Journal of Chromatography, 452 (1988) 409–418
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 793

IMPROVED SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION OF URINARY CATECHOLAMINES AND 5-S-L-CYSTEINYL-L-DOPA

TIEHUA HUANG*, JEFFREY WALL and POKAR KABRA*
Department of Laboratory Medicine, University of California, San Francisco, CA 94143 (U.S.A.)

SUMMARY

We describe a rapid, precise, accurate liquid chromatographic procedure for determining urinary catecholamines and 5-S-L-cysteinyl-L-dopa. The catecholamines (norepinephrine, epinephrine, and dopamine) and 5-S-L-cysteinyl-L-dopa are extracted from 1.0 ml of urine together with internal standards, by using a Bond-Elut strong cation-exchange (SCX) and an affinity phenylboronic acid (PBA) extraction column in series. The eluate obtained from PBA column is then chromatographed on a reversed-phase C_{18} column with a mobile phase containing pentane- and heptanesulfonate as ion-pair reagents. The detection is achieved with an amperometric detector set at an oxidation potential of +0.55 V. The chromatography is complete in less than 8 min for catecholamines and less than 5 min for cysteinyldopa. The method can measure less than 2 μg/l for catecholamines and 5 μg/l for cysteinyldopa. Analytical recoveries of catecholamines and cysteinyldopa added to urine pool ranged from 90–107%. Between run coefficient of variation ranged from 4.7 to 8%. None of the drugs and catecholamines metabolites tested interfered with the assay.

INTRODUCTION

Laboratory measurements of norepinephrine, epinephrine, dopamine and/or their metabolites are primarily useful for the diagnosis of catecholamine-secreting neurochromaffin tumors (pheochromocytomas, neuroblastomas), metabolic disorders, manic-depressive psychoses, and essential hypertension. The neurochromaffin tumors may produce excessive amounts of catecholamines or their metabolites[1]. Measurement of urinary catecholamines and vinylmandelic acid is best applied to follow-up testing in patients with an elevated metanephrines or those subjects highly suspect for pheochromocytoma[2]. Urinary 5-S-L-cysteinyl-L-dopa was recently evaluated as tumor marker for malignant melanoma and found to be a good estimate of the increased tumor burden in patients with metastases[3].

Liquid chromatography with electrochemical detection (LC–ED) has gained

* Visiting scientist from Railway Medical College, Nanjing, China.

0021-9673/88/$03.50

wide acceptance as a preferred method for the measurement of urinary catecholamines and 5-S-L-cysteinyl-L-dopa[4,5]. A number of procedures utilizing cation exchange resin[6], boric acid gel[7], alumina[8], alumina and boric acid gel[9], Bond-Elut cation-exchange column[10], and Sep-Pak extraction cartridges[11] have been used for the isolation of catecholamines from urine and plasma. A single-step extraction procedure using alumina, cation-exchange columns, or boric acid gel has proved inadequate, because endogenous peaks interfered with the catecholamine analysis[12,13]. As a result, current LC–ED methods generally require elaborate, tedious, and time consuming sample preparation steps for LC–ED[14].

Here, we describe a solid phase extraction method employing bonded phase sorbents for the isolation of catecholamines and 5-S-L-cysteinyl-L-dopa from urine. The selective isolation of catecholamines was accomplished by using two different kinds of Bond-Elut columns (strong cation exchange, and affinity mode). This chromatographic mode sequencing resulted in the selective isolation of catecholamines and 5-S-L-cysteinyl-L-dopa. The urine sample is first passed through a strong cation-exchange (SCX) Bond-Elut column which selectively retains the cationic species including catecholamines and its basic metabolites from the urine. This is followed by the elution of cationic catecholamines onto an affinity phenylboronic acid (PBA) column, where covalent bond formation with the *cis*-diol group of catecholamines takes place in a neutral or alkaline medium. Therefore, the PBA column selectively retains the *cis*-diol containing catecholamines, while other polar and non-polar cationic constituents are washed off with aqueous or polar organic solvent washes. This relatively simple, rapid and specific sample preparation scheme has dramatically improved the quality of chromatographic analysis.

