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AMINE METABOLITE PROFILE OF NORMAL AND UREMIC URINE USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the simultaneous analysis of phenolic amines and aliphatic amines in human urine is described. The amine metabolites in urine were extracted using Dowex 50W-X8 cationic resin, derivatized and analyzed by a gas chromatographic—mass spectrometric—computer system. The amine metabolites profile of 5 ml of urine was obtained with good gas chromatographic separation. The gas chromatographic method described here separates urinary phenolic amines, di- and polyamines and methylguanidine in a single chromatographic separation. The urinary levels of methylguanidine, putrescine, cadaverine, spermidine, *p*-tyramine, dopamine, and 3-methoxytyramine were quantitated by using a mass spectrometric technique. In uremic patients, only the urinary excretion of methylguanidine was increased in comparison with normal subjects, although the urinary excretion of other amines was decreased in uremic patients.

INTRODUCTION

Recently, gas chromatography—mass spectrometry (GC—MS) has been used to screen a number of metabolites in blood or urine. In particular, the analysis of organic acid in urine has been pursued by many investigators, and information

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has been obtained as to the diagnosis of patients with metabolic disorders [1—3]. However, very few studies have been devoted to the application of the profiling amine analysis to clinical diagnosis, since the amine levels were very low. It is important to analyze the urinary amines which are the intermediate metabolites of amino acids and possess biogenic activity.

LeGatt et al. [4] reported a new method for the simultaneous extraction and GC separation of trace amines: 2-phenylethylamine, *m*-tyramine, *p*-tyramine, *p*-octopamine, normetanephrine, and 3-methoxytyramine. Nelson et al. [5] analyzed 3-O-methylated catecholamine in human urine using ion-pair extraction and GC with electron-capture detection; they quantitated the amount of normetanephrine, metanephrine, and 3-methoxytyramine. Kawai and Tamura [6] quantitated urinary norepinephrine, epinephrine, and dopamine as the trifluoroacetyl derivatives using GC. The polyamines also have been analyzed using GC [7—9], ever since Russel et al. [10] reported an elevated polyamine concentration in the urine of cancer patients. However, a profiling analysis of all the amines, including phenolic and aliphatic amines, has not yet been attempted, except in our previous study [11].

The present study was undertaken to screen simultaneously phenolic amines and aliphatic amines in uremic urine using GC—MS.

MATERIALS AND METHODS

Urine samples

Twenty-four-hour urine samples were collected in containers containing 50 ml of 3 *N* hydrochloric acid and stored at −20°C until analyzed.

Sample preparation

As an internal standard, 40 nmol of N-3-aminopropyl-1,3-diaminopropane were added to 5 ml of urine. To hydrolyze the conjugated amines, 5 ml of concentrated hydrochloric acid were added to the samples and heated at 100°C for 16 h. The sample was evaporated to dryness and dissolved in phosphate buffer (pH 8) including 0.7 mol sodium chloride, and applied to Dowex 50W-X8 (H^+, 10 × 0.8 cm). After washing with 5 ml of water, 50 ml of phosphate buffer, and 30 ml of 1 *N* hydrochloric acid, the absorbed amines were eluted with 100 ml of 6 *N* hydrochloric acid. The eluate was evaporated to dryness with a rotary evaporator. For acylation, 200 μl of anhydrous ethyl acetate and 200 μl of pentafluoropropionic anhydride were added to the dry residue, and the amines were acylated at 70°C for 15 min. After cooling the sample was dried with a stream of nitrogen at room temperature and redissolved in 100 μl of anhydrous ethyl acetate. A 2-μl aliquot of this solution was subjected to GC—MS.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a JGC-20K gas chromatograph, a JMS D-300 double focusing mass spectrometer, and a JMA 2000 data processing system (JEOL, Tokyo, Japan). The gas chromatograph was equipped with a 3% OV-1 glass column (2 m × 2 mm I.D.). The column

temperature was programmed from 75 to 260°C at 6°C/min. The carrier gas was helium with a flow-rate of 30 ml/min. Electron-impact ionization mass spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 300 μA, ion source temperature 200°C, and accelerating voltage 3 kV.

