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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOR THE SIMULTANEOUS DETERMINATION OF THE METHOXYLATED AMINES, NORMETANEPHRINE, METANEPHRINE AND 3-METHOXYTYRAMINE, IN URINE

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SUMMARY

A simple method for the simultaneous analysis of normetanephrine, metanephrine and 3-methoxytyramine (both free and conjugated) in human urine by reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection has been developed. Existing methods have been optimized for extraction by study of analytical parameters. The hydrolysed urines are purified and concentrated by successive passages on two ion-exchange resins and ammoniacal elution to eliminate interference from pigments or related chemical compounds. The methoxyamines are separated by high-performance liquid chromatography on a reversed-phase column. Detection and quantitation are achieved with an electrochemical detector using a vitreous carbon electrode. Samples can be injected at 25-min intervals. Reference values of adults and children are given.

INTRODUCTION

The need for rapid analysis of 3-O-methylated catecholamines arises from their important biological role. Various methods have been developed for their detection; however, some lack the necessary sensitivity and others are too complicated for routine analysis. The relatively low concentrations of monoamine metabolites have necessitated the use of sensitive analytical techniques such as gas chromatography—mass spectrometry [1] which requires expensive equipment, or high-performance liquid chromatography (HPLC) with fluorescence detection [2] or electrochemical detection (ElCD) [3—8].

We report here a procedure for urinary metabolites, which has the adequate sensitivity and accuracy otherwise exhibited by the more complex HPLC

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separation and ElCD systems. Reversed-phase chromatography is eminently suitable for separation of 3-O-methyl derivatives [9]. HPLC on microparticulate reversed-phase columns [10] offers a powerful technique for separating related compounds which are water-soluble but with some hydrophobic character. ElCD offers a major increase in sensitivity and is also somewhat selective for these metabolites [11—13]. A large peak was encountered in certain urine samples which interfered with the accurate measurement of 3-methoxytyramine; to eliminate all interference, we have made appropriate modifications to the extraction procedure [14—20] from a small quantity of acidified urine and under varying chromatographic conditions. Also, nanogram levels of 3-O-methylated amines have been determined in urine. The simplicity of the procedure favours multiple sample analysis.

EXPERIMENTAL

Apparatus

The analyses were performed on a Hewlett-Packard 1080 A chromatograph with an electrochemical detector (Tacussel Systems) consisting of: Faraday cage enclosing a cell-Tacussel DELC with a glassy carbon electrode (working electrode), a Pt electrode (auxiliary electrode), a Ag/AgCl electrode (reference electrode) and polarographic analyser Tacussel PRG-E.

The chromatograph comprises an analytical column (300 mm × 3.9 mm I.D.) packed with μBondapak C_{18} (10 μm particle size), and combined with a 30 × 3.9 mm I.D. precolumn filled with μBondapak C_{18}/Corasil, all from Waters Assoc. (Milford, MA, U.S.A.). Peak areas were obtained using a Hewlett-Packard integrator.

Chromatographic conditions

The flow-rate was adjusted to 1.5 ml/min, and the temperature of the column compartment and the eluents was 25°C. Eluent A contained a buffer consisting of 2 vols. of 0.02 *M* citric acid and 1 vol. of 0.02 *M* $NaHPO_4$ $2.5 \cdot 10^{-3}$ *M* sodium octylsulfonate and $5 \cdot 10^{-5}$ *M* disodium EDTA were added to 100 ml of buffer [13]. The mixture was filtered under vacuum through a 0.40-μm Millipore HA type filter before use. Eluent B was methanol (special for chromatography) from E. Merck (Darmstadt, G.F.R.). The mobile phase contained 90% of buffer (eluent A) and 10% of methanol (eluent B).

Materials

D,L-Metanephrine hydrochloride (B grade; MN), D,L-normetanephrine hydrochloride (B grade; NMN) and 3-methoxytyramine hydrochloride (A grade; MT) were all from Calbiochem (San Diego, CA, U.S.A.). D,L-α-methyl DOPA (3,4-dihydroxyphenylalanine) was from Sigma (St. Louis, MO, U.S.A.). Water was demineralized and distilled in an all-glass apparatus. Stock solutions of standards and external standard (100 μg/ml) were prepared in 0.01 *N* hydrochloric acid. Octane sulfonic acid sodium salt was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Ethylenediaminetetraacetic acid (EDTA) disodium salt was from E. Merck (Darmstadt, G.F.R.). All other reagents were reagent grade from E. Merck: 4 *N* and 5 *N* ammonium hydroxide, 0.1 *M*

sodium acetate, and borate buffer containing 3.11% (w/v) of boric acid adjusted to pH 8.8 with 0.5 *N* sodium hydroxide. Millipore filters type GS, pore size 0.22 μm, were from Millipore Corporation (Bedford, MA, U.S.A.). The resins used are Dowex 50W-X2 (100—200 mesh) obtained from Fluka (Buchs, Switzerland) and Bio-Rex 70 (50—100 mesh, Na^+) from Bio-Rad (Richmond, CA, U.S.A.).

