ELSEVIER

Journal of Chromatography B, 664 (1995) 287–293

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Automated analysis of urinary catecholamines by high-performance liquid chromatography and on-line sample pretreatment

Aronne Pastoris*, Lidia Cerutti, Roberta Sacco, Laura De Vecchi, Andrea Sbaffi

Laboratorio Analisi, Ospedale SS. Trinità, Viale Zoppis 10, 28021 Borgomanero, Italy

First received 28 July 1994; revised manuscript received 10 October 1994; accepted 19 October 1994

Abstract

A simple and automated solid-phase extraction for the selective and quantitative HPLC analysis of free catecholamines in urine is described. The urinary catecholamines react with diphenylboric acid, giving a complex at pH 8.5 which is strongly retained on a PLRP-S cartridge; elution is accomplished with the same mobile phase used for HPLC analysis. Separation is performed by ion-pair reversed-phase HPLC, with sodium heptanesulphate as counter-ion, and a totally end-capped C_{18} analytical column. Quantitation is achieved with an electrochemical detector. A Spark Holland Prospekt system controls the on-line solid-phase extraction, preconcentration and direct elution to the LC column. Chromatography run-time is 10 min and the total time to process one urine sample is ca. 12 min.

1. Introduction

The catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA) are important neurotransmitters in the human body.

The regulation of physiological processes by catecholamines is mediated by both sympathetic nerves and the adrenal medulla. Most catecholamines are excreted in urine as deaminated metabolites: vanilmandelic acid (VMA), homovanillic acid (HVA) and hydroxymethoxyphenylglycol (HMPG). A small fraction is excreted unchanged or as O-methylated amines (metanephrines).

There are several tumor types that cause increased catecholamine excretion: pheochromocytoma, neuroblastoma, ganglioneuroblastoma and ganglioneuroma. Most pheochromocytomas contain predominantly norepinephrine, with consequently increased concentration of NE in urine. Excretion of dopamine and its metabolites is usually not significant in patients with pheochromocytoma. The excretion of catecholamines and their metabolites is always increased in neuroblastoma and often increased in ganglioneuroma. NE, VMA, DA and HVA may be excreted in large amounts. Increased excretion of DA and HVA is particularly characteristic of neuroblastoma [1].

Analysis of the above-mentioned catecholamines in urine is an important parameter for the differential diagnosis and monitoring of these

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00490-0

carcinomas [2,3]. Various separation methods have been used as clean-up step for the analysis of the catecholamines in biological fluids: solvent extraction [4], adsorption on alumina [5], ion-exchange [6] and complexation with diphenylboric acid [7].

Owing to its simplicity, its reproducibility, and with respect to automated use, the last approach shows most suitable features. We adopted the buffering and complexation method used by Grossi et al. [7], studying a new methodological approach for analytical application of the Prospekt System. The complexing agent is available as the diphenylboric acid–ethanolamine complex, which is dissociated into a negatively charged diphenylborate and ethanolamine in alkaline medium; at these pH conditions the diphenylborate forms a negatively charged complex with the vicinal hydroxyl groups of catecholamines.

The complex is strongly retained on PLRP-S and PRP-1 cartridges in alkaline medium and can be easily eluted with the same mobile phase employed for the HPLC analysis.

2. Experimental

2.1. Chemicals and materials

Norepinephrine bitartrate salt, epinephrine bitartrate salt, dopamine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide, heptane sulfate sodium salt were purchased from Sigma (St. Louis, MO, USA).

Diphenylboric acid–ethanolamine complex was from Aldrich-Chemie (Steinheim, Germany). EDTA disodium salt, potassium dihydrogen phosphate were obtained from J.T. Baker (Deventer, Netherlands).

Ammonium hydroxide, ammonium chloride, acetonitrile (HPLC grade), methanol (HPLC grade) and water (HPLC grade) were purchased from Farmitalia Carlo Erba (Milan, Italy). Lyphochek Normal and Abnormal Urine Control and Bio-Rex 70 microcolumns were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

The PLRP-S, PRP-1, C_{18}, C_{18}-OH low hydrocarbon and C_{18} high hydrophobic extraction reversed-phase cartridges, containing 20 mg of resin, were from Spark Holland (Emmen, Netherlands).

