Journal of Chromatography, 377 (1986) 145—154
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3044

DETERMINATION OF FREE AND TOTAL CATECHOLAMINES AND SALSOLINOL IN URINE BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AFTER A ONE-STEP SAMPLE CLEAN-UP

J. ODINK*, H. SANDMAN and W.H.P. SCHREURS

Department of Clinical Biochemistry, TNO-CIVO Toxicology and Nutrition Institute, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

(First received October 4th, 1985; revised manuscript received November 8th, 1985)

SUMMARY

A simple method is presented for the routine determination of free and total catecholamines and salsolinol in urine. For the analysis of total catecholamines and salsolinol, the urine samples are first hydrolysed. The compounds are isolated by a one-step sample clean-up by cation-exchange chromatography and separated by ion-pair reversed-phase liquid chromatogaphy and detected electrochemically. The method is suited for routine analysis. Pretreatment of ten samples for the high-performance liquid chromatographic analysis of free and total catecholamines and salsolinol is carried out in 4 h. A single analysis is completed in 30 min. The absolute losses of the compounds were less than 10%, except for epinephrine (20%). The detection limits were 0.9, 2.4, 4.8 and 6 nmol/l for norepinephrine, epinephrine, dopamine and salsolinol, respectively, in native urine. The values were twice as high for hydrolysed urine. The sample clean-up appears to be rather specific: apart from catecholamines and salsolinol, only those structurally related amines that contain a vicinal hydroxyl group are isolated.

INTRODUCTION

Dopamine (DA), norepinephrine (NE) and epinephrine (E) play an important role in the central and peripheral nervous system. Clinically, they are important for the diagnosis of tumours excreting these compounds [1]. Furthermore, they have been studied in relation to essential hypertension [2]. Recently, evidence has been presented to the effect that, apart from the concentrations of free catecholamines, the levels of the conjugated compounds

0378-4347/86/$03.50

may also provide a clinically useful biochemical index for categorizing hypertension and following its treatment [3].

During the past few years, many methods using high-performance liquid chromatography (HPLC) and electrochemical or fluorimetric detection have been described for the analysis of free catecholamines in urine. Only a few were suitable for the analysis of total (free + conjugated) catecholamines in hydrolysed urine samples, although significant losses of the compounds were reported due to hydrolysis and/or sample clean-up [4, 5].

The purpose of this study was to develop a method for the analysis of both free and total catecholamines, using a simple sample clean-up with minimal losses of the compounds of interest. Special attention has been paid to possible interference by structurally related compounds. In the HPLC elution profiles of the majority of hydrolysed urine samples, salsolinol (SAL), a metabolite of DA with α-adrenergic agonist activity, was detectable. Its formation as a consequence of alcohol consumption has been suggested [6]. Therefore, we thought it worthwhile to include SAL in the assay.

EXPERIMENTAL

Apparatus

HPLC analyses were performed using a system incorporating a Perkin Elmer Series 10 pump, a Perkin Elmer ISS 100 autoinjector and a Bioanalytical Systems amperometric detector, consisting of an LC-4B control unit, a TL-5A amperometric flow-cell with glassy carbon, stainless-steel and Ag/AgCl electrode as working, auxilliary and reference electrode, respectively (Perkin Elmer, Gouda, The Netherlands). The injector and detector were connected on-line to a Perkin Elmer 3230 Labdata system equipped with LIMS/CLAS software.

Stainless-steel Hyperchrome HPLC columns, 7.5 cm × 4.6 mm I.D. (Salm & Kipp, Breukelen, The Netherlands), were packed with ODS-Hypersil 3 μm (Shandon Southern Products, Astmoor, U.K.) at 63 MPa by the balanced-density slurry technique, on a column-packing installation designed at our institute, using a Haskel pump Type DSTV-150 (Ammann Technik, Stuttgart, F.R.G.).

Chemicals

Norepinephrine, epinephrine, 3,4-dihydroxybenzylamine (DHBA) and dopamine were obtained from Sigma (St. Louis, MO, U.S.A.). Salsolinol was a gift from Hoffmann-La Roche (Mijdrecht, The Netherlands) and α-methyldopamine (α-MDA) from Merck, Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.). Other catecholamine metabolites and related compounds were also obtained from Sigma. 1-Decylsulphonate was obtained from Aldrich Europe (Beerse, Belgium). Columns, prefilled with cation-exchange resin (Bio-Rex® 70, 4 cm × 0.8 cm I.D.), were from the catecholamine kit (No. 4107803) supplied by Bio-Rad (Utrecht, The Netherlands).