EXPERIMENTAL

Apparatus

Analysis was performed on a Series 3B (Perkin-Elmer, Norwalk, CT, U.S.A.) liquid chromatograph equipped with a Model 7105 (Rheodyne, Cotati, CA, U.S.A.) injector equipped with a 150-μl sample loop, a LC-4B electrochemical detec tor (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Model BD 41 (E and K Scientific Products, Saratoga, CA, U.S.A.) recorder, and a 150 × 4.6 mm Ultrasphere ODS 5 μm column (Beckman, San Ramon, CA, U.S.A.) maintained at 35°C in a Model LC 100 column oven (Perkin-Elmer). A Vac-Elut apparatus and Bond-Elut SCX and Bond-Elut PBA extraction columns containing 100 mg of respective bonded phases were obtained from Analytichem International, Harbor City, CA, U.S.A. The mobile phase for catecholamine analysis was prepared by dissolving 300 mg of heptanesulfonate, 100 mg of disodium salt of EDTA, 13.8 g of $NaH_2PO_4 \cdot H_2O$ (0.1 *M*), and 15 ml of acetonitrile in 1 l water. The pH of the mobile phase was adjusted to 3.0 with 0.1 *M* phosphoric acid. The mobile phase for 5-S-L-cysteinyl-L-dopa was prepared by dissolving 100 mg of pentanesulfonate, 50 mg of disodium EDTA, 2 ml of diethylamine, and 20 ml of acetonitrile in 1 l of water. The pH of the mobile phase was adjusted to 2.2 with 0.1 *M* phosphoric acid. The flow-rate for both analyses was set at 1.0 ml/min.

Reagents and standards

All inorganic reagents were analytical grade. Methanol and acetonitrile (HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Deionized water

was used for preparing reagents and solutions. The saturated sodium carbonate solution was prepared by adding 30.0 g of sodium carbonate to 100 ml of water and shaking it vigorously. The 1 *M* perchloric acid solution was prepared by adding 43 ml of 11.6 *M* perchloric acid to 457 ml water. The 1 *M* hydrochloric acid solution was prepared by adding 42 ml of 12 *M* hydrochloric acid to 458 ml of water. The 1 *M* sodium hydroxide solution was prepared by dissolving 4 g of sodium hydroxide into 100 ml of water. The 1 *M* dipotassium hydrogenphosphate buffer was prepared by dissolving 174 g of dipotassium hydrogenphosphate to 1 l of distilled water.

The stock norepinephrine (300 μg/ml), epinephrine (100 μg/ml), dopamine (900 μg/ml), and the internal standard 3,4-dihydroxybenzylamine (100 μg/ml) were prepared by dissolving 36.5 mg of norepinephrine hydrochloride, 18.2 mg of epinephrine bitartrate, 111.1 mg of dopamine hydrochloride, and 15.8 mg of 3,4 dihydroxybenzylamine hydrobromide (Sigma, St. Louis, MO, U.S.A.) in 100 ml of 0.1 *M* hydrochloric acid, respectively. These standards are stable at 4°C for six months. The working standards of norepinephrine, epinephrine, dopamine, and internal standard containing 6.0 μg/ml of norepinephrine, 2.0 μg/ml of epinephrine, 18.0 μg/ml of dopamine and 2.0 μg/ml of internal standard were prepared in 0.1 *M* hydrochloric acid. The urine standards were prepared by adding 10, 20, 30 and 50 μl of norepinephrine, epinephrine and dopamine working standard solutions to increase the urine concentrations by 60, 120, 180 and 300 ng/ml for norepinephrine; 20, 40, 60 and 100 ng/ml for epinephrine; and 180, 360, 540 and 900 ng/ml for dopamine.

