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SIMULTANEOUS QUANTITATION OF CATECHOLAMINES AND O-METHYLATED METABOLITES IN URINE BY ISOCRATIC ION-PAIRING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A simple high-performance liquid chromatographic procedure was developed for the simultaneous determination of catecholamines and metanephrines in urine. One-step sample preparation was achieved with Bio-Rex 70 ion-exchange resin. The extract was assayed on a C_{18} reversed-phase column. Dihydroxybenzylamine was used as an internal standard. The eluent was monitored by an electrochemical detector with an oxidation potential of +0.85 V. The use of 1-heptanesulphonic acid in the mobile phase permitted the separation of norepinephrine, epinephrine, dopamine, normetanephrine and metanephrine in a single chromatogram. The corresponding detection limits were 5, 9, 14, 10 and 30 nmol/l, respectively. For the between-day precision, the coefficients of variation at physiological and pathological concentrations were less than 11%. Compounds with similar chemical structures and drugs commonly prescribed for the treatment of hypertension were assayed and found not to cause interferences in the chromatogram. The assay is reliable and is suitable for the analysis of clinical specimens. Reference values were established for normotensive Chinese patients with no neurological or endocrine disorders and also for patients suffering from essential hypertension.

INTRODUCTION

The measurement of the 24-h urinary excretion of cateholamines and their metabolites, metanephrines and vanillylmandelic acid (VMA) is recommended for the diagnosis of pheochromocytoma[1,2]. There is no general agreement as to which analyte is the most important for diagnosis. Several investigators favour VMA[3,4], while others recommend metanephrines[5,6] or norepinephrine[7,8]. A report in which the literature up to 1984 is reviewed indicated that no single urinary test is foolproof for the diagnosis of pheochromocytoma[9]. For every test, false positives have been reported for patients with essential hypertension, and false negatives have been reported for patients with confirmed pheochromocytoma. Some workers[10] recommend that all catecholamines and their metabolites be measured.

The quantitation of catecholamines and metanephrines by high-performance

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liquid chromatography (HPLC) coupled with electrochemical detection (ED) have largely replaced the older spectrophotometric, fluorimetric, radiometric and gas chromatographic procedures. However, most published methods permit the simultaneous measurement of only either norepinephrine (NE), epinephrine (EP) and dopamine (DA)[11,12]; normetanephrine (NM) and metanephrine (MN)[13,14]; or VMA and homovanillic acid (HVA)[15,16]. It would obviously be advantageous if several of these compounds could be assayed with a single sample preparation and chromatographic procedure. In this paper we describe a simple, reliable and rapid procedure for the simultaneous extraction and quantitation of urinary NE, EP, DA, NM and MN.

EXPERIMENTAL

Apparatus

The HPLC system consisted of an M6000A pump and data module (Waters Assoc., Milford, MA, U.S.A.), an LC-4B amperometric detector with a glassy carbon electrode and silver–silver chloride reference electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and an MS830 column heater and MS1660 autosampler (Kontron Instruments, Zurich, Switzerland). Separations were carried out on a 250 × 4 mm I.D. stainless-steel Hibar column, packed with 10-μm LiChrosorb C_{18} (Merck, Darmstadt, F.R.G.).

Chemicals

NE, EP, DA, NM, MN and 3,4-dihydroxybenzylamine hydrochloride (DHBA) were obtained from Sigma (St. Louis, MO, U.S.A.). 1-heptanesulphonic acid from Kodak (Rochester, NY, U.S.A.), acetonitrile from Fisher Scientific (Pittsburgh, PA, U.S.A.) and Bio-Rex 70 cation-exchange resin (100–200 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other chemicals were of analytical-reagent grade or better and were supplied by Merck.

Urine specimens

Twenty-four-hour urine specimens were collected from hospitalized Chinese patients. One group consisted of 40 patients who were normotensive and had no neurological or hormonal disorders. The other group consisted of 17 patients suffering from essential hypertension. Samples were collected in dark glass bottles containing 750 ml of 2 *M* sulphuric acid as a preservative. Sample were refrigerated during collection and thereafter at 4°C. Analysis was performed within 7 days after collection.

Sample preparation

Bio-Rex 70 cation-exchange resin was conditioned and converted to the NH_4^+ form by washing successively with 3 *M* hydrochloric acid, 3 *M* sodium hydroxide solution, 3 *M* acetic acid, 1 *M* ammonium acetate and 0.1 *M* ammonium acetate buffer (pH 6.5). It was buffered to pH 6.5 with 0.1 *M* ammonium acetate and stored at 4°C. The resin was then allowed to reach room temperature, and the slurry was packed into polystyrene tubes of 0.7 I.D. cm to form a resin bed 3 cm high. The columns were washed with 10 ml of deionized water and drained immediately prior to the application of urine.