Collection, storage and treatment of urine samples

Urine samples were collected in polyethylene containers. The pH was

adjusted to 4 with concentrated hydrochloric acid. Samples were stored at −20°C. Before the analysis, the samples were thawed and centrifuged for 5 min at 2000 *g* to remove solid particles.

Samples for the analysis of the free compounds were treated as follows. To 5 ml of urine, 30 μl of DHBA (100 μmol/l) and 15 ml of Na_2H_2EDTA (1 g/l) were added. The pH was then raised to 6.50 ± 0.05 with sodium hydroxide (0.5 mol/l).

To hydrolyse the conjugates, 30 μl of DHBA (100 μmol/l), 100 μl of a solution containing sodium ascorbate (50 g/l) and Na_2H_2EDTA (50 g/l), and 250 μl of hydrochloric acid (12 mol/l) were added to 2.5 ml of urine (final pH of 1). The samples were then heated in capped glass vials at 100°C for 20 min, unless indicated otherwise. After cooling, 15 ml of Na_2H_2EDTA (1 g/l) were added and the pH was raised to 6.50 ± 0.05 with sodium hydroxide (0.5 mol/l).

Samples thus pretreated were poured onto a cation-exchange column. After washing with distilled water (2 × 10 ml), the compounds were eluted with 5 × 3 ml of boric acid (40 g/l) and collected in tubes containing 0.15 ml of glacial acetic acid.

HPLC separation and detection

Analyses of catecholamines, metabolites and analogues were carried out by injecting 50-μl aliquots (solutions containing a mixture of reference compounds or boric acid eluates) into the ODS-Hypersil 3-μm column. The column was eluted isocratically at a flow-rate of 1.8 ml/min with a solution containing $(NH_4)_2SO_4$ (5 g/l), acetic acid (10 ml/l), Na_2H_2EDTA (0.5 g/l), 1-decyl-sulphonate (0.5 g/l) and methanol (10 ml/l), pH 2.9. The effluent was monitored electrochemically at a potential of +550 mV vs. Ag/AgCl. For the analysis of 3-O-methylated precursors and metabolites (Table III) a potential of +730 mV vs. Ag/AgCl was used.

Concentrations of catecholamines and SAL were calculated from peak heights. DHBA was used as internal standard (I.S.).

RESULTS

Applicability of the procedure

Typical elution profiles of a standard solution and of a native and a hydrolysed pooled urine sample are shown in Fig. 1. The HPLC procedure provided an excellent separation of the compounds of interest.

The electrochemical response was linear up to at least 75 pmol for NE and E and 450 pmol for DA and SAL. Assuming a signal-to-noise ratio of at least 3, the detection limits of the assay described in this paper were 15, 40, 80 and 100 fmol for NE, E, DA and SAL, respectively (Fig. 2). This corresponds to 0.9, 2.4, 4.8 and 6 nmol/l for NE, E, DA and SAL, respectively, in native urine. Since half the volume was used, these values are twice as high for hydrolysed urine. In practice, these limits suffice for catecholamine determinations in 24-h urine. Generally, SAL is detectable in hydrolysed samples. It can be detected occasionally in native urine, indicating that the compound is present mainly in the urine as a conjugate.

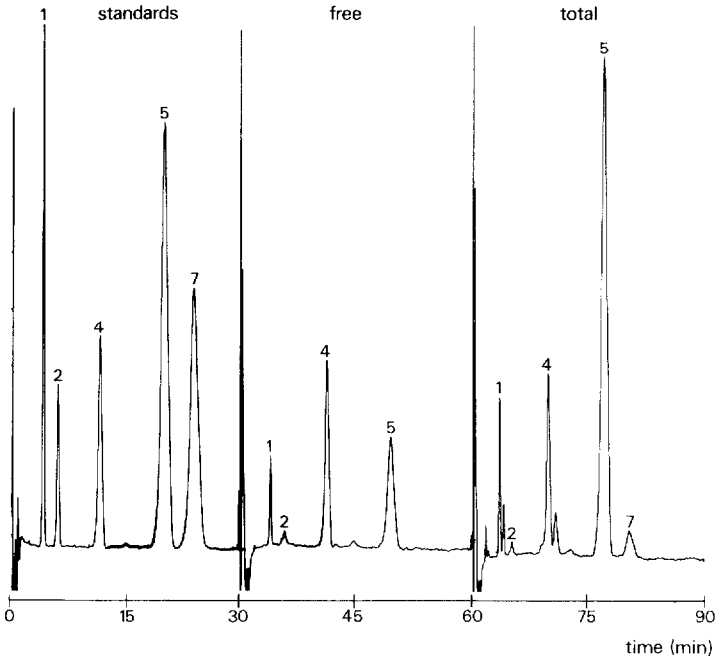

Fig. 1. HPLC elution profiles of a mixture of reference compounds and of boric acid eluates from 5 ml of native and 2.5 ml of hydrolsed pooled urine. Peaks: 1 = NE; 2 = E; 4 = DHBA; 5 = DA; 7 = SAL.