Synthesis and purification of 5-S-L-cysteinyl-L-dopa and 5-S-D-cysteinyl-L-dopa

The procedure of Agrup *et al.*[15] was modified for the synthesis of 5-S-L-cysteinyl-L-dopa and 5-S-D-cysteinyl-L-dopa. The progress of the reaction was monitored by LC set up for measuring 5-S-L-cysteinyl-L-dopa, since both L-dopa and cysteinyldopa are detectable by electrochemical detector. The synthesis was carried in a 50 ml beaker to which 2.5 mg of tyrosinase (Sigma), 7.5 mg of L-dopa, and 10 mg of either L-cysteine or D-cysteine, were added as required. To the reaction mixture, 10 ml of 0.5 *M* potassium dihydrogenphosphate buffer at pH 6.5 was added and mixture stirred with a magnetic mixer. At approximately 10-min intervals, a 10-μl aliquot of this mixture, was removed and diluted with 10 ml of water, and a 10-μl aliquot was injected onto the LC. The L-dopa eluted just before cysteinyldopa. As the reaction progressed towards completion, the intensity of cysteinyldopa peak increased while L-dopa peak gradually decreased. When the reaction was complete, no more L-dopa was detectable by the LC system.

The cysteinyldopa was isolated from the other components of synthesis mixture by passing it through the PBA affinity columns. The PBA columns have affinity for cysteinyldopa and L-dopa. However, because all the L-dopa had been consumed by the excess cysteine, only cysteinyldopa was eluted from the affinity columns. The cysteinyldopa was isolated from ten PBA columns placed on the Vac Elut chamber, and activated by passing one column volume of methanol and 1 *M* dipotassium hydrogenphosphate buffer. The synthesis mixture (about 1 ml) was passed through each column, which was then washed with 2 volumes of water. Each column was eluted with 500 μl of 0.1 *M* hydrochloric acid and the eluate collected into 10 × 75 mm collecting tubes. The contents of the ten collecting tubes were pooled and an aliquot of the pool was then injected onto LC to asses the purity of cysteinyldopa.

The concentration of cysteinyldopa was calculated from its molar absorptivity of 2800 at 292 nm. The concentrated cysteinyldopa was then diluted to 100 μg/ml with 0.2 *M* hydrochloric acid containing 100 mg/l of ascorbic acid and stored frozen at −20°C until needed.

Standards for 5-S-L-cysteinyl-L-dopa analysis

A reference standard containing a solution of 200 ng/ml of 5-S-L-cysteinyl-L-dopa and 5-S-D-cysteinyl-L-dopa (internal standard) was prepared by adding 100 μl of stock 5-S-L-cysteinyl-L-dopa and 5-S-D-cysteinyl-L-dopa to 50 ml of 0.2 *M* hydrochloric acid containing 100 mg/l of ascorbic acid as preservative. The reference standard was aliquoted into 500 μl portions and frozen at −20 °C for subsequent use. The standards are stable for six months when kept frozen, and two days when kept at room temperature. The working internal standard containing 200 ng/ml of 5-S-D-cysteinyl-L-dopa was prepared by diluting 1 ml of 100 μg/ml of stock internal standard with 500 ml of 0.2 *M* hydrochloric acid containing 100 mg/l ascorbic acid. After mixing, a 0.5-ml volume was aliquoted and frozen at −20°C for subsequent use. The standard is stable for six months, when kept frozen, and two days when kept at room temperature.

Controls. The urine blank was prepared by adjusting the pH of urine above 10 with 1 *M* sodium hydroxide and allowing it to stand for few days at room temperature. This urine was analyzed for endogenous 5-S-L-cysteinyl-L-dopa and usually contained less than 10 ng/ml. For the low control (100 μg/l), 0.5 ml of 100 μg/ml stock 5-S-L-cysteinyl-L-dopa standard was added to 500 ml of blank urine; and for high control (500 μg/l), 2.5 ml of the stock 5-S-L-cysteinyl-L-dopa standard was added to 497.5 ml of blank urine. The controls were aliquoted into 1-ml portions, and frozen and stored at −20°C. The controls are stable for six months.

Specimens. A 24-h urine sample was collected in a container with 10 ml of 6 *M* hydrochloric acid. Catecholamines and 5-S-L-cysteinyl-L-dopa are stable in acidified urine for upto a week if stored at 4°C. Patients should avoid exposure to sunlight as much as possible for two weeks preceding the collection of urine sample due to the increased synthesis of 5-S-L-cysteinyl-L-dopa by exposure to sun light.