The resin Dowex 50W-X2 was first washed with numerous baths of glass-distilled water, then regenerated by washing with successive volumes of 4 *M* hydrochloric acid, 2 *M* sodium hydroxide and 4 *M* hydrochloric acid. The pH of the resin was rigorously adjusted to pH 5 and then washed with numerous baths of glass-distilled water just before use. The resin was poured into a Pyrex glass column.

The resin Biorex 70 may be cycled [13] by washing with successive volumes of 3 *M* hydrochloric acid, 3 *M* sodium hydroxide, 3 *M* acetic acid, 1.0 *M* ammonium acetate (pH 6.5) and 0.1 *M* ammonium acetate (pH 6.5). The pH was adjusted to 6.5 during the last wash if necessary. Just before use the resin was poured into a polypropylene Bio-Rex column (Bio-Rad).

Urine samples were collected over hydrochloric acid and acidified to pH 1 [21]. Methoxyamines are less sensitive to oxidation than catecholamines [22], thus acidified urine (pH 1) may be stored for at least a month without addition of EDTA or ascorbic acid.

Method

Urine samples (10 ml) were adjusted to pH 1 with 6 *N* HCl and hydrolysed at 100°C for 20 min [15, 21, 23, 24]. The hydrolysed urines were diluted to 40 ml with distilled water [16], the pH was readjusted to 6, and the amines were adsorbed on a 10 × 1 cm column of Dowex W-X2 H^+ resin. Then the column was washed with 10 ml of 0.1 *N* sodium acetate and 20 ml of water. The methoxyamines were eluted with 18 ml of 5 *N* ammonium hydroxide. The eluate was evaporated to dryness under vacuum at 40°C. The dry deposit was dissolved in 4 ml of borate buffer (pH 8.8). This eluate can not be used directly because it contains a large amount of urinary pigments. A second purification was necessary. The solution was diluted to 45 ml with 1% (w/v) EDTA solution, adjusted to pH 6.5 and passed through a short column (3.2 × 0.7 cm of Bio-Rex 70 resin). The column was washed with 10 ml of distilled water. The 3-O-methoxylated amines were eluted with 30 ml of 4 *N* ammonium hydroxide. Then 50 μl of α-methyl DOPA solution as an external standard were added. The eluate was evaporated to dryness under vacuum at 40° C. The dry deposit was dissolved in 2 ml of borate buffer (pH 8.8) and filtered on Millipore filters; 40 μl of this solution were injected onto the column. All experiments were carried out with citrate—phosphate buffer—methanol (90:10, v/v) by isocratic elution at a flow-rate of 1.5 ml/min. The present compounds were determined by an external standard method.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

The aim of this work has been to separate the three amines from a single

urine sample and to determine them with minimal interference from pigments or related chemical compounds. By coupling Dowex 50W-X2 and Bio-Rex 70 resins a better purification was achieved than with the use of one resin. One essential condition for quantitative adsorption of 3-O-methylated amines on Dowex 50W-X2 and good exchange of organic compounds on the resin was a low saline content of the sample [4]. The separation and the peak heights or areas of catecholic compounds on reversed-phase packed columns depended strongly on the methanol composition and the pH of the mobile phase, and the oxidizing potential (vs. Ag/AgCl).

In order to obtain the required separation the pH was optimised. Fig. 1 shows the variation of the retention times of 3-O-methylated amines with the pH of the eluent when the percentage of methanol was held constant. A pH of 3.2 seems to be suitable to achieve a good separation of the three compounds.