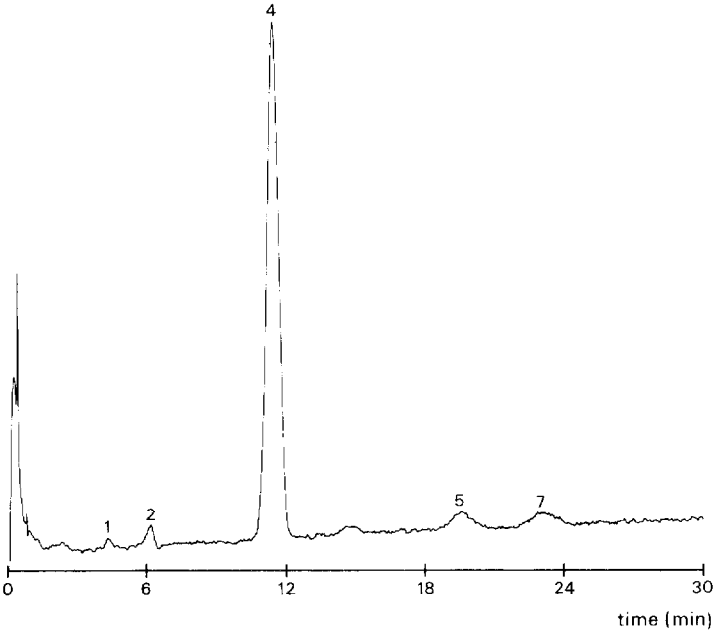

Fig. 2. HPLC elution profile of NE, E, DA and SAL at the detection limits (15, 40, 80 and 100 fmol, respectively). Peaks: 1 = NE; 2 = E; 4 = DHBA (3 pmol); 5 = DA; 7 = SAL.

Optimization of the hydrolysis conditions

Catecholamines and SAL are easily oxidized. In order to prevent the losses, sodium ascorbate was added to the incubation medium as a reductant. As shown in Fig. 3, maximal values were obtained between 10 and 30 min heating (pH 1, 100°C) for the catecholamines and SAL. The concentration of DHBA added to the urine remained constant during this period. Prolonged heating caused small losses of NE. For routine analysis, a heating period of 20 min was chosen. Hydrolysis without the addition of a reductant resulted in significant losses of the compounds.

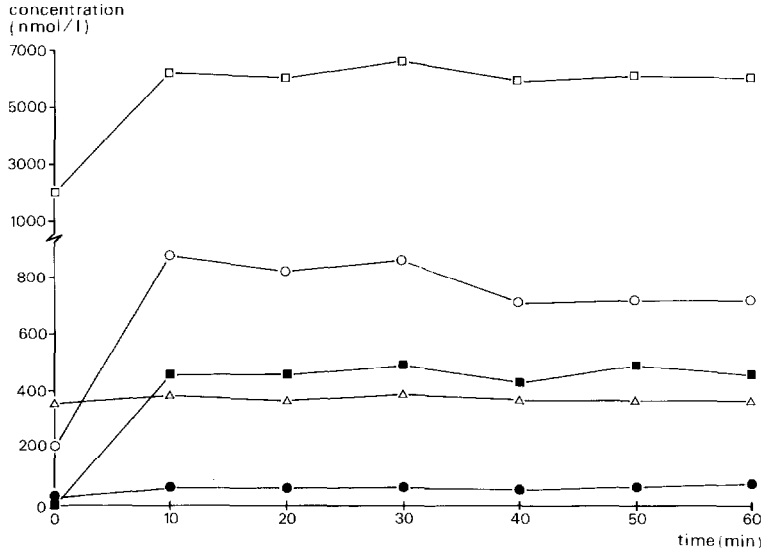

Fig. 3. Time course of acid hydrolysis (pH 1.0) at 100°C of pooled urine. (○) NE; (●) E; (△) DHBA; (□) DA; (■) SAL.