Extraction procedure for catecholamines

To 1.0 ml of appropriate standard, control, or unknown urine into labeled 13 × 100 mm disposable glass tube, was added 50 μl of working internal standard (3,4-dihydroxybenzyl amine) solution and 5 ml of water. The pH of the sample was adjusted to 6.5–7.0 with 1 *M* and/or 0.05 *M* sodium hydroxide. The SCX and PBA columns were placed on the top of Vac-Elut chamber and connected to a source of reduced pressure. Both columns were activated by washing them with three column volumes of 1 *M* hydrochloric acid, followed by two column washes of methanol and 0.01 *M* ammonium acetate (pH 7.3). A reservoir was placed above the activated SCX column into which urine was poured and it was connected to the vacuum source. The SCX columns were washed with two column volumes of methanol followed by two column volumes of 0.01 *M* ammonium acetate. The SCX columns were eluted with 3 × 500 μl of 1 *M* perchloric acid. The eluate was neutralized with 400 μl of saturated sodium carbonate, and then transferred to the activated PBA column. The PBA columns were washed with two column volumes of methanol followed by two column

volumes of water. Finally, the PBA columns were eluted with 2 × 500 μl of 0.1 *M* perchloric acid solution. The eluate was mixed, then a 40–80-μl aliquot was injected onto the LC. The column was eluted with the mobile phase at a flow-rate of 1.0 ml/min at 35°C. The detection is achieved with an amperometric detector set at an oxidation potential of +0.55 V. The quantitation of catecholamines is based on the peak-height ratio of analyte to internal standard.

Extraction procedure for 5-S-L-cysteinyl-L-dopa

For each standard, control, and unknown, a SCX column was placed on the Vac-Elut chamber. The columns were activated by passing one volume of methanol and one volume of 0.1 *M* hydrochloric acid. To 0.5 ml of each standard, control, or test sample placed into a 16 × 100 mm tube was added 0.5 ml of the working internal standard. The solution was mixed briefly, then the contents of each tube was transferred onto an activated SCX Bond-Elut column. The columns were washed with one volume of 0.1 *M* hydrochloric acid. The SCX columns were removed from the Vac-Elut chamber. A set of PBA Bond-Elut columns were placed on the Vac-Elut chamber and activated with one volume of methanol and one volume of 1 *M* dipotassium hydrogen phosphate. The SCX columns were then placed in order, on top of the PBA columns, and washed with two column volumes of 1 *M* dipotassium hydrogenphosphate. The SCX columns were removed from the PBA columns, and the PBA columns were washed with water. A set of 10 × 75 mm collecting tubes were placed under the PBA columns and eluted with 0.5 ml of 0.1 *M* hydrochloric acid with 10 mg/l ascorbic acid. The collecting tubes were vortex-mixed, and an aliquot of 20 μl was injected onto the LC. The column was eluted with the mobile phase at a flow-rate of 1.0 ml/min at 35°C. The detection is achieved with an amperometric detector set at an oxidation potential of +0.55 V.

RESULTS

Recovery and linearity

Catecholamines were added to an urine pool in amounts equivalent to 10–600 μg/l for norepinephrine, 4–100 μg/l for epinephrine, and 30–1800 μg/l for dopamine. A constant amount of 3,4-dihydroxybenzyl amine (internal standard) was added to each sample, which was then processed as described above. Concentrations and peak height ratios were linearly related over these ranges. The correlation coefficients were 1.000, 0.995 and 0.998, respectively. Analytical recoveries for catecholamines are given in Table I. Similarly, 5-S-L-cysteinyl-L-dopa was added to an urine blank pool in amounts equivalent to 31–2000 μg/l, which were then processed as described above. Concentrations and peak height ratios were linearily related over this range. The correlation coefficient was 1.000. Analytical recovery for 5-S-L-cysteinyl-L-dopa is given in Table I.

Sensitivity and precision

The minimum limit of detection for the assay is 1 μg/l for norepinephrine and epinephrine, and 2 μg/l for dopamine. The signal-to-noise ratio was >3 at these concentrations. The minimum limit of detection for 5-S-L-cysteinyl-L-dopa is 5 μg/l, at a signal-to-noise ratio of 3. Repeated analysis of pooled urine samples spiked with

TABLE I

RECOVERY OF CATECHOLAMINES AND 5-S-L-CYSTEINYL-L-DOPA FROM URINE

$n = 5$.