The effect of percentage of methanol on the retention times (Fig. 2A) and peak heights (Fig. 2B) was studied. Standards of NMN, MN and MT were

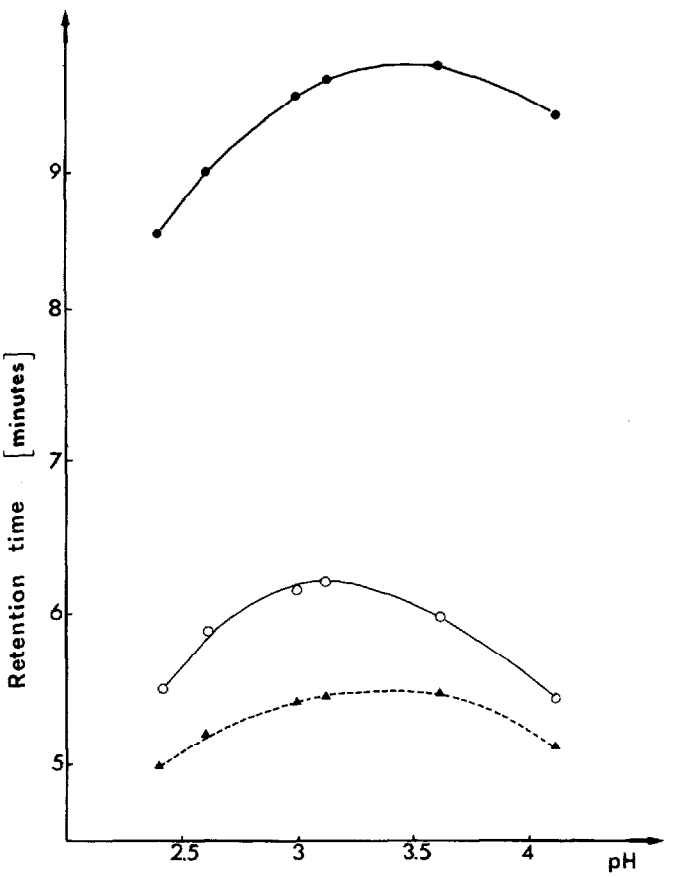

Fig. 1. Relationship between the retention times and the pH of the mobile phase. Column μBondapak C_{18}, mobile phase 90:10 (v/v) mixture of the 0.02 *M* citrate—phosphate buffer and methanol with octane sulfonic acid ($2.3 \cdot 10^{-3}$ *M*); pH adjusted to the different values with phosphoric acid or sodium hydroxide; flow-rate 1.5 ml/min; temperature 25°C; electrode potential +0.9 V vs. Ag/AgCl reference electrode. Symbols: ▲, NMN; ○, MN; ●, MT.

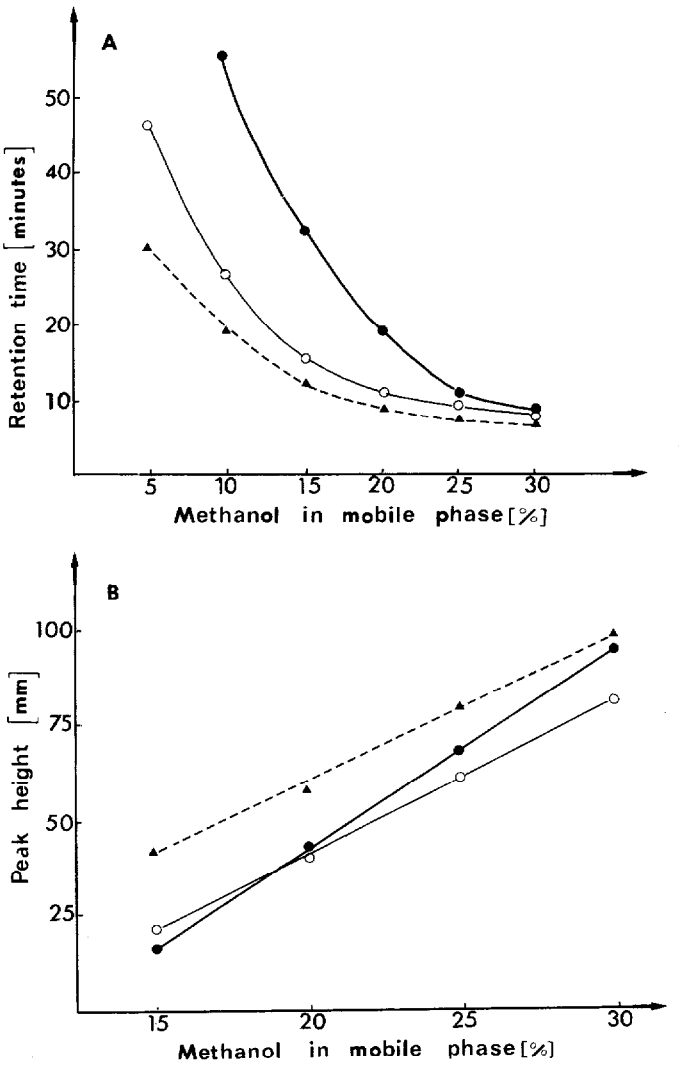

Fig. 2. Retention times and peak heights as a function of the percentage of methanol in the mobile phase. Symbols as in Fig. 1.