Quantitative determination of amines

The calibration curves for the amines identified in the urine were obtained by adding a known amount of standard to 5 ml of deproteinized plasma. After addition of 40 nmol of N-3-aminopropyl-1,3-diaminopropane, the solution was hydrolyzed at 100°C for 16 h and applied to Dowex 50W-X8 resin column using the same procedure as with the urine sample. The calibration curves relating the concentration of methylguanidine, putrescine, cadaverine, *p*-tyramine, dopamine, 3-methoxytyramine, and spermidine to the ratio of the peak height of N-3-aminopropyl-1,3-diaminopropane, were obtained from the mass chromatogram. Ion *m/e* 246 was used for the quantitation of methylguanidine and ion *m/e* 176 for the quantitation of putrescine and cadaverine. Ion *m/e* 266 was used for the quantitation of *p*-tyramine, and ion *m/e* 428 for that of dopamine, and ion *m/e* 296 for quantitation of 3-methoxytyramine. Ion *m/e* 204 was used for the quantitation of spermidine, and for the monitoring of an internal standard, N-3-aminopropyl-1,3-diaminopropane.

RESULTS

The elution profile of putrescine, *p*-tyramine, 3-methoxytyramine, and dopamine on the Dowex 50W-X8 column was estimated. The washing buffer and elution buffer are the same as in the extraction procedure of Inoue and Mizutani [12], except for the elution volume of 6 *N* hydrochloric acid which was used for extraction of polyamine in tissue. The elution volume of putrescine was 13 ml of 6 *N* hydrochloric acid, but it was found that phenolic amines

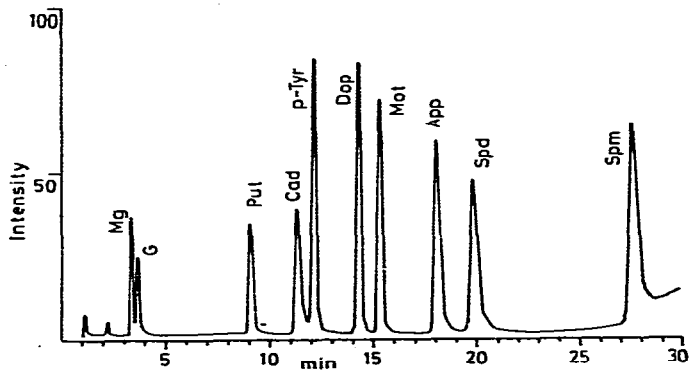

Fig. 1. Gas chromatogram of standard prepared with 50 nmol of each of the amines. Peaks: pentafluoropropyl derivatives of: Mg, methylguanidine; G, guanidine; Put, putrescine; Cad, cadaverine; p-Tyr, *p*-tyramine; Dop, dopamine; Mot, 3-methoxytyramine; App, N-3-aminopropyl-1,3-diaminopropane; Spd, spermidine; Spm, spermine. GC conditions were as follows: column, 3% OV-1 on Gas-Chrom Q (80—100 mesh), 2 m × 2 mm I.D.; column temperature, 75°C to 260°C at 6°C/min.

such as *p*-tyramine, 3-methoxytyramine, and dopamine were eluted with 100 ml of 6 *N* hydrochloric acid. The results suggest that it is possible to extract polyamines and phenolic amines simultaneously. Furthermore, sodium ion which was retained on the resin during the first washing was removed by washing with 1 *N* hydrochloric acid. The eluate without sodium ion was suitable for derivatization of the amines and GC analysis.

Fig. 1 shows a chromatogram of standard samples with 50 nmol of each of the amines. A good GC separation was obtained. The conditions were described under Gas chromatography—mass spectrometry.