Norepinephrine			*Epinephrine*			*Dopamine*			*5-S-L-Cysteinyl-L-dopa*		
Added (μg/l)	*Recovered (μg/l)*	*Recovery (%)*	*Added (μg/l)*	*Recovered (μg/l)*	*Recovery (%)*	*Added (μg/l)*	*Recovered (μg/l)*	*Recovery (%)*	*Added (μg/l)*	*Recovered (μg/l)*	*Recovery (%)*
0*	72.2	0	0*	24.3	0	0*	244	0	31.2	28	90
10	82.2	100	4	30.5	107	30	267	99	62.5	59	94
25	97.0	98	10	32.2	95	180	447	106	125	114	91
60	126	95	20	42.9	97	360	639	106	250	251	100
120	205	107	40	61.8	96	540	777	99	500	489	98
300	374	100	60	79.3	94	900	1062	93	1000	991	99
600	669	99	100	129	103	1800	2063	101	2000	1945	97

* Blank.

catecholamines at two different levels and at the endogenous levels gave within-day coefficients of variation (C.V.) ranging from 2 to 8.3%. The day-to-day C.V. values ranged from 3.4 to 7.4%. Similarly, repeated analysis of pooled urine sample including the endogenous level of 5-S-L-cysteinyl-L-dopa at two different concentrations give within-day C.V. values ranging from 1.5 to 2.5%, while day-to-day C.V. values ranged from 4.7 to 8%.

Interferences

We evaluated potential interference in this assay by chromatographing pure solutions of drugs, catecholamine metabolites and/or samples of urine containing various catecholamines and their metabolites. Drugs tested but not detected under these conditions were: acetaminophen, amitryptyline, caffeine, carbamazepine, chloramphenicol, chlordiazepoxide, diazepam, ethosuximide, gentamicin, imipramine, pentobarbital, phenobarbital, phenytoin, primidone, salicylate, secobarbital, and theophylline. The catecholamine metabolites 3,4-dihydroxyphenylalanine (DL-DOPA), DL-3-O-methyl-DOPA, 4-hydroxy-3-methoxyphenylglycol, DL-α-methyl-DOPA, 3,4-dihydroxymandelic acid, vinylmandelic acid, homovanillic acid, normetanephrine and metanephrine did not interfere with this analysis.

DISCUSSION

For LC analysis of catecholamines and 5-S-L-cysteinyl-L-dopa in urine, a preliminary extraction step is required. For this purpose, various extraction methods utilizing cation-exchange resins, adsorption chromatography on alumina and affinity chromatography on boronic acid gels have been extensively used. However, many of these extraction methods suffer from endogenous interferences and low and erratic recovery of the desired analyte from the urine. Additionally, extraction methods using thin-layer chromatography, ion-exchange resins, and adsorption on alumina are time consuming and require careful evaluation of analytical recovery.

The PBA gels have been used for the isolation of catecholamines by several investigators[9,16,17]. Kagedal and Peterson[17] isolated 5-S-L-cysteinyl-L-dopa from urine by using phenyl boronate gel with good recovery. However, further improvement in the selectivity of extraction procedure was achieved by combining phenylboronate clean-up with a cation exchange extraction. However, the clean-up procedure was slow due to the unstability of bed structure at reduced or positive pressure. Bonded phase silicas are useful material as sorbents for sample preparation for several reasons. They retain rigidity under positive or reduced pressure, thereby maintaining the sorbent bed dimensions. Because of the bed stability, they allow a rapid change in solvent polarity without contraction or swelling of silica. In addition, a wide variety of selective bonded phases can be synthesized on the silica surface for the isolation/extraction of different catecholamines and their metabolites[16]. The key to obtaining reproducible and high recoveries of a desired analyte is to identify the right kind of bonded-phase silica that is most selective for that compound. This selectivity is dependent on the differences in affinity between the desired analyte and interferences in the matrix for the sorbent.