A 5-ml aliquot of urine was transferred to a scintillation vial and mixed with 50 μl of internal standard (10 mg of DHBA in 10 ml of 0.1 *M* hydrochloric acid), 100 μl of Na_2EDTA solution (0.1 g/ml) and 100 μl of freshly prepared sodium metabisulphite solution (0.1 g/ml). The pH of the mixture was adjusted to 6.5 $\pm$ 0.1 by adding 5, 1 and 0.1 *M* sodium hydroxide solution successively. The mixture was transferred to a Bio-Rex 70 column and allowed to drain. The column was then washed with 10 ml of deionized water and eluted with 7 ml of 4 *M* formic acid. The eluate was collected and a 100-μl aliquot was injected directly into the column.

Chromatographic conditions

A buffer solution, composed of 300 m*M* sodium dihydrogenphosphate, 4 m*M* 1-heptanesulphonic acid and 0.2 g/l Na_2EDTA was titrated to pH 2.5 with orthophosphoric acid, then filtered throuh a 0.22-μm Type HA filter (Millipore, Bedford, MA, U.S.A.). The mobile phase was prepared by mixing 98 parts by volume of the above buffer with 2 parts of acetonitrile. The mobile phase was degassed by sparging with helium for *ca.* 1 min before use. A flow-rate of 2.2 ml/min was used. The column temperature was maintained at 40°C during the analysis. A potential of +0.85 V was applied across the electrode of the electrochemical detector.

Calculations

A set of calibration standard solutions, each containing 5, 20, 80, 200 or 400 μmol/l of NE, EP, DA, NM and MN, was prepared in 0.1 *M* hydrochloric acid and stored at −70°C. Working solutions were prepared by diluting 50 μl of each calibration standard solution with 50 μl of internal standard solution and 6.9 ml of 4 *M* formic acid. The working concentrations corresponded to those found in the eluate obtained from 5 ml of urine, with analyte concentrations in the range 50–4000 nmol/l. Calibration graphs and regression equations were constructed, based on peak-height ratios between catecholamines and the internal standard. The concentrations of catecholamines in urine specimens were calculated from the regression equation after adjusting for the relative recovery between each catecholamine and the internal standard.

Method validation

The absolute recovery was evaluated by adding known amounts of standards and DHBA to pooled urine at concentrations of 50, 200, 800 and 3200 nmol/l. Ten samples from each spiked preparation was extracted by the described procedure, except that the DHBA–4 *M* formic acid solution was replaced with 4 *M* formic acid. The peak heights were compared with those obtained by injecting standards of known concentrations.

The analytical recovery was assessed by adding known amounts of standards at four different concentrations to urine samples from twelve individuals. The results obtained were compared with those obtained with the original samples.

The within-run precision was determined by extracting and assaying replicate samples from three pools of urines with different concentrations of the compounds. The between-day precision was determined at three different concentrations by assaying aliquots of two pools of urine, which were stored at −70°C.

RESULTS AND DISCUSSION

Fig. 1 shows voltammograms obtained with the chromatographic parameters described. A potential of +0.85 V was chosen for maximum signal sensitivity and stability. Injections of standards, prepared in 4 *M* formic acid, indicated that the signals produced by the detector were linear at urine concentrations from 35 to 7000 nmol/l for each analyte (Fig. 2).

The one-step extraction procedure was reliable and reproducible. No significant difference in absolute recovery was observed at the different concentrations examined. The mean absolute recoveries ± the standard error of the mean for NE, EP, DA, NM, MN and DHBA were 79.7 ± 0.88%, 75.0 ± 1.26%, 89.7 ± 1.73%, 74.5 ± 1.42%, 62.1 ± 1.35% and 89.4 ± 0.77%, respectively. Analysis of duplicates urine samples with all five analytes at different concentrations demonstrated that the extraction procedure was linear for concentrations between 100 and 8000 nmol/l.