chromatographed using methanol—citrate—phosphate buffer pH 3.2 with increasing concentrations of methanol, as indicated in Fig. 2A and B. The addition of methanol to the buffer was necessary to reduce retention times. In our system 10% of methanol was found to be sufficient to achieve adequate resolution of the 3-O-methylated compounds.

Fig. 3 shows the relative detector response for assumed NMN, MN and MT peaks as a function of the oxidizing potential vs. Ag/AgCl reference electrode. The response at 0.9 V was set at 100% for each standard. Figs. 4 and 5 show that under the conditions outlined above the three O-methylated amines could be resolved within less than 25 min, at a flow-rate of 1.5 ml/min. The retention times for NMN, MN and MT were 8, 10 and 20 min, respectively. A constant

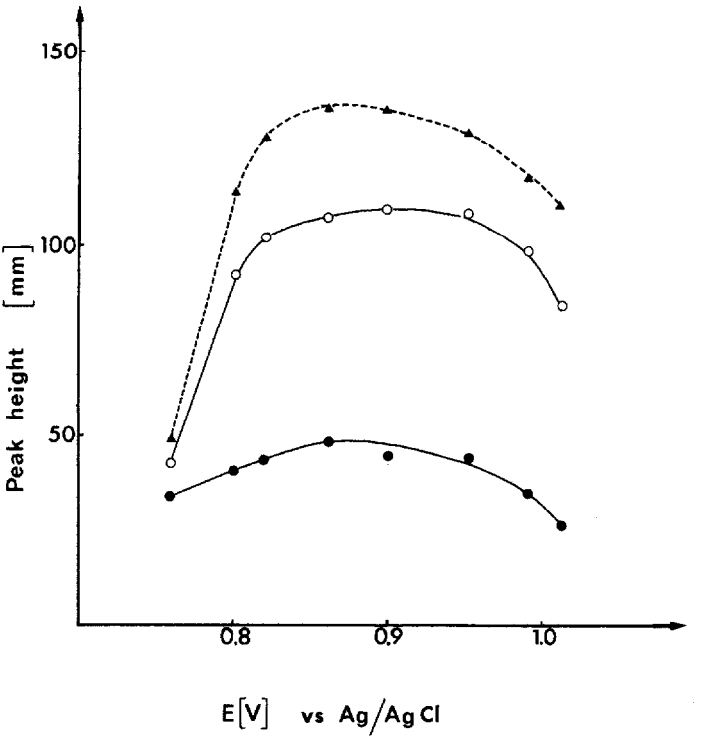

Fig. 3. Relationship between peak heights and the oxidizing potential, vs. Ag/AgCl. Symbols as in Fig. 1.

column temperature (25°C) was found to be necessary to obtain constant retention times. This result shows that different chromatographic conditions can lead to similar separations. A further consequence was that one can compensate for the loss of resolution of a column by decreasing the methanol concentration, or by changing both and adjusting the pH. In this case peak broadening occurs.

The linearity of both the extraction procedure and detector response (determined from peak area) was verified for each methoxyamine over the anticipated range of assay. The former was investigated by assaying pooled urine to which known amounts of NMN, MN and MT had been added and determining the peak areas obtained for each compound. Calibration curves were plotted for each compound (Fig. 6). In each case a linear relationship between methoxyamine concentration and peak area was observed over the concentration ranges studied. The equations for the calibration curves obtained were as follows:

NMN: $y = 0.166x - 0.78$

MN: $y = 0.153x - 0.05$

MT: $y = 0.165x - 0.01$

Each point on the calibration curve was established from the mean of five determinations. The linearity of detector response was confirmed by the injec-