The length and the internal diameter of the cartridges were 10 and 2.0 mm, respectively; the PLRP-S and PRP-1 were packed with a 15–25 μm polymer-based material and the others with a 40-μm silica-based material.

2.2. Apparatus

The sample handling system consisted of three modules: the solvent delivery unit (SDU), the Marathon autosampler with a 200-μl loop and the control module Prospekt (Spark Holland) which contained two high-pressure switching valves, the cartridge transport and sealing mechanism. The unit was equipped with a Model 210A manual injector (Beckman Instruments, Fullerton, CA, USA), which allows direct injection without a cartridge.

The chromatographic system consisted of a Model 126 solvent delivery module System Gold, programmed by a Model 424 system controller (Beckman Instruments); a dual-electrode coulometric ESA detector Model 5100A (Bedford, MA, USA) equipped with a 5021 conditioning cell and a 5011 high-sensitivity cell; a 5 μm 250 × 4.6 mm I.D. Ultrasphere IP (ion-pair) C_{18} column (Beckman Instruments) and a 30 × 4.6 mm I.D. C_{18} column (Brownlee Labs Applied Biosystem, Santa Clara, CA, USA), operated at room temperature; an integrator Model C-R3A (Shimadzu Corporation, Kyoto, Japan).

The signal was monitored at the following potentials: +350 mV (oxidizing electrode), +100 mV (screen electrode) and −300 mV (quantifying electrode). The gain of the detector was 100, equivalent to a full-scale of −1.0 μA, and the response-time was 4 s. Chromatograms were plotted at an integrator attenuation of 8.

2.3. Mobile phase

The eluent consisted of 50 m*M* potassium dihydrogen phosphate (containing 1 m*M* sodium-

heptane sulphate and 0.07 m*M* EDTA)–methanol–acetonitrile (100:8:15, v/v).

The apparent pH was adjusted to 3.2 (at room temperature) with 1.5 *M* orthophosphoric acid. The mobile phase was degassed twice under vacuum by filtration through a 0.2-μm Millipore membrane. The flow-rate was 0.8 ml/min.

2.4. Preparation of standards

Stock solutions of norepinephrine (NE, 1 g/l), epinephrine (E, 1 g/l), dopamine (DA, 4 g/l) and the internal standard dihydroxybenzylamine (DHBA, 1.26 g/l) were prepared in 0.1 mol/l hydrochloric acid and were stored at −20°C.

Working solutions were obtained by adding to an acidified normal urine 0, 10, 50, 100, 300 μg/l of norepinephrine and epinephrine and 0, 125, 250, 500, 1200 μg/l of dopamine. These standards were prepared daily.

2.5. Methods

Urine specimens (24 h) were collected in dark polyethylene bottles containing 10 ml of 6 *M* HCl (final pH 1–3), and aliquots were frozen if not assayed within 24 h.

The sample was diluted 4-fold with 2.0 *M* ammonia–ammonium chloride buffer pH 8.5, containing 0.5% EDTA, 0.1% diphenylborate (PBA) and 18.0 μg/l internal standard. Then the sample was transferred into a glass vial and positioned on the autosampler tray for the automated clean-up and injection operated by the Prospekt system (Fig. 1).

The Prospekt controller unit controls the Marathon autosampler, the SDU, all switching valves and the cartridge exchange, by means of a time program. The Marathon autosampler collects samples from the vials and fills a 200-μl loop.

The SDU is used to condition the cartridge with solvents, to wash the cartridge and to transfer the sample-loop contents onto the clean-up cartridge. The Prospekt controller performs the column-switching to eliminate the interfering peaks produced by the excess of complexing agent, which elute after the catecholamine peaks and which increase the turnover time between analyses.

To realize this set-up, two on-line analytical columns have been used, the first 3 cm and the second 25 cm in length. After elution of the catecholamines from the short column onto the

