ELSEVIER

Journal of Chromatography B, 655 (1994) 121–126

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Determination of free urinary catecholamines by high-performance liquid chromatography with electrochemical detection

Pirkko Volin[a,b]

[a]*United Laboratories Ltd., P.O. Box 222, SF-00381 Helsinki, Finland*
[b]*Analytical Chemistry Division, Department of Chemistry, University of Helsinki, P.O. Box 6, SF-00014 Helsinki, Finland*

(First received January 19th, 1993; revised manuscript received January 7th, 1994)

Abstract

The present paper compares a manual high-performance liquid chromatographic (HPLC) method with electrochemical detection (ED) with an on-line HPLC-ED method for the determination of free catecholamines (CAs) (norepinephrine, epinephrine, and dopamine) in urine. The manual method involves a two-stage extraction procedure with home-packed columns filled with weakly acidic cation-exchange resin followed by adsorption onto alumina while the on-line method uses a strong cation-exchange polymer as precolumn material which allows the simultaneous determination of free CAs and metanephrines (MNs) in urine. Reference control urine samples analysed with both methods gave levels of CAs within the acceptable reference ranges. Dihydroxybenzylamine was used as an internal standard in both methods and the separation was performed by ion-pairing reversed-phase HPLC.

1. Introduction

Norepinephrine (NE), epinephrine (E) and dopamine (DA), the three naturally occurring catecholamines (CAs), are neurotransmitters in the brain and peripheral sympathetic nerves. E is quantitatively produced by the adrenal medulla, while NE is mainly generated by the sympathetic nervous system. An excess production of either NE or E (or both of them) is one of the diagnostics for neural crest tumour pheochromocytoma associated hypertension [1,2]. Also other neural crest tumours such as neuroblastoma and ganglioneuroma are associated with an abnormal secretion of CAs in tissue and body fluids. An increase in urinary dopamine has been reported to be associated with malignant pheochromocytoma and neuroblastoma [3].

The analysis of the metabolites of CAs, total metanephrines (MNs), has been described to be the best discriminator between essential and pheochromocytoma associated hypertension [4]. In a normal situation metanephrine (MN) is a major excretion product of E, while normetanephrine (NMN) represents less than 5% of the total NE excretion products in urine [5]. According to Crout *et al.* [6] and Rosano *et al.* [2], pheochromocytomas can, however, show an increase either in the NE or E content in urine or in their metabolites, MNs, depending on the size of the tumour and its metabolic content. Combination of the measurement of CAs and

0378-4347/94/$07.00
SSDI 0378-4347(94)00029-5

MNs might therefore be of special value, at least as a screening procedure, for the elimination of false negative findings in patients with pheochromocytoma.

A review on a variety of techniques for the determination of CAs and their metabolites has been published by Kågedal and Goldstein [7]. Although the chromatographic methods used for the analysis of CAs and MNs have continuously been improved, only a few studies have been published on the simultaneous analysis of urinary CAs and MNs by HPLC [8–11].

The aim of this study was to compare an on-line method with a manual one for the determination of urinary CAs, and to evaluate the possibilities for the simultaneous determination of CAs and MNs in urine.

2. Experimental

2.1. Instrumentation

In *the manual dual extraction method* the chromatographic system consisted of a Model 200 SSI pump (State College, PA, USA) and an ESA Coulochem Model 5100 coulometric detector equipped with the analytical cell (Model 5011), the conditioning cell (Model 5021), and the guard cell (Model 5020), all from ESA (Bedford, MA, USA). The samples were injected with an Autosampler Model 655A-40 from Hitachi (Tokyo, Japan). The peak heights were recorded on a Hewlett-Packard Model 3393A integrator and 9114B Disc Drive (Avondale, PA, USA).

In *the on-line method* the automated sequential trace enrichment apparatus ASTED (Gilson Medical Electronics, Villiers-le-Bel, France), where the loop on the Rheodyne injection valve was replaced by a stainless-steel trace enrichment cartridge (TEC), was used instead of the autosampler. TEC was packed with 15 mg of 10-μm diameter Separon HEMA-BIO 300 SB (2-hydroxyethylmethacrylate sulphobutyl) from Tessek (Czech Republic and Denmark).

2.2. Chemicals and reagents

E, NE hydrochloride and 1-heptanesulfonic acid sodium salt monohydrate were obtained from Fluka (Buchs, Switzerland), DA (3-hydroxytyramine hydrochloride) from Calbiochem (La Jolla, CA, USA), and the internal standard 3,4-dihydroxybenzylamine hydrobromide, MN hydrochloride, NMN hydrochloride and Trisma base from Sigma (St. Louis, MO, USA). Acids and buffer substances were all from Merck (Darmstadt, Germany). Methanol was obtained from Orion (Helsinki, Finland). Water and mobile phases were purified through a Milli Q system (Millipore, Bedford, MA, USA). The cation-exchange resin (Bio-Rex 70, 50–100 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA, USA); it was prepared as described earlier [12].