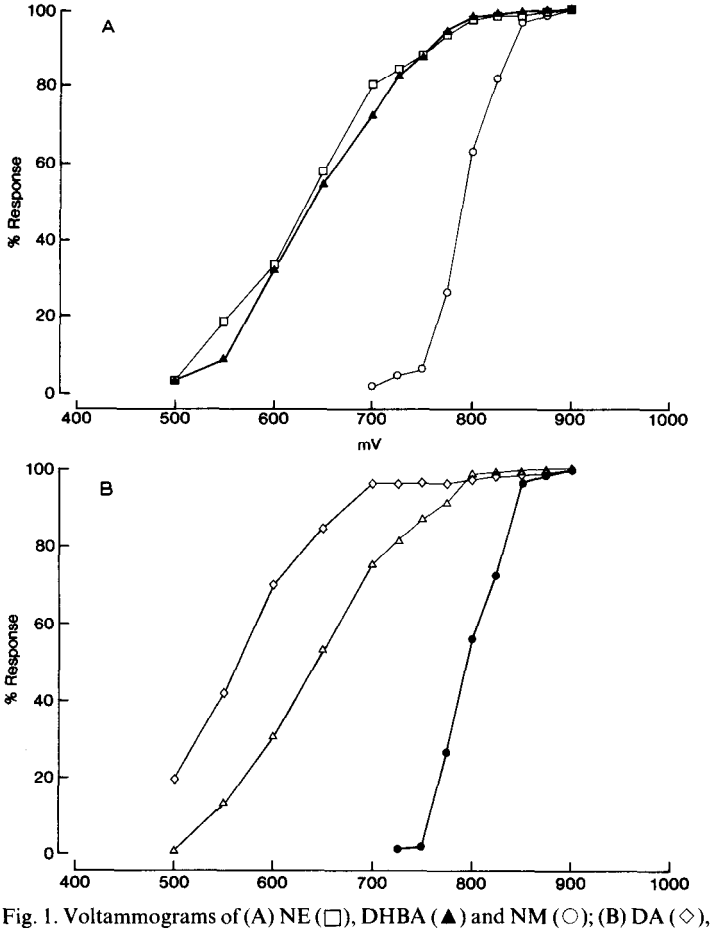

Fig. 1. Voltammograms of (A) NE (□), DHBA (▲) and NM (○); (B) DA (◇), EP (△) and MN (●). Units are expressed as a percentage of the maximum response, obtained at an applied potential of +0.9 V.

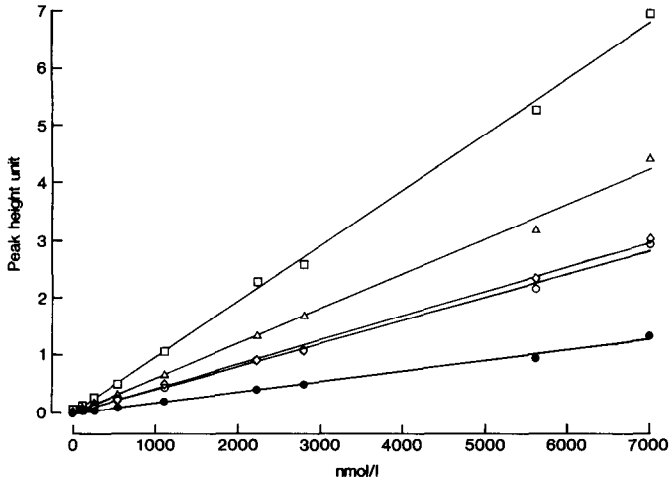

Fig. 2. Linearity of peak-height response relative to urinary concentrations from 35 to 7000 nmol/l. Chromatographic conditions as described in the text. (□) NE; (△) EP; (◇) DA; (○) NM; (●) MN.

The analytical recoveries of the compounds at different concentrations are presented in Table I. The mean analytical recoveries for NE, EP, DA, NM and MN were 99.7, 97.0, 89.5, 99.6 and 102.7%, respectively.

TABLE I

ANALYTICAL RECOVERY

Known amounts of standards were added at four different concentrations to twelve urine samples from different individuals. The results were compared with those obtained with the original specimens.

Compound	*Added concentration (nmol/l)*	*Recovery (%)*	*S.D. (%)*	*n*
NE	50	110.6	5.4	6
	200	96.3	6.4	12
	800	98.5	6.6	12
	2000	97.9	1.1	6
EP	50	90.8	8.9	6
	200	97.5	7.6	12
	800	98.8	3.3	12
	2000	98.7	1.8	6
DA	100	92.2	9.3	6
	400	92.3	9.9	12
	1600	88.5	6.8	12
	4000	86.0	2.4	6
NM	50	118.3	11.8	6
	200	95.2	9.5	12
	800	101.1	10.8	12
	2000	105.6	3.5	6
MN	100	110.5	6.9	6
	400	96.7	12.2	12
	1600	100.2	11.1	12
	4000	93.5	4.4	6

TABLE II

WITHIN-RUN PRECISION

Replicate samples from three pools of urine with different concentrations of compounds were assayed as a batch.