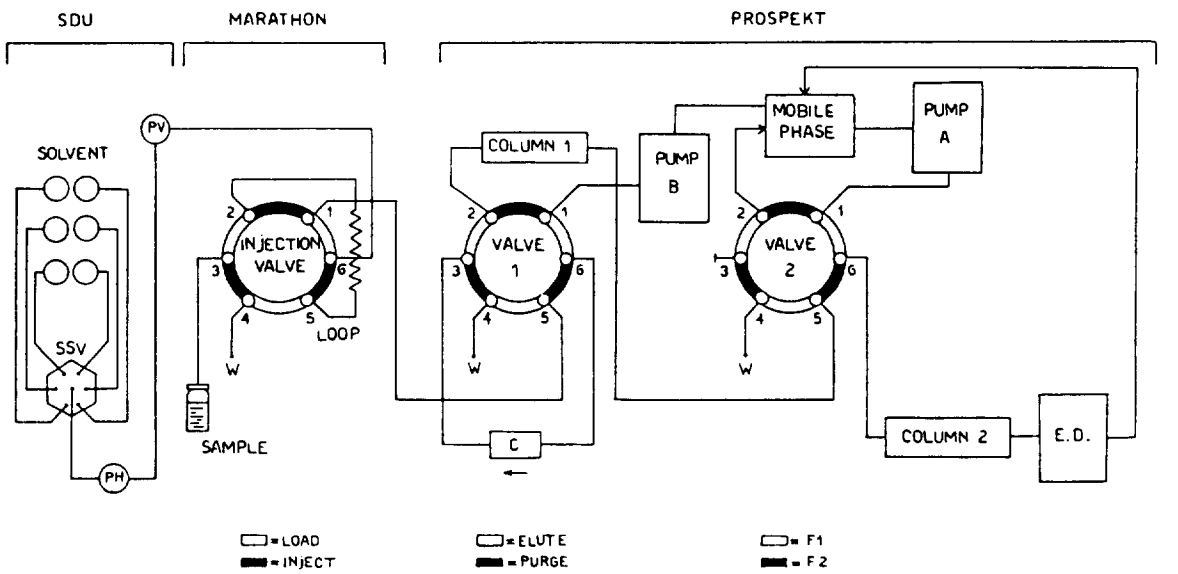

Fig. 1. Scheme of the system. SSV = solvent selecting valve; PH = pump head; C = clean-up cartridge; ED = electrochemical detector.

longer one, valve no. 2 switches, permitting elution of interferences from the first column to waste, while the catecholamines are eluted from the second column to the detector.

Two isocratic pumps continuously supply the mobile phase to the analytical columns: pump B delivers the mobile phase to both columns until the column-switching takes place, then it pumps the solvent only through the short column, while the long column is flushed with pump A.

2.6. Sample preparation program

The sample preparation program is a time-based program, which means that at during an established time a particular event will be executed. Fig. 1 and Table 1 explain the events that take place in the sample pretreatment. Before the start of the time program, the Prospekt changes the clean-up cartridge and the autosampler fills the loop with sample, and then the following steps are performed:

–Time 00:00. The injection valve is in the load position, so the pretreatment solvents do not flow through the loop.

–Time 00:02. Methanol activates the clean-up cartridge for 1 min.

–Time 01:00. The cartridge is washed with water for 30 s.

–Time 01:30. The 0.2 *M* ammonia–ammonium buffer pH 8.5, containing 0.05% EDTA, normalizes the pretreatment resin.

–Time 03:00. The injection valve switches to the inject position, so the previous buffer flows through the loop and transports the sample to the cartridge.

–Time 04:00. The injection valve turns to the load position and the methanol–0.2 *M* ammonia–ammonium buffer solution pH 8.5 (20:80, v/v) washes the cartridge and eliminates some interfering peaks.

–Time 04:15. The water eliminates the excess of alkaline buffer.

–Time 06:30. While the SDU stops, valve 1 switches to the elute position and the sample is eluted to the analytical column by the mobile phase. At this time the integrator starts (Aux.1 is switched on and off).

–Time 07:00. The elution process stops.

–Time 08:15. Valve 2 starts the column-switch-

Table 1
Time program

	S.D.U.		MARATHON	PROSPEKT												
TIME	SOLV.	PURGE FLOW	INJECTION VALVE	CHANGE CARTRIDGE	VALVE 1	VALVE 2	VALVE 3	AUXILIARIES						BCD OUT	END TIME	COMMENT
H:MM:SS		ml/min	F1 : load F2 : inject	F1 Execute	F1 : elute F2 : purge	F1 : ••• F2 : •••	F1 : ••• F2 : •••	1	2	3	4	5	6			
00:00:00			F1		F2	F2										1 : METHANOL
00:00:01	1															2=WATER
00:00:02		1.0														3=0.2M BUFFER
00:01:00	2															4=0.2M BUFFER -
00:01:30	3															METHANOL
00:03:00			F2													
00:04:00	4		F1													
00:04:15	2															
00:06:30		0			F1			ON								
00:06:31								OFF								
00:07:00					F2											
00:08:15						F1										
00:11:30						F2										
00:11:31															END	

ing to eliminate reagent interfering peaks from the first analytical column.