Stock solutions were prepared separately at concentrations of 2.0, 4.0 and 32.0 mM in 0.1 M perchloric acid for E, NE, and DA, respectively. They were stable at −20°C for six months. A working standard solution was prepared by diluting each stock solution to 67, 133 and 1067 μM, respectively, with 0.1 M perchloric acid and for the on-line method by diluting to 400, 800 and 6400 μM, respectively, with 60 mM sodium chloride–10 mM sulphuric acid. NMN and MN stock solutions were prepared separately at a concentration of 18.0 and 17.0 mM in 0.1 M HCl. The final concentrations of analytes ranged from 0.075 to 0.375 μM for E, from 0.130 to 0.650 μM for NE, from 1.075 to 5.375 μM for DA, from 0.550 to 8.800 μM for NMN and from 0.590 to 9.440 μM for MN. The internal standard solution for the on-line method (21 μM) was diluted from the stock solution (7 mM) with 0.01 M sulphuric acid.

In *the manual method* Tris/EDTA-buffer was prepared by dissolving 30.6 g of Trisma base and 5.0 g of EDTA-disodium salt in 100 ml of water. The pH was adjusted to 8.6 with hydrochloric acid. The phosphate buffer, pH 7.0 was prepared by dissolving disodiumhydrogenphosphate (24 mM), potassiumdihydrogenphosphate (8.7 mM), and EDTA-disodium salt (27 mM) in

water. The pH was adjusted to 7.0 with 5 *M* sodiumhydroxide. The alumina (Woelm, Neutral Activity Grade I) was acid-washed according to Anton and Sayre [13]. In *the on-line method* ammonium phosphate solution was prepared by dissolving diammoniumhydrogenphosphate (0.5 *M*) in 800 ml of water. After that the pH was adjusted to 8.3 with orthophosphoric acid and the volume diluted to 1 l with water.

2.3. Chromatographic conditions

The manual method

The separation of urinary CAs was optimized using an ESA Catecholamine HR-80 column (80 × 4.6 mm I.D., 3 μm) protected by a pre-column filter with a frit (55-0068 from ESA). The mobile phase was prepared by mixing first sodium dihydrogen orthophosphate (51 m*M*), heptane sulfonic acid sodium-salt (1.1 m*M*), and EDTA-disodium salt (0.2 m*M*) in 5 l of water from which 4775 ml was taken and mixed with 225 ml of methanol. The pH was adjusted to 3.5 with orthophosphoric acid. The flow-rate of the mobile phase was 1.0 ml/min. The detector potentials were set at +0.35 V, +0.05 V, and −0.26 V for the conditioning cell, detector 1, and detector 2, respectively.

The on-line method

The same separation column as in the manual method was used. The mobile phase was prepared by dissolving diammonium hydrogen orthophosphate (125 mM), EDTA-disodium salt (196 μM), and heptane sulfonic acid (500 μM) in 800 ml of water. The pH was adjusted with orthophosphoric acid to 3.5. After addition of 5.0 ml of ethanol the volume was made up to 2 l with water. The flow-rate was 1.5 ml/min. The detector potentials were set at +0.90 V, +0.10 V, and +0.45 V for the guard cell, detector 1, and detector 2, respectively.

2.4. Sample collection

Twenty-four-hour urine specimens were collected in polyethylene bottles containing 10 ml of 6 *M* HCl as preservative. On completion of the collection, the pH was adjusted to below 3 with 6 *M* HCl. The volume of the specimen was measured, and an aliquot was removed for analysis and stored at 4°C. Under these conditions the specimens were stable for at least one month.

2.5. HPLC assays

Manual method

To a 5-ml aliquot of the 24-h sample of centrifuged (10 min at 2500 *g*) urine 20 ml of phosphate buffer, pH 7.0, and 100 μl of the internal standard solution were added, and the pH was adjusted to 6.5 with 1 *M* NaOH and/or HCl. The mixture was applied to a home-packed, short (3 × 0.7 cm I.D.) polyethylene Bio-Rex column (Bio-Rad) plugged with a frit. After washing with 10 ml of water, 1.1 ml of 0.7 *M* H_2SO_4 was added, and the CAs were eluted with 4 ml of 2 *M* $(NH_4)_2SO_4$ into glass tubes of 5-ml volume containing 50 mg acid-washed alumina. To the eluate 500 μl of Tris-EDTA buffer (pH 8.6) was added, and the mixture was shaken vigorously on a rotary mixer for 10–15 min. The supernatant water layer was aspirated. After the alumina was washed by shaking vigorously with 3 ml of distilled water, the CAs were eluted with 160 μl of 0.1 *M* $HClO_4$ on a rotary mixer for 10–15 min. After centrifugation for 10 min at 2000 *g*, an aliquot of 20 μl of the eluate was injected onto the chromatographic system.