Recovery and precision

Recoveries were determined by the addition of known amounts of the free compounds to urine samples of healthy individuals. The results (not adjusted for the I.S.) are summarized in Table I. The analysis of the free compounds showed absolute recoveries of at least 95%. Hydrolysis hardly affected these values except for the value of E, for which an overall loss of 20% was observed.

To determine the precision of the assay, a pooled urine sample was analysed on different days, in duplicate, under routine conditions. Precision was evaluated from the analysis of variance. The results (adjusted for the I.S.) are presented in Table II.

Interference by structurally related compounds

The described analysis was tested for possible interferences from structurally related compounds. For this purpose, the chromatographic behaviour of a number of catecholamine metabolites and analogues on the cation-exchange column was investigated. The compounds in the eluates were separated by HPLC and detected electrochemically. The results are summarized in Table III.

TABLE I

RECOVERIES OF THE COMPOUNDS ADDED TO URINE SAMPLES OF HEALTHY INDIVIDUALS

In the assay of the free compounds, the endogenous concentrations were increased by 200, 100, 600, 500 and 500 nmol/l for NE, E, DHBA, DA and SAL, respectively. In the assay of the total compounds, these values were twice as high. The data are not adjusted for the I.S.

Assay	Compound	Recovery (mean ± S.D.) (%)
Free (n = 10)	NE	97.5 ± 4.0
	E	94.9 ± 4.6
	DHBA	95.9 ± 2.9
	DA	96.8 ± 7.2
	SAL	95.0 ± 4.7
Total (n = 10)	NE	91.8 ± 2.6
	E	79.3 ± 5.0
	DHBA	94.7 ± 6.5
	DA	93.0 ± 7.2
	SAL	96.3 ± 3.7

TABLE II

PRECISION OF THE ASSAYS FOR FREE AND TOTAL CATECHOLAMINES AND SAL

The data are adjusted for the I.S.

Assay	Compound	Concentration (nmol/l of urine)			
		Mean	$S.D._{between}$	$S.D._{within}$	$S.D._{overall}^{*}$
Free (n = 54)	NE	151	13.1	6.1	14.4
	E	36	3.9	2.5	4.6
	DA	620	85	48	98
Total (n = 17)	NE	502	41	21	46
	E	61	8.8	7.1	11.3
	DA	5748	708	276	760
	SAL	464	68	31	75

*Precision of a single assay: $(S.D._{overall})^2 = (S.D._{between})^2 + (S.D._{within})^2$.

As was to be expected, acid metabolites were not retained by the resin and thus did not interfere with the assay. Similar results were obtained for the alcoholic metabolites and the amino acids.

Under the conditions used, all amines tested were retained by the resin. Only those compounds that contained a vicinal hydroxyl group eluted with boric acid. A good separation of coeluting compounds was observed in the HPLC elution profile (Fig. 4). N-MDA was not baseline-resolved from DA and SAL; however, this compound is not excreted in detectable amounts in urine of normal individuals.

Tetrahydropapaveroline (THP), a condensation product of DA reported to be present in the urine of patients on 3,4-dihydroxyphenylalanine (L-DOPA)

TABLE III

CHROMATOGRAPHIC PROPERTIES OF CATECHOLAMINES AND STRUCTURALLY RELATED COMPOUNDS ON THE CATION-EXCHANGE RESIN

Compound	Amino group	Carboxyl group	Vicinal hydroxyl group	Retained at pH 6.50	Eluted with boric acid
Acid metabolites					
Vanillactic acid	—	+	—	—	N.A.*
Vanilpyruvic acid	—	+	—	—	N.A.
Homovanillic acid	—	+	—	—	N.A.
Vanilmandelic acid	—	+	—	—	N.A.
Vanillic acid	—	+	—	—	N.A.
3,4-Dihydroxyphenylacetic acid	—	+	+	—	N.A.
3,4-Dihydroxymandelic acid	—	+	+	—	N.A.
Alcoholic metabolites					
3,4-Dihydroxyphenylglycol	—	—	+	—	N.A.
3-Methoxy-4-Hydroxyphenyl-glycol	—	—	+	—	N.A.
Amino acids					
3-Methoxytyrosine	+	+	—	—	N.A.
3,4-Dihydroxyphenylalanine	+	+	+	—	N.A.
α-Methyl-3,4-dihydroxyphenyl-alanine	+	+	+	—	N.A.
Amines					
Normetanephrine	+	—	—	+	—
Metanephrine	+	—	—	+	—
3-Methoxytyramine	+	—	—	+	—
Norepinephrine	+	—	+	+	+
Epinephrine	+	—	+	+	+
α-Methylnorepinephrine	+	—	+	+	+
3,4-Dihydroxybenzylamine	+	—	+	+	+
Dopamine	+	—	+	+	+
N-Methyldopamine	+	—	+	+	+
Salsolinol	+	—	+	+	+
α-Methyldopamine	+	—	+	+	+
Isoproterenol	+	—	+	+	+
Tetrahydropapaveroline	+	—	+	+	+