Extraction on bonded-phase silica columns, especially the PBA extraction column, is more selective and efficient than ion-exchange resin clean-up for catechola-

mine isolation. PBA bonded silica in its ionized form binds covalently to compounds containing vicinal *cis*-diol groups such as those found in the catecholamines. The catecholamines are then eluted from the PBA columns with strong acid. The recovery and reproducibility are excellent. There are several advantages to using bonded-phase silica extraction phases over other isolation techniques (alumina-cation-exchange resin, and boronic acid gels) for catecholamines and 5-S-L-cysteinyl-L-dopa. In addition to interference-free analysis, the extraction procedure could be adapted for batch processing by using a Vac-Elut chamber. This vacuum chamber device allowed us to process ten urine samples simultaneously in less than 20 min. Under the extraction conditions described in this report, both SCX and PBA columns can withstand two column volumes of methanol washes, so that the non-polar components could be washed off these columns without affecting the recovery of catecholamines. Another distinct advantage of this extraction protocol is that both extraction columns (SCX and PBA) can be reused for at least eight urine analyses without affecting the recovery ($>90\%$), reproducibility (coefficient of variation $<8\%$), and selectivity of extraction columns. The ion-pair reversed-phase chromatographic analysis of catecholamines and 5-S-L-cysteinyl-L-dopa was optimized by varying the concentration of acetonitrile, ion-pair reagent, and pH of the mobile phase. The chromatograms obtained by these procedures are illustrated in Figs. 1 and 2. The three catecholamines and the

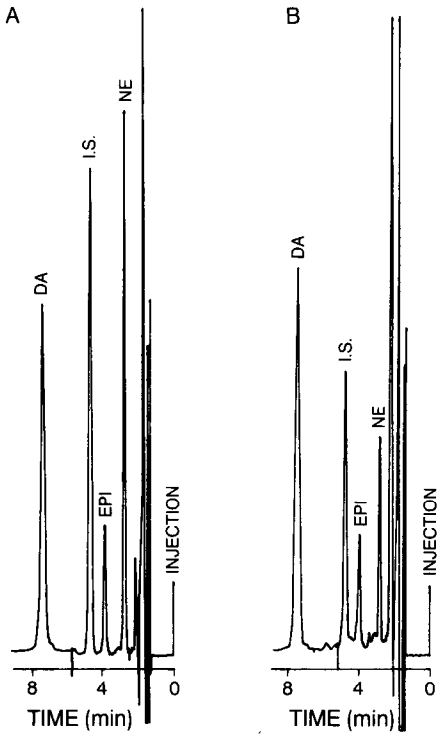

Fig. 1. (A) Chromatogram of urine standard containing 177 μg/l of norepinephrine (NE), 48 μg/l of epinephrine (EPI), and 224 μg/l of dopamine (DA). (B) Chromatogram of urine sample from a patient containing 72.2 μg/l of NE, 24.3 μg/l of E, and 244 μg/l of DA.

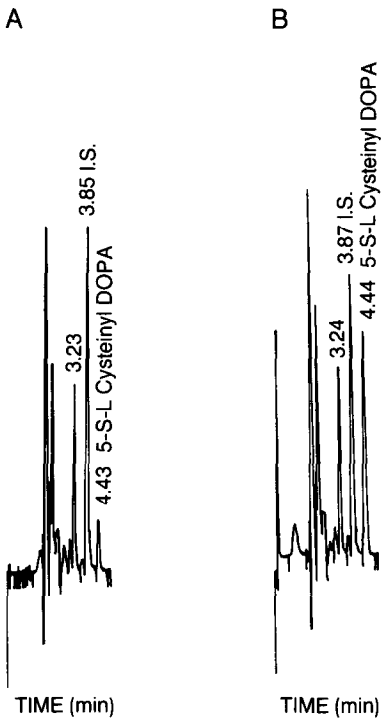

Fig. 2. Chromatograms and of patient urine samples containing (A) 40 μg/l and (B) 198 μg/l of 5-S-L-cysteinyl-L-dopa.

internal standard are separated in less than 8 min. Similarly, 5-S-L-cysteinyl-L-dopa and its internal standard 5-S-D-cysteinyl--L-dopa are separated in less than 5 min.