On-line method

The urine sample was centrifuged (10 min at 2500 *g*) and an aliquot of 200 μl was mixed with 50 μl of diammonium hydrogen phosphate (pH 8.3) and 25 μl of the internal standard solution. Thereafter 200 μl of the mixture was loaded onto the TEC. This was followed by 2 ml of 0.01 *M* diammonium hydrogen phosphate (pH 8.3). The analytes retained on the TEC were eluted onto the analytical column by the HPLC mobile phase. The TEC was then reequilibrated with 200 μl of 1 *M* sulphuric acid followed by 800 μl of diammonium hydrogen phosphate (pH 8.3).

With each series of samples four calibration

standards were analysed. The CAs in urine were quantitated by the peak-height ratio method with 3,4-dihydroxybenzylamine as the internal standard. The peaks were identified by their retention times. CA excretion over the 24-h period was calculated by multiplying the determined concentrations in μM by the total urine volume. Urine Control Normal and Abnormal from Bio-Rad were used for within-run and between-run accuracy and precision studies. The control at the normal concentration level was injected after every five samples during an overnight run and the control at the abnormal concentration level once.

3. Results and discussion

The combination of cation-exchange resin and alumina has been shown to produce the most sensitive and specific chromatograms for urinary CAs [14], and the combination of cation- and anion-exchange resins for urinary MNs [12]. As sample pretreatment and enrichment prior to injection onto the chromatograph is one of the most time-consuming steps of these methods, involving considerable sources of error, an on-line method was used in the present study as a screening technique for the detection of pheochromocytoma. With a strong cation-exchange polymer as solid-phase extraction material simultaneous on-line measurements of both CAs and MNs in urine were possible. Normal and abnormal urine control samples were analysed by manual and on-line methods and the values were compared with suggested reference ranges. The results show acceptable precision and accuracy for all CAs with both methods. The analysed values for MN by the on-line method were within the reference range while those for NMN were slightly below the limits. The within- and between-run coefficients of variation (C.V.s) obtained by both methods were less than 10% (Table 1).

Fig. 1 shows a chromatogram of CAs from a urine specimen obtained from a healthy adult using the manual dual extraction method. For the between-run precision, analysed during two months by this procedure, the C.V.s at physiological concentrations were less than 7%. The

Table 1
Within- and between-run precision of the manual and on-line methods

Compound	Manual method				On-line method			
	n	x (μM)	C.V.(%)		n	x (μM)	C.V. (%)	
			Within-run	Between-run			Within-run	Between-run ($n = 4$)
Lyphochek normal								
NE	20	0.25	4.2	4.2	8	0.21	5.1	5.4
E	20	0.10	2.3	3.9	8	0.08	5.7	9.7
DA	20	0.84	5.3	6.5	8	0.97	5.6	5.8
NMN	–	–	–	–	5[a]	1.25	6.5	7.3
MN	–	–	–	–	5[a]	0.44	8.7	8.7
Lyphochek abnormal								
NE	20	1.14	3.7	6.1	5	1.10	5.3	5.5
E	20	0.43	2.1	5.2	5	0.41	5.3	6.5
DA	20	2.85	4.9	6.2	5	2.45	5.7	6.1
NMN	–	–	–	–	5[a]	4.10	7.5	7.1
MN	–	–	–	–	5[a]	1.73	7.9	8.4

[a]Hydrolysed samples.

Fig. 1. Typical chromatogram of a urine sample from a healthy adult assayed by the manual HPLC method. Volume of extract injected: 20 μl. The detector potentials were set at +0.35 V, +0.05 V, and −0.26 V for the conditioning cell, detector 1, and detector 2, respectively.

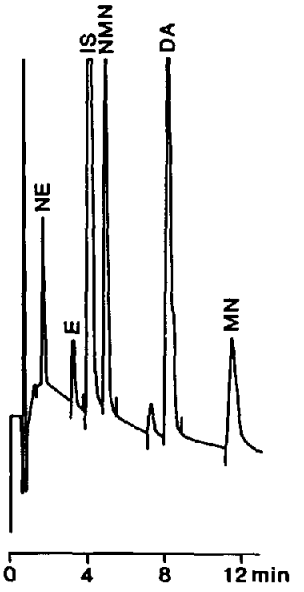

Fig. 2. Chromatogram of a urine sample of a patient with pheochromocytoma assayed by the on-line HPLC method. The detector potentials were set at +0.90 V, +0.10 V, and +0.45 V for the guard cell, detector 1, and detector 2, respectively.

recoveries were assessed by adding known amounts of standards at four different concentrations to urine from seven individuals. The added amounts ranged from 10 to 150 μg/l. The spiked urines were carried through the entire procedure. The results obtained were compared to those obtained with the original samples. The recoveries of added CAs ranged from 87 to 102%. Concentrations of 25 nmol/l NE, 40 nmol/l E, and 65 nmol/l DA could be quantitated with C.V.s less than 9%, at a signal-to-noise ratio of 3. The procedure was found to be linear at least up to 5 μmol/l for each CA.