Compound	*n*	*Mean concentration (nmol/l)*	*Coefficient of variation (%)*
NE	10	120	6.6
	6	316	2.7
	6	932	3.7
EP	10	34.4	10.5
	6	224	3.9
	6	825	1.7
DA	10	755	3.9
	6	1121	3.7
	6	4193	2.3
NM	10	147	9.6
	6	329	5.3
	6	1011	3.6
MN	10	75.3	9.2
	6	447	6.6
	6	1665	3.7

Data on within-run and between-day precision are presented in Tables II and III, respectively. The coefficients of variation were all below 11%. A signal-to-noise ratio of 3 was adopted as the detection limit of the assay. The detection limits for NE, EP, DA, NM and MN in urine were 5, 9, 14, 10 and 30 nmol/l, respectively.

TABLE III

BETWEEN-DAY PRECISION

Aliquots of two pools of urine were stored at −70°C. Twenty-two sets were assayed over a period of 60 days.

Compound	*n*	*Mean concentration (nmol/l)*	*Coefficient of variation (%)*
NE	22	297	5.8
	22	799	6.3
EP	22	116	6.4
	22	423	5.4
DA	22	506	5.9
	22	2359	5.8
NM	22	268	10.1
	22	620	8.3
MN	22	151	8.9
	22	667	10.5

Twelve compounds with structures similar to those of catecholamines and drugs commonly prescribed for the treatment of hypertension were assayed by this procedure. Chlopromazine, L-DOPA, epinine, 5-hydroxytryptamine, labetalol, methyldopa, 3-metoxytyramine, 3-methoxytyrosine, metropolol, phenoxybenzamine, propanolol and tyramine were found to produce no interference with the specified method.

The method described is very robust. It has been employed in our laboratory for the routine analysis of patients urine for more than 8 months. Our workload consisted of 15–25 specimens per week. Urine samples for quality control were also assayed with each batch of analyses. During this period, no significant deterioration of the detector response was observed and repolishing or replacement of the glassy carbon electrode was not necessary. The C_{18} column continued to produce good separations after 8 months of service.

Reference values established with specimens obtained from 40 non-hypertensive Chinese adults having no neurological or hormonal disorders and from 17 patients with essential hypertension are presented in Table IV.

Fig. 3 shows typical chromatograms obtained with a 24-h urine sample from a healthy individual, extracted with and without added DHBA. No significant endogenous peak was found at the retention time of the internal standard (DHBA).

A chromatogram obtained from a patient with pheochromocytoma, confirmed by surgery, is presented in Fig. 4. The determination of NE, EP, DA, NM and MN was completed in 10 min. However, a total chromatography time of 25 min is recommended, because 5-hydroxytryptamine and 3-metoxytyramine were also extracted by this technique and appeared as late peaks with retention times of 20 and 23 min, respectively.

Pheochromocytoma is a rare neural-crest tumour, which in about 10% of cases is malignant. Early diagnosis and subsequent surgical removal usually lead to prompt recovery[2]. It is generally held that a predominant increase in EP indicates an adrenal tumour[17]. Although urinary-free dopamine mainly reflects renal conversion of plasma DOPA by renal tubular aromatic amino acid decarboxylase, an increase in urinary

TABLE IV

REFERENCE VALUES FOR URINARY CATECHOLAMINES AND METANEPHRINES

Analyses were performed on 24-h urine specimens collected from 40 Chinese patients who were normotensive and had no neurological or endocrine disorders and from 17 Chinese patients suffering from essential hypertension. The upper limits were calculated from the mean + 2 S.D.

Compound	*Normal (n = 40)*		*Essential hypertension (n = 17)*	
	Range (nmol per 24 h)	*Upper limit (nmol per 24 h)*	*Range (nmol per 24 h)*	*Upper limit (nmol per 24 h)*
NE	63–416	440	85–557	535
EP	19–113	110	13–137	145
NM	18–212	240	64–370	350
MN	38–315	275	38–420	370
DA	221–2410	2570	220–3603	3810