Wu and Gornet[19] compared three different extraction techniques (PBA, cation-exchange resin, and alumina) for the isolation of catecholamines from urine, and concluded that neither cation-exchange resin or alumina alone is suitable for routine use without altering the chromatographic conditions. Chromatographic interferences were observed for both cation exchange resin and alumina isolation, although the degree of interference varied from sample to sample. Both the PBA, and the combined cation-exchange resin alumina produced chromatograms with minimal interferences in most samples. However, by using both alumina and a cation-exchange step in a clean-up procedure, the recovery of catecholamines is adversely affected.

The use of silica-bonded PBA and SCX extraction columns in series helped solve many of the above mentioned problems. The major advantage of the silica-bonded phases, especially when connected to a vacuum manifold, is the shorter turn around time so that more than 80 samples can be processed in a work day. The chromatographic mode sequence (PBA in series with SCX extraction column) resulted in the selective isolation of catecholamines and 5-S-L-cysteinyl-L-dopa. The combined PBA and SCX isolation scheme described by us is simple, rapid and selective for the extraction and analysis of catecholamines and 5-S-L-cysteinyl-L-dopa.

REFERENCES

1 S. C. Chattoraj and N. B. Watts, in N. B. Tietz (Editor), *Text Book of Clinical Chemistry*, W. B. Saunders, Philadelphia, PA, 1986, p. 997.
2 S. E. Gitlow, M. Mendlowitz and Betrani, *Am. J. Cardiol*, 26 (1970) 270.
3 G. Agrup, P. Agrup, T. Andersson, L. Hafstrom, C. Hansson, S. Jacobsson, P. E. Jonsson, H. Rorsman, A. M. Rosengren and E. Rosengren, *Acta Dermatoverner (Stockholm)*, 59 (1979) 381.
4 A. M. Krstulović, *J. Chromatogr.*, 229 (1982) 1.
5 S. Allenmark, *J. Liq. Chromatogr.*, 5, *Suppl.* 1 (1982) 1.
6 G. M. Tyce, N. S. Sharpless and C. A. Owen, *Biochem. Pharmacol.*, 21 (1972) 2409.
7 A. J. Speek, J. Odink, J. Schrijver and W. H. P. Scheurs, *Clin. Chim. Acta*, 128 (1983) 103.
8 P. T. Kissinger, R. M. Riggin, R. L. Alcorn and L. D. Rau, *Biochem. Med.*, 13 (1975) 299.
9 T. P. Moyer, N. S. Jiang, G. M. Tyce and S. G. Sheps, *Clin. Chem.*, 25 (1979) 256.
10 K. N. Frayn and P. F. Maycock, *Clin. Chem.*, 29 (1983) 1426.
11 D. S. Goldstein, *J. Chromatogr.*, 275 (1983) 174.
12 D. D. Koch and G. L. Polzin, *J. Chromatogr.*, 386 (1987) 19.
13 C. Lucarelli, P. Betto, G. Ricciarello, M. Giambenedeth, F. Sciarra, C. Tosticroce and P. L. Mottironi, *Chromatographia*, 24 (1987) 423.
14 E. Baraldi, D. Cavani, S. Seghedoni, G. Chiossi, L. Roli, A. T. Scacchatti and G. F. Baraghini, *Chromatographia*, 24 (1987) 407.
15 G. Agrup, L. E. Edholm, H. Rorsman and E. Rosengren, *Acta Dermatovener (Stockholm)*, 63 (1983) 59.
16 L. S. Yago and T. J. Good, in P. M. Kabra and L. J. Marton (Editors), *Clinical Liquid Chromatography*, Vol. 1, CRC Press, Boca Raton, FL., 1984, p. 197.
17 B. Kagedal and A. Petterson, *Clin. Chem.*, 29 (1983) 2031.
18 K. Oka, M. Sekiya, H. Osada, K. Fujita, T. Kato and T. Nagatsu, *Clin. Chem.*, 28 (1982) 646.
19 A. H. B. Wu and T. G. Gornet, *Clin. Chem.*, 31 (1985) 298.