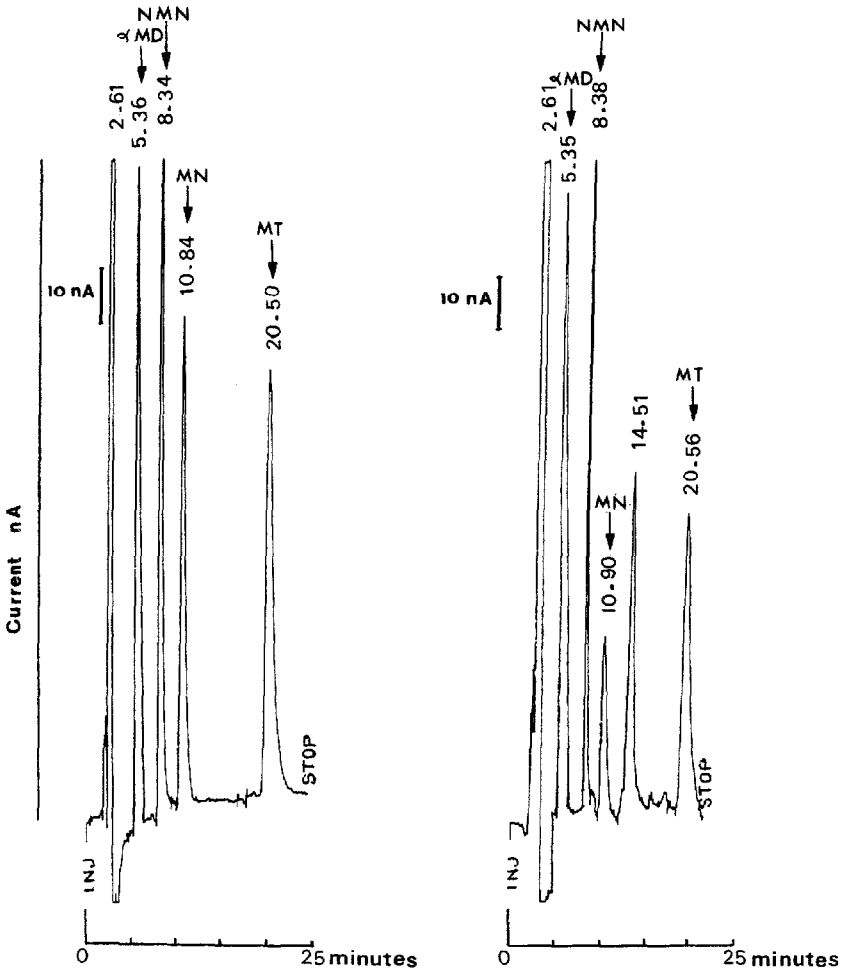

Fig. 4. Chromatogram of a standard mixture of 40 ng of normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MT), with α-methyl DOPA (α-MD) as an external standard.

Fig. 5. Chromatogram of a urine extract from a normal subject.

tion of known amounts of methoxyamine standards directly onto the chromatograph. Response for each compound was found to be linear over the range investigated (0—0.4 nmol).

Reference values

Forty urine specimens from normal children (twenty boys and girls) between the ages of 2 and 6 years and (twenty boys and girls) between the ages of 6 and 13 years were analysed. The reference values are presented in Table I. Thirty urine specimens from normal volunteers (healthy laboratory staff) (fifteen males and fifteen females) between the ages of 20 and 50 years were analyzed and found to yield a mean NMN excretion of 85.2 μmol/mol creatinine, a mean