Fig. 2 shows chromatograms of amines in the urine of a normal subject (Fig. 2A) and a patient with renal failure (Fig. 2B). Methylguanidine, guanidine, putrescine, cadaverine, *p*-tyramine, dopamine, 3-methoxytyramine, and spermidine were detected in normal and uremic urine. These amines were identified by comparison with mass spectra and retention times of pentafluoropropyl derivatives of authentic compounds in our laboratory.

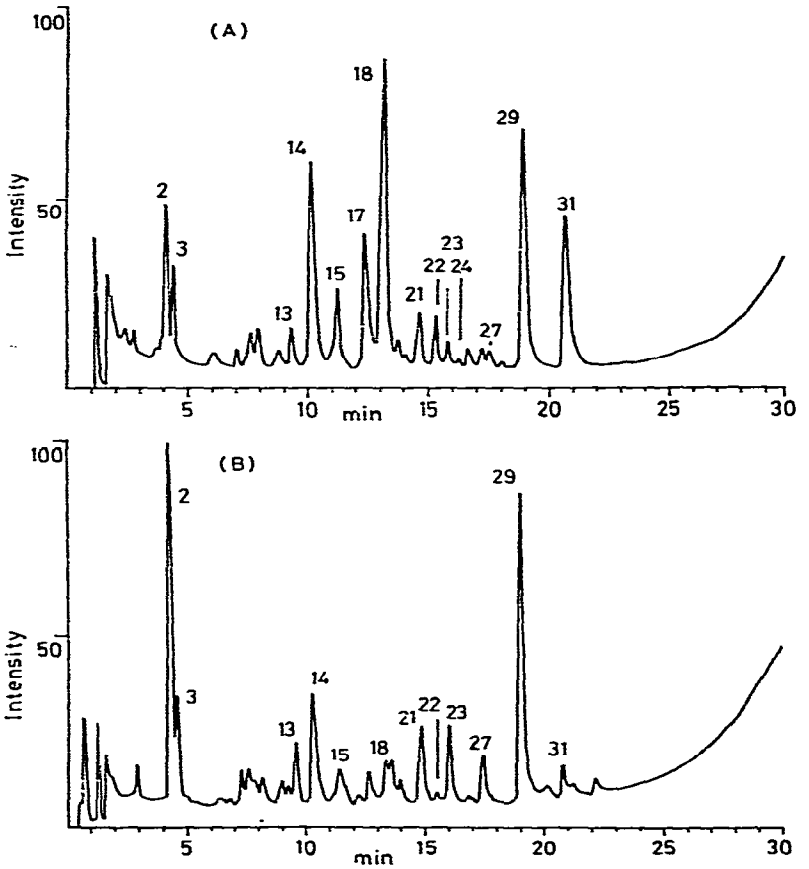

Fig. 2. Gas chromatograms of the urinary amines of (A) a normal subject and (B) a patient with renal failure. The extract was subjected to pertafluoropropyl derivatization and separated on a 3% OV-1 glass column.Peaks: 2, methylguanidine; 3, guanidine; 14, putrescine; 17, cadaverine; 18, *p*-tyramine; 22, dopamine; 24, 3-methoxytyramine; 29, N-3-aminopropyl-1,3-diaminopropane (internal standard); 31, spermidine.

