinary catecholamines measured by HPLC with ED IS = internal standard (a) Urine sample pretreated with alumina alone, (b) same urine sample pretreated with additional ion-exchange chromatographic step, (c) same urine sample pretreated as in (b) and separated under the chromatographic conditions described below, except that the concentration of the octanesulphonic acid used as an ion-pairing reagent was only 1 m*M* Mobile phase, 0 1 *M* formic acid containing 2% (v/v) methanol, 0 2% (v/v) diethylamine, 2 m*M* EDTA, 2 m*M* octanesulphonic acid, pH adjusted to 3 6 with 5 *M* sodium hydroxide, flow-rate, 1 0 ml/min, temperature, ambient, detector potential, 0 8 V vs Ag/AgCl, sensitivity, 10 nA full-scale

raising the pH of the mobile phase has a pronounced effect on the degree of hydrophobic interaction and retention times [14], it will also result in an increase in salt content in the mobile phase and thus shorten the column life To ensure an accurate and precise routine determination of CA in different matrices of urine samples, an additional step using ion-exchange chromatography is essential Fig 1b shows the results for same urine sample eluted through an anion-exchange column before alumina extraction and separated under the same chromatographic conditions as in Fig. 1a NE, E and DA were totally free from interferences. The use of 1 m*M* instead of 2 m*M* octanesulphonic acid reduces the analysis time significantly, as shown in Fig 1c Fig 2a shows that when a pure standard solution was processed by the same procedure, no sig-

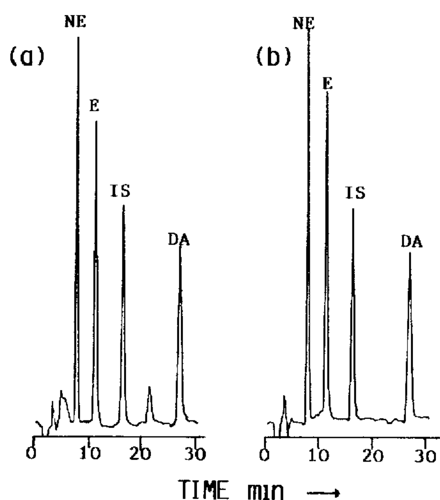

Fig 2 (a) Chromatogram of a standard mixture containing 50 ng/ml each of NE, E and DHBA as internal standard (IS) and 100 ng/ml DA, eluted through an anion-exchange column before alumina extraction, (b) same standards treated with alumina alone Chromatographic conditions as in Fig 1

nificant decrease in sample concentration was observed compared with extraction with alumina alone (Fig 2b)

The use of diethylamine and EDTA in the mobile phase has the effect of eliminating the interactions of basic compounds with residual silanol group [15], therefore minimizing peak tailing in reversed-phase HPLC analysis

Analysis time

Adjustment of the urine pH with a glass electrode makes the results more reliable but impracticable in routine analysis, as it is tedious and favours the oxidative decomposition of NE and E In this analysis, urine samples were adjusted form pH <2–6 1 and then to 8 5 by addition of Tris buffer (0 5 *M*, pH 6.3 and 11) Addition of 20 g/l EDTA to the Tris and acetate buffer protects against the autoxidation of catecholamines This is the best and most rapid procedure for pH adjustment. This method was verified by repetitive analyses of samples

The average sample preparation time was about 1 h for 12 samples The analysis time was 22 min per injection and about 60 samples could be analysed within 24 h when an automatic sample injector was used

Sensitivity and analytical recovery

The use of the internal standard technique reduces the variability and improves the accuracy The calibration graphs were linear for concentrations of NE and E in the range 1–200 ng/ml and for DA in the range 2–400 ng/ml

Typical regression equations and correlation coefficients were $y=0.0226x+0.0045$ ($r=0.9998$) for NE, $y=0.0179x-0.0032$ ($r=0.9997$) for E and $y=0.0122x+0.0001$ ($r=0.9996$) for DA, where y is the ratio of peak height of the CA to that of the over internal standard and x is the concentration of the CA. The coefficients of variation of the linearity and slope of the calibration graphs for between-day analyses were less than 0.2 and 2% ($n=4$), respectively.

The within-assay precision of the method was obtained by processing four aliquots of a pooled urine sample spiked with various concentrations of NE, E and DA. The day-to-day coefficient of variation was calculated from values for spiked samples assayed on four consecutive days. The data in Table I indicate that the precision of the method is good, the within-assay coefficient of variation being less than 5% and the day-to-day coefficient of variation less than 7%.

The absolute recoveries of NE, E and DA and also DHBA (internal standard) added to urine sample at various concentrations were ca. around 66, 71, 63 and 76%, respectively. After correction of recovery based on the internal standard level, the overall analytical recoveries of CAs added to urine were 95% for NE, 91% for E and 88% for DA (Table I).

The proposed procedure using ED was compared with detection using a Shi-

TABLE I

PRECISION AND RECOVERY OF ANALYSIS

Original levels: NE, 16.1 ng/ml; E, 5.4 ng/ml; DA, 97.6 ng/ml. All sample were processed as described under Experimental.

Compound	Added (ng/ml)	Mean recovery ($n=4$) (%)	Coefficient of variation ($n=4$)	
			Within-day	Between-day
NE	10	87	2.5	5.5
	25	93	3.1	4.7
	50	107	3.1	11.1
	100	94	1.4	5.7
Mean		95	2.5	6.8
E	10	90	2.4	6.8
	25	88	7.4	6.4
	50	95	3.9	2.3
	100	90	5.8	2.1
Mean		91	4.9	4.4
DA	20	88	4.2	6.9
	50	88	4.8	1.8
	100	92	3.3	3.0
	200	84	0.9	4.6
Mean		88	3.3	4.1

madzu Model RF-535 fluorescence detector Close agreement was observed between the two detection methods; the correlation coefficients (r) were 0 99, 0 93 and 0 97 for NE, E and DA, respectively ($n=9$)

The detection limits at a signal-to-noise ratio of 3 were 1 ng/ml for NE and E, 2 ng/ml for DA

Interference studies

Ion exchange has been shown to be more selective and superior to solvent extraction for the determination of organic acids [16]. Anion-exchange chromatography as used in this study removes most of the interfering substances and fewer disturbances were observed in subsequent analyses.

No drugs were used directly for testing interferences However, many of the urine samples analysed were collected from patients under medication and the results obtained did not show interferences

CONCLUSION

The method presented here is not new as such, but modified in various important details so as to make HPLC with ED applicable to routine analysis with an accurate and precise determination of various types of catecholamines in urine, free from interferences and with good reproducibility

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