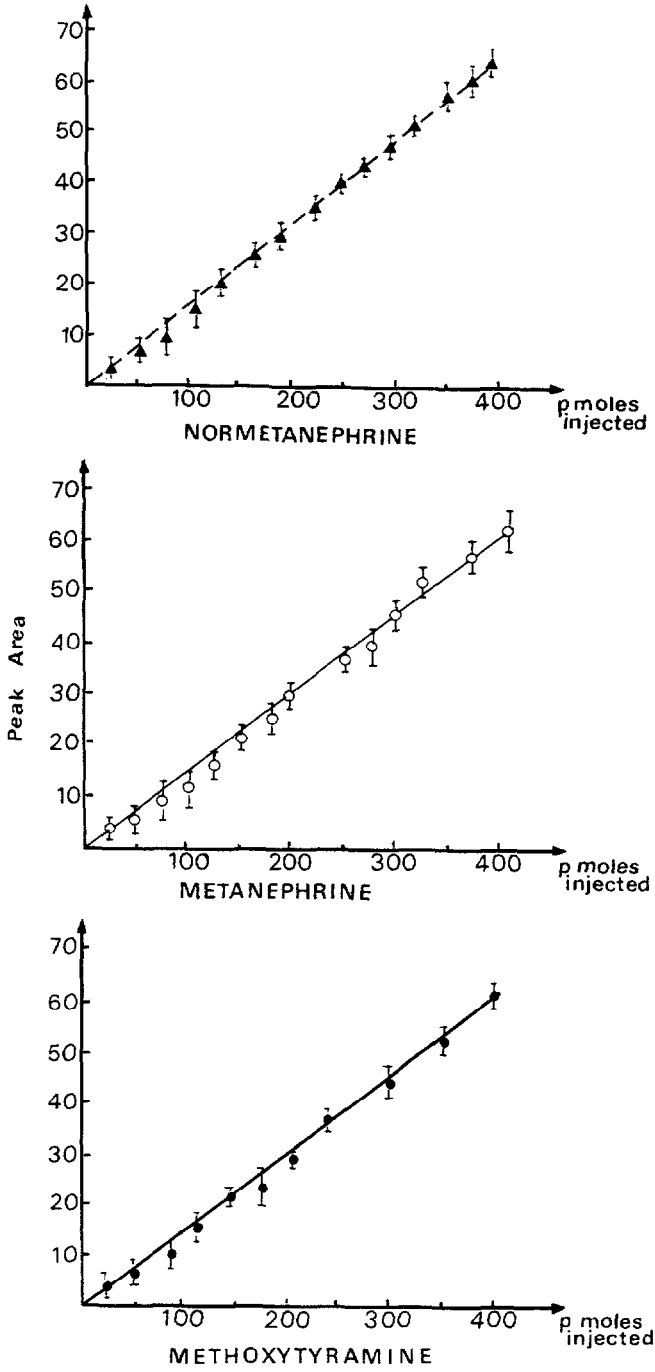

Fig. 6. Calibration curves for the determination of normetanephrine, metanephrine and methoxytyramine in urine by the assay procedure described.

TABLE I

URINARY NORMETANEPHRINE, METANEPHRINE AND METHOXYTYRAMINE IN 40 NORMAL CHILDREN AND 30 NORMAL ADULTS

Values are given in μmol/mol creatinine, mean ± S.D.

	Age group		
	2—6 years (n = 20)	6—13 years (n = 20)	20—50 years (n = 30)
Normetanephrine	203 ± 41	93.5 ± 8.5	85.2 ± 38.9
Metanephrine	204 ± 32	109 ± 29	73.2 ± 19.2
Methoxytyramine	183 ± 30	88.5 ± 18.5	59.6 ± 15.9

TABLE II

EXCRETION OF NORMETANEPHRINE, METANEPHRINE AND METHOXYTYRAMINE IN PATIENTS WITH PHEOCHROMOCYTOMA AND ESSENTIAL HYPERTENSION

Values are given in μmol/mol creatinine.

	Normetanephrine	Metanephrine	Methoxytyramine
Pheochromocytoma (n = 4)	3322	2562	174
	4018	2067	219
	4375	3346	145
	5026	3596	221
Essential hypertension* (n = 22)	200.5 ± 87.1	153 ± 69	112 ± 87

*Values expressed as mean ± S.D.

MN excretion of 73.2 μmol/mol creatinine and a mean MT excretion of 59.6 μmol/mol creatinine (Table I).

Medications were suppressed. Bananas, tea, coffee, tomatoes and vanilla-containing food were omitted.

A typical chromatogram for a normal urine is illustrated in Fig. 5.

Pathology

The excretion patterns of four patients with pheochromocytoma prior to therapy are presented in Table II. NMN, MN and MT excretions are respectively 49.1, 39.5 and 3.2 times higher than the reference values.

Twenty-two urine specimens from patients with essential arterial hypertension (twelve males, ten females) without other pathology able to modify catecholamine metabolism and who were on a normal diet, were analysed and found to yield a mean NMT excretion of 200.5 μmol/mol creatinine, a mean MN excretion of 204 μmol/mol creatinine and a mean MT excretion of 183 μmol/mol creatinine. NMN, MN and MT excretion are respectively 2.3, 2.7 and 3.07 times higher than the reference values.

CONCLUSION

In conclusion, the present method for analysis of urinary 3-O-methylated amines appears to be relatively simple and reliable. This technique is applicable to studies of derangements in catecholamine metabolism such as is found in patients with pheochromocytoma as well as to studies of hypertensive patients whose catecholamine metabolism may be minimally different from normal. Although this technique can at present be used for the determination of urinary NMN, MN and MT, it is being extended to the quantitation of other biologically important amines such as octopamine and tyramine. This methodology should find wide application in both clinical and research laboratories.

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