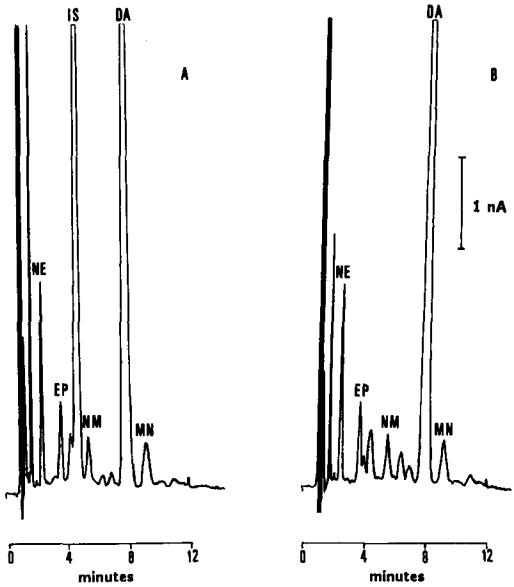

Fig. 3. (A) Representative chromatogram of a urine specimen obtained from a healthy individual, extracted after the addition of internal standard. (B) Chromatogram from the same specimen, extracted without added internal standard. Chromatographic conditions as described in the text.

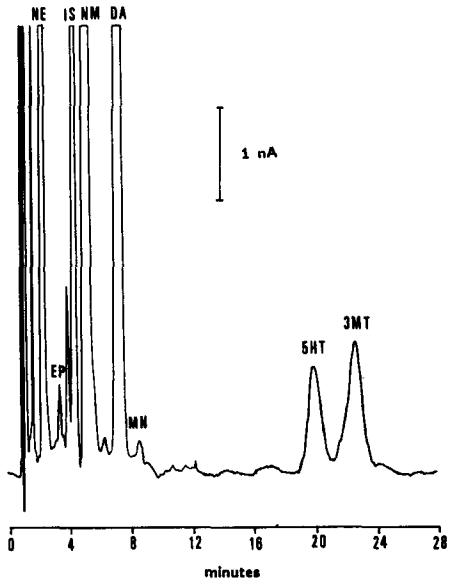

Fig. 4. Chromatogram of a urine specimen from a patient with surgically confirmed pheochromocytoma. The concentrations of NE, EP, DA, NM and MN were 7138, 87, 3713, 14245 and 59 nmol/l, respectively (urine volume, 1200 ml). 5HT = 5-hydroxytryptamine; 3MT = 3-metoxytyramine.

dopamine has been reported to be associated with malignancies[1,18]. As no single urine test has been proved to be completely reliable, screening for all urinary catecholamines and metabolites, namely EP, NE, DA, MN, NM, VMA and HVA, is recommended[10]. The development of a procedure that permits the simultaneous quantitation of most of these compounds is not simple. Differences in the chemical and partition characteristics of these compounds make it difficult to devise a single extraction and analytical procedure for their simultaneous quantitation. Different sorbents for the extraction of urinary catecholamines and metanephrines have been used, including alumina[19,20], cation-exchange resins[21,22] and boric acid gel[23,24], singly or in combination[22,25]. Other workers have used on-line sampling pre-treatment with adsorbents packed in small columns[26,27]. Odink *et al.*[28] used Bio-Rex 70 as adsorbent and boric acid as eluent, but failed to recover NM and MN. In our procedure, elution from Bio-Rex 70 with 4 *M* formic acid gave good recoveries of all of the catecholamines and metanephrines.

Parker *et al.*[29] developed an HPLC metod that is applicable to the analysis of NE, EP, MN, NM, VMA and 5-HIAA. However, three different methods of sample preparation were required, depending on the catecholamines or metabolites being quantitated. Joseph[30] employed butane boronic acid as a group-specific pairing agent by HPLC on a C_{18} column with ED and fluoresence detection and was able to quantitate eleven catecholamines, indoleamines and related precursors and metabolites in brain tissue, including NE, EP, VMA and HVA. Wester *et al.*[31] described the simultaneous determination of seventeen of the major monoamine neurotransmitters, precursors and metabolites, including NE, EP, DA, NM, MN, VMA and HVA. Their HPLC–ED procedure was applicable to extracts from brain tissue and cerebrospinal fluid[32]. However, the sample preparation procedure for urine was not described by either Joseph[30] or Wester *et al.*[31]. It is uncertain whether their procedures are applicable to urine which usually contains a large amount of endogenous metabolites and electroactive phenolic compounds. Only one method has been published on the simultaneous determination of urinary catecholamines and their O-methylated metabolites. Abeling *et al.*[33] employed Amberlite CG-50 and HPLC with fluoresence detection, which required a 10-fold concentration of the eluate by lyophilization prior to HPLC.

With the advantages of being simple, rapid, robust and capable of the simultaneous quantitation of NE, EP, DA, NM and MN, our method is ideal for application in clinical laboratories. The reference values in normotensive Chinese and hypertensive patients obtained in this study agree well with those reported previously[33].

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