*N.A. = Not applicable.

treatment [7], was omitted from Fig. 4 because its retention time exceeded 3 h under our chromatographic conditions.

Isoproterenol, a drug with bronchodilatory activity, also eluted in the boric acid fraction. However, if present in urine, it is separated on the HPLC column.

α-Methyl-DOPA (α-MDOPA), a frequently prescribed antihypertensive drug, was not retained by the resin. Its metabolites, α-MDA and α-methylnorepinephrine (α-MNE), however, eluted in the boric acid fraction. If present, they are well separated from the other compounds in the HPLC elution profiles of native and hydrolysed urine samples (Fig. 5).

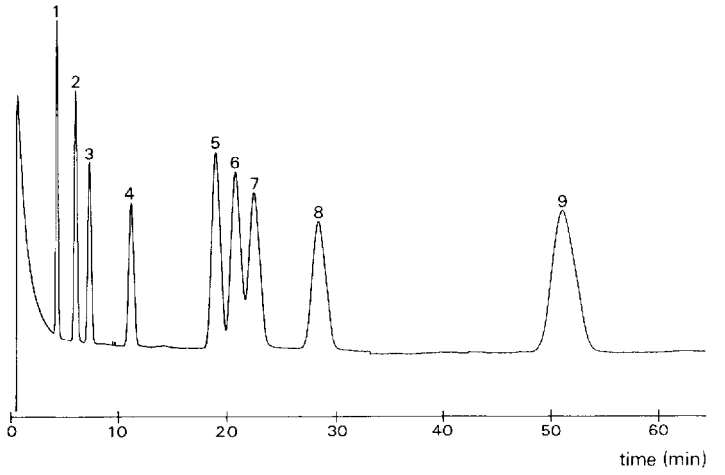

Fig. 4. HPLC elution profile of standards coeluting in the boric acid eluate. Peaks: 1 = NE; 2 = E; 3 = α-MNE; 4 = DHBA; 5 = DA; 6 = N-MDA; 7 = SAL; 8 = isoproterenol; 9 = α-MDA.

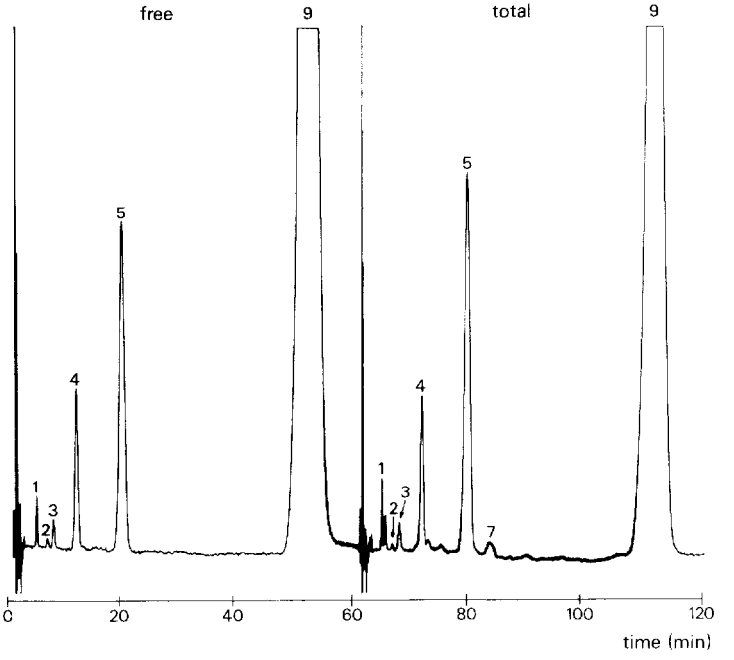

Fig. 5. HPLC elution profiles of boric acid eluates from 5 ml of native and 2.5 ml of hydrolysed urine from a patient using α-MDOPA. Peaks: 1 = NE; 2 = E; 3 = α-MNE; 4 = DHBA; 5 = DA; 7 = SAL; 9 = α-MDA.