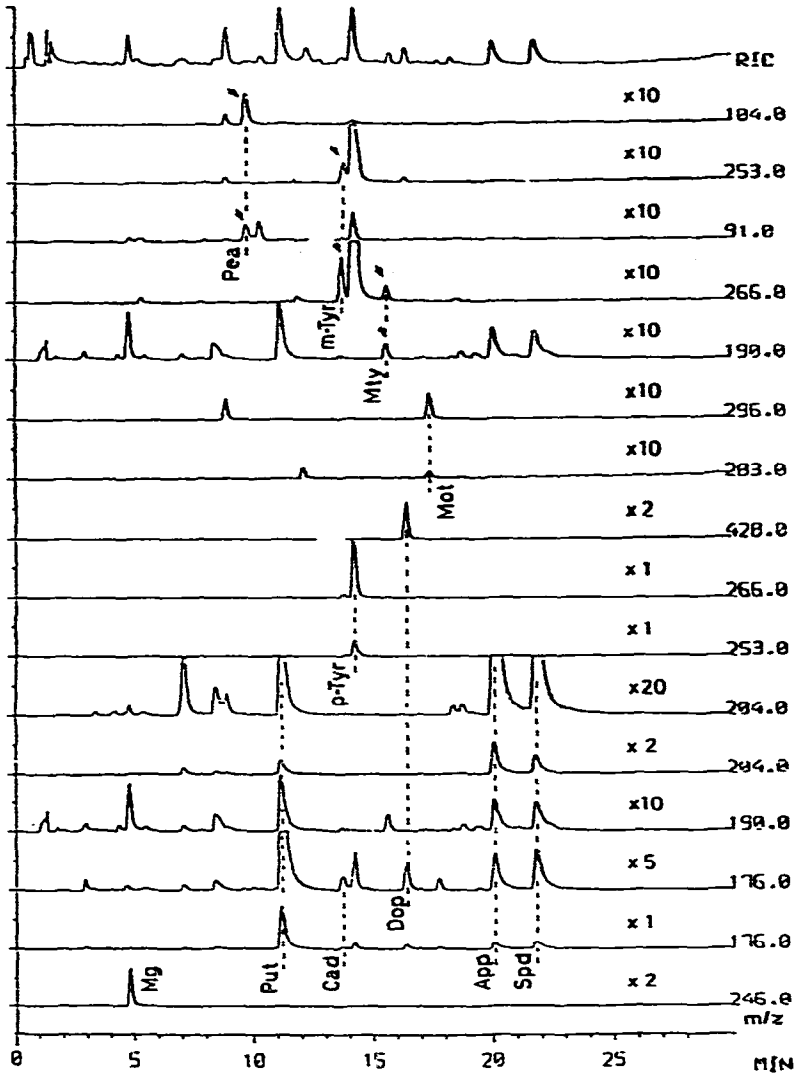

Fig. 3. Mass chromatogram of the urinary amines of a normal subject. Ions: *m/e* 246 for the detection of methylguanidine, *m/e* 176, 190 and 204 for putrescine and cadaverine, *m/e* 266 and 253 for *p*-tyramine, *m/e* 428 and 176 for dopamine, *m/e* 296 and 283 for 3-methoxytyramine, *m/e* 204, 190 and 176 for spermidine and N-3-aminopropyl-1,3-diaminopropane (internal standard), *m/e* 104 and 91 for 2-phenylethylamine (Pea), *m/e* 266 and 253 for *m*-tyramine (m-Tyr), *m/e* 190 and 266 for N-methyltyramine (Mty). For abbreviations, see Fig. 1.

The quantitation of urinary amines was performed using a mass chromatogram (Fig. 3, see Quantitative determination of amines). Table I shows the urinary excretion of seven amines in normal subjects and uremic patients. The levels of methylguanidine in urine of uremic patients were increased in comparison with that of normal urine, whereas those of other amines were decreased one-fourth to one-fifth.

TABLE I

URINARY EXCRETION OF SEVEN AMINES IN NORMAL SUBJECTS AND UREMIC PATIENTS, μmol/24 h (μmol/g CREATININE)

For abbreviations see Fig. 1.