The results for NE, E, and DA with the on-line method were in agreement with the reference ranges given by Bio-Rad: 0.21 μ*M* (0.16–0.26 μ*M*), 0.08 μ*M* (0.060–0.099 μ*M*) and 0.97 μ*M* (0.85–1.08 μ*M*) for the normal control and 1.10 μ*M* (0.983–1.291 μ*M*), 0.41 μ*M* (0.383–0.470 μ*M*) and 2.45 μ*M* (2.602–3.10 μ*M*) for the abnormal control, respectively. Values for normal and abnormal urine levels of total NMN showed slightly lower levels compared to the manufacturer's report: 1.25 μ*M* (1.78–2.09 μ*M*) and 4.10 μ*M* (5.5–6.5 μ*M*), respectively, while those for MN were within the reference ranges: 0.44 μ*M* (0.37–0.54 μ*M*) and 1.73 μ*M* (1.7–2.3 μ*M*), respectively. When the chromatographic conditions stated in the previous report [12] were used, the values for NMN were also within acceptable limits. The within- and between-run C.V.s were all less than 10% (Table 1). Recovery was measured by comparing spiked aliquots of the urine pool at two levels to direct injections of standards containing known quantities of CAs and MNs. The calculated recoveries were in the range of 90–107% for all analytes. The limits of quantitation for CAs and MNs were 25 nmol/l and 0.2 μmol/l, respectively, and the method was linear at least with concentrations up to 2.5, 0.5, 15, 17 and 6 μmol/l for NE, E, DA, NMN and MN, respectively.

As shown in Table 2, the excretion of all CAs (analysed with the manual method) increased with age when expressed in μmol, and slightly decreased when expressed in μg/mg of creatinine.

Advancements in methodology for catechol-

Table 2
Excretion limits for free CAs in 24-h urine of normal children and adults

Compound	Excretion limit				
	0–2 years ($n = 10$)		3–16 years ($n = 10$)		Adults ($n = 20$)
	μmol	μg/mg of creatinine	μmol	μg/mg of creatinine	μmol
NE	0.10	0.07	0.30	0.06	0.60
E	0.03	0.02	0.06	0.01	0.10
DA	1.00	0.58	2.50	0.52	5.00

amine assays have enabled increased understanding of the adrenergic mechanisms. The on-line method which allows simultaneous analysis of catecholamines and metanephrines might be of interest for the diagnosis of neural crest tumours and also for the detection of abnormalities in catecholamine metabolism.

4. Acknowledgements

The author is grateful to Michel Palu and Heikki Sutinen from Polymer Labs. Associates for generously supplying the ASTED apparatus for this study.

4. References

[1] G.A. Smythe, G. Edwards, P. Graham and L. Lazarus, *Clin. Chem.*, 38 (1992) 486.
[2] T.G. Rosano, T.A. Swift and L.W. Hayes, *Clin. Chem.*, 37 (1991) 1854.
[3] D.F. Davidson, *Ann. Clin. Biochem.*, 24 (1987) 494.
[4] P. Moleman, *Clin. Chim. Acta*, 189 (1990) 19.
[5] I.J. Kopin, *Pharmacol. Rev.*, 37 (1985) 333.
[6] J.R. Crout, J.J. Pisano and A. Sjoerdsma, *Am. Heart J.*, 61 (1961) 375.
[7] B. Kågedal and D.S. Goldstein, *J. Chromatogr.*, 429 (1988) 177.
[8] H. Nohta, E. Yamaguchi, Y. Ohkura and H. Watanabe, *J. Chromatogr.*, 493 (1989) 15.
[9] Y.P. Chan and T.S. Siu, *J. Chromatogr.*, 459 (1988) 251.
[10] N.G. Abeling, A.H. van Gennip, H. Overmars and P.A. Voûte, *Clin. Chim. Acta*, 137 (1984) 211.
[11] B. Green, J.D.H. Cooper and D.C. Turnell, *Ann. Clin. Biochem.*, 26 (1989) 361
[12] P. Volin, *J. Chromatogr.*, 578 (1992) 165.
[13] A.H. Anton and D.V. Sayre, *J. Pharmacol. Exp. Ther.*, 138 (1962) 360.
[14] A.H.B. Wu and T.G. Gornet, *Clin. Chem.*, 31 (1985) 298.