DISCUSSION

In the analysis of catecholamines with HPLC, a two-step sample clean-up is usually applied. Recently, procedures have been published using a one-step sample clean-up. Smedes et al. [8] described a rapid and simple method for the

extraction of catecholamines from native urine and plasma with phenylboric acid. In our hands, application of the procedure to the analysis of total catecholamines resulted in interfering HPLC peaks.

Our group [9] reported good results on the analysis of free catecholamines in urine with Affi-Gel® 601 (immobilized boric acid). Preliminary experiments showed that the procedure could also be applied to catecholamines in hydrolysed urine samples. However, in practice, this method appeared to be impracticable owing to variations in the properties of different batches of Affi-Gel 601.

Recent reports [4, 5] indicate that cation-exchange chromatography is suitable as a one-step sample clean-up for HPLC analysis of free and total catecholamines. However, in the analysis of total catecholamines, absolute losses of the compounds were of the order of 20%.

In this study, cation-exchange chromatography was also used. Under the described conditions, catecholamines, SAL and DHBA were retained quantitatively by the resin and eluted almost quantitatively with boric acid. This, in combination with the optimized hydrolysis procedure, reduced absolute losses of all compounds, except for total E, to less than 10%.

The precision observed in this study was lower than generally reported. However, for the evaluation of the data presented, it should be noted that the urine sample analysed was not spiked. Furthermore, the data of all routine analyses performed over a period of five months were used for the calculation of the precision.

The results of our study on the chromatographic properties of catecholamines and structurally related compounds indicate that the sample clean-up on the cation-exchange resin is rather specific: only amines are retained, while a vicinal hydroxyl group seems to be a prerequisite for elution with boric acid. Elution is most likely to be caused by the formation of a complex with borate [10] in the mobile phase. In fact, the extraction properties strongly resemble those of phenylboric acid [8] and immobilized boric acid [9].

As pointed out elsewhere [9, 11], the use of α-MDOPA may interfere with the assay of E, owing to the formation of α-MNA: they have almost the same elution time in the HPLC elution profile. In the procedure presented here, E and α-MNE were separated completely. This was accomplished by the use of 3-μm instead of 5-μm particles and by the use of decylsulphonate instead of the more generally used ion-pair forming agents with a shorter aliphatic chain.

The presence of SAL in human urine has been demonstrated by gas chromatography–mass spectrometry [6]. Although we did not produce an absolute proof, several arguments are in favour of the assumption that the compound eluting after DA in the HPLC profile was SAL. The peak had the same retention time as SAL, it had the same half-wave potential (+415 mV under the conditions used) and it was eluted from the cation-exchange column with boric acid.

We conclude that the combination of the rather specific and easy to handle one-step sample clean-up procedure, the high resolving power of the HPLC system used and the high sensitivity of the electrochemical detection provide a fast and simple method for the determination of free and total catecholamines and salsolinol in urine.

REFERENCES

1 G. Stenström, B. Sjögren and J. Waldenström, Acta Med. Scand., 214 (1983) 145.
2 S. Goldstein, Hypertension, 5 (1983) 86.
3 S.R. Snider and O. Kuchel, Endocr. Rev., 4 (1983) 291.
4 E.D. Schleicher, F.K. Kees and O.H. Wieland, Clin. Chim. Acta, 129 (1983) 295.
5 N.G. Abeling, A.H. Van Gennip, H. Overmars and P.A. Voûte, Clin. Chim. Acta, 137 (1984) 211.
6 F. Bloom, J. Barchas, M. Sandler and E. Usdin (Editors), Beta-carbolines and Tetrahydroisoquinolines, Proceedings of a Workshop, La Jolla, CA, December 12–13, 1981, Alan R. Liss, New York, 1982.
7 M. Sandler, S.B. Carter, K.R. Hunter and G.M. Stern, Nature, 41 (1973) 439.
8 F. Smedes, J.C. Kraak and H. Poppe, J. Chromatogr., 231 (1982) 25.
9 A.J. Speek, J. Odink, J. Schrijver and W.H.P. Schreurs, Clin. Chim. Acta, 128 (1983) 103.
10 S. Higa, T. Suzuki, A. Hayas, I. Isuge and Y. Yamamura, Anal. Biochem., 77 (1977) 18.
11 D.F. Davidson and J. Fitzpatrick, Ann. Clin. Biochem., 22 (1985) 297.