–Time 11:30. The column-switching stops.

–Time 11:31. The sample preparation program and the chromatographic analysis end.

The run-time for the first sample is 18.00 min, while for the following samples this is reduced to 11.30 min, since the sample clean-up is performed during the previous chromatographic run.

3. Results and discussion

3.1. Clean-up cartridges

We have tested the efficiency of five types of clean-up reversed-phase cartridges and found that both polymer-based resins PLRP-S and PRP-1 show good recoveries, while silica-based packings C_{18}, C_{18}-OH low hydrocarbon and C_{18} high hydrophobic show insufficient performance (Table 2).

We adopted PLRP-S resins owing to their higher and more uniform recoveries for all catecholamines.

3.2. Complexing reagent concentration

The 2 *M* ammonia–ammonium buffer pH 8.5, containing 0.5% EDTA and 0.1% PBA affords optimal buffering and complexing action and shows the best recoveries between batches of cartridges; a higher ionic strength causes a loss of diphenylborate–norepinephrine complex, while a lower PBA concentration may be insufficient to form the complex with the catecholamines.

3.3. Counter-ion effect and column-switching

Pretreatment with diphenylborate in alkaline medium is characterized by high specificity and stability for catecholamines, but the complex is totally dissociated into free catecholamines and complexing agent under our ion-pair LC conditions (pH 3.2). Though the complexation and clean-up steps have good performances, the following LC analysis of free catecholamines gives two interfering peaks owing to the reagent: one coelutes with the catecholamines, making quantitation unfeasible, the other elutes 32 min after the dopamine peak, increasing the turnover time between analyses. This effect mainly occurred with the use of sodium dodecyl sulphate as counter-ion. A long column was not sufficient to separate the interferences and also increased chromatographic analysis time.

Changing to a shorter counter-ion, such as sodium heptane sulphate and keeping the organic modifier constant, the retention times of the catecholamines decreased relative to those of the interfering compounds.

This behaviour permits the use of the column-switching technique, as described previously. Chromatograms obtained from standard and urine samples are shown in Fig. 2.

Table 2
Absolute recovery ($n = 10$)

	Amount added (μg/l)	Recovery (%)				
		PLRP-S	PRP-1	C_{18}	C_{18}-LH	C_{18}-HH
NE	200	97 ± 2.9	93 ± 3.0	60 ± 6.0	36 ± 8.1	1.6 ± 15.0
E	100	99 ± 2.0	99 ± 3.1	71 ± 5.1	59 ± 7.5	1.9 ± 12.0
DHBA	180	99 ± 2.0	99 ± 2.1	75 ± 4.9	50 ± 8.0	12.0 ± 8.0
DA	500	98 ± 2.5	97 ± 3.2	85 ± 4.7	61 ± 6.5	1.9 ± 13.5

C_{18}-LH = C_{18}-OH low hydrocarbon.
C_{18}-HH = C_{18} high hydrophobic.

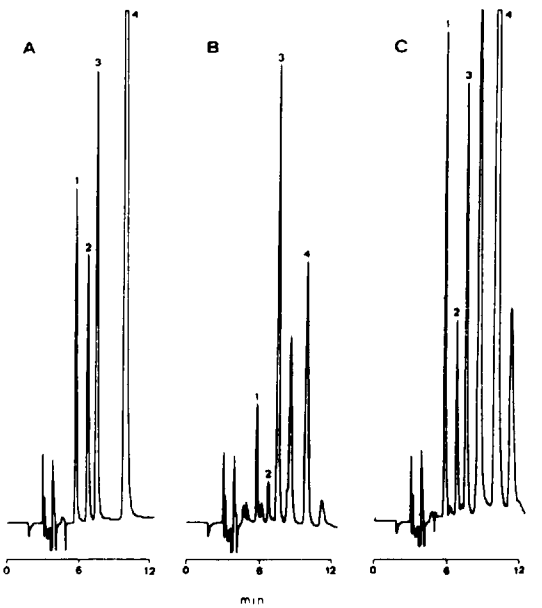

Fig. 2. Chromatograms obtained from a standard mixture (A), a normal urine (B) an abnormal urine (C), using the on-line sample pretreatment system. (A) Containing 100 μg/l NE, 100 μg/l E, 180 μg/l DHBA, 323 μg/l DA; (B) containing 29 μg/l NE, 13 μg/l E, 79 μg/l DA; (C)containing 142 μg/l NE, 72 μg/l E, 640 μg/l DA. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = dihydroxylbenzylamine; 4 = dopamine. The full-scale −1 μA, other conditions as in Experimental.

The performance of the two analytical columns is unchanged after processing about 300 samples, because the clean-up cartridge acts as a protective filter and does not increase the back-pressure owing to its relatively large particle size.

3.4. pH Effect

We tested the pH of mobile phase in the range 2.8–3.8 with intervals of 0.2 pH units. At pH values above 3.6 we noted a decrease of the electrochemical signal of the epinephrine peak; at pH 3.2 we obtained the best performance relative to the recovery and the chromatographic resolution between NE, E and L-DOPA; in fact L-DOPA interferes with NE at pH 3.0 and with E at pH 2.8.

3.5. Recovery, precision and linearity

Table 2 shows the absolute recoveries of norepinephrine, epinephrine, dopamine and the internal standard from urine spiked with 200, 100, 500, 180 μg/l respectively. The peak areas of NE, E, DA and DHBA were measured in chromatograms obtained after manual injection of the four compounds dissolved in 0.1 *M* hydrochloric acid without extraction, and they were compared with those obtained after solid-phase extraction from urine spiked with known amounts of catecholamines.

Table 3 shows the precision of the automated analysis obtained using Lyphochek Normal and Abnormal Urine Controls.

The calibration graphs are linear from 0 to 300 μg/l for norepinephrine (mean $r = 0.9998$) and epinephrine (mean $r = 0.9999$) and between 0 and 1200 μg/l for dopamine (mean $r = 0.9999$); the intercept of the regression line is close to zero ($n = 6$).

A correlation study between manual extraction (x) on Bio-Rex 70 cation-exchange resin (Bio-Rad method) and the column-switching automated method (y) was performed on 150 urine samples giving $y = 0.998x + 1.050$, $r = 0.9985$ for NE; $y = 0.996x + 0.080$, $r = 0.9990$ for E; $y = 1.011x - 0.902$, $r = 0.9974$ for DA.

Considering a signal-to-noise ratio of 3, the

Table 3
Precision of the automated catecholamines analysis

	Within-day		Between-day	
	Mean value (μg/l)	R.S.D. (%)	Mean value (μg/l)	R.S.D. (%)
Normal urine (n = 35)				
NE	33	2.0	32	3.3
E	13	1.7	14	3.5
DA	70	2.3	72	4.3
Abnormal urine (n = 35)				
NE	163	3.4	161	4.4
E	77	1.1	80	3.4
DA	420	2.0	411	2.6

limits of catecholamine detection were 1.3, 2.0, and 3.0 μg/l for norepinephrine, epinephrine and dopamine, respectively.

In urine, the catecholamines–diphenylborate complex is stable for more than 6 h at room temperature and at least for 24 h at 4°C.

Acknowledgements

We are grateful to Beckman Analytical (Cassina de Pecchi, MI, Italy) for supplying the Prospekt System and the clean-up cartridges. We also extend our thanks to Mr. Maurizio Lugli for excellent technical assistance.

References

[1] L. Landsberg and J.B. Young, in J.D. Wilson and D.W. Foster (Editors), *Textbook of Endocrinology*, W.B. Saunders Company, Philadelphia, PA, 1985, Ch.23, p. 891.
[2] T.G. Rosano, *Clin. Chem.*, 30 (1984) 301.
[3] T.G. Rosano, T.A. Swift and L.W. Hayes, *Clin. Chem.*, 37 (1991) 1854.
[4] F. Smedes, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 231 (1982) 25.
[5] G. Melcalf, *Anal. Biochem.*, 57 (1974) 316.
[6] M. Hamaji and T. Seki, *J. Chromatogr.*, 163 (1979) 329.
[7] G. Grossi, A.M. Bargossi, C. Lucarelli, R. Paradisi, C. Sprovieri and G. Sprovieri, *J. Chromatogr.*, 541 (1991) 273.