	Put	Cad	Spd	p-Tyr	Dop	Mot	Mg	Creatinine (mg/dl)	Urine volume (l)
Control (n=9)	17.52±8.65	2.74±3.07	7.51±1.98	12.05±9.23	3.75±1.67	1.35±0.89	26.4±10.2		
$\bar{X}$±S.D.	(13.60±3.60)	(2.14±2.16)	(6.33±2.02)	(9.40±5.48)	(3.00±0.86)	(1.12±0.66)	(22.6±10.8)		
Uremia (n=7)									
I.S.	2.60(7.19)	1.36(3.76)	1.47(4.06)	2.39(6.60)	2.46(6.81)	0.48(1.32)	51.6(142.8)	47.6	0.76
M.S.	3.41(6.96)	0.18(0.37)	1.54(3.14)	3.42(6.97)	0.75(1.54)	0.34(0.70)	36.9(75.4)	92.5	0.53
T.I.	1.94(11.20)	0.53(3.44)	0.30(1.78)	0.74(4.28)	0.22(1.28)	0.09(0.55)	36.2(208.5)	36.2	0.48
S.Y.	1.49(6.88)	1.19(5.54)	0.45(2.12)	7.46(34.47)	0.24(1.14)	0.10(0.49)	40.2(186.0)	40.0	0.54
R.S.	3.70(16.27)	0.25(1.14)	0.59(2.64)	0.38(1.71)	0.12(0.57)	0.06(0.27)	31.3(139.3)	44.3	0.51
S.M.	4.68(8.49)	1.31(2.37)	1.13(2.06)	4.38(7.97)	0.81(1.47)	0.37(0.67)	39.6(72.1)	83.5	0.66
T.N.	1.82(3.31)	N.D.*	1.03(1.87)	1.50(2.73)	0.37(0.67)	0.58(1.05)	109.1(196.7)	38.5	1.44
$\bar{X}$±S.D.	2.80±1.08**	0.68±0.53***	0.93±0.45**	2.89±2.28§	0.71±0.75**	0.28±0.19§	49.2±25.0§		
	(8.61±3.80)	(2.37±1.86)	(2.52±0.76)	(9.24±10.5)	(1.92±2.02)	(0.72±0.32)	(145.8±51.5)		

* N.D. = not detected.

** $P<0.001$.

*** Not significant.

§ $P<0.05$.

DISCUSSION

Phenolic amines and aliphatic amines were profiled in urine and good recovery and reproducibility were obtained using GC—MS. MS which detects specific ions, in fact detected very small amounts of amines, although with less sensitivity than with selected ion monitoring. Fig. 3 shows a mass chromatogram of normal urine. The peaks at *m/e* 91 and *m/e* 104 appearing at a retention time of 9.8 min were considered to be the fragment ions of 2-phenylethylamine by comparing with the mass spectra and retention times of authentic compound. The peaks at *m/e* 190 and *m/e* 266 appearing at the retention time of 15.6 min were considered to be the fragment ions of N-methyltyramine by comparing with the mass spectra cited in the literature [13]. *m*-Tyramine was also detected at the retention time of 13.7 min in front of *p*-tyramine [13]. Many amine metabolites were extracted and separated using the present procedure.

In patients with renal failure, many metabolites which are normally excreted in urine are retained in blood, causing various uremic symptoms. Methylguanidine is noted to be one of the uremic toxins. It is also known that the levels of polyamines and tyramine are elevated in blood of uremic patients [14,15]. The present finding that only the level of methylguanidine was increased in uremic urine is very striking. Stein et al. [16] also reported that the urinary excretion of methylguanidine was increased in uremia. It is considered that the increase of polyamine and tyramine in uremic blood results from decreased excretion, whereas increase of methylguanidine results form increased production.

The present finding as to the normal urinary *p*-tyramine level, 12.05 ± 9.23 μmol/24 h (9.40 ± 5.48 μmol/g creatinine), was about two times higher than in previous reports [13, 17—19] in which the unconjugated *p*-tyramine in the urine was quantitated. Our results that normal urinary putrescine and spermidine levels were 17.52 ± 8.65 μmol/24 h (13.60 ± 3.60 μmol/g creatinine) and 7.51 ± 1.98 μmol/24 h (6.33 ± 2.02 μmol/g creatinine) were in agreement with those of Marton et al. [20] and Denton et al. [7]. The fact that the normal urinary dopamine level was 3.75 ± 1.67 μmol/24 h (3.00 ± 0.86 μmol/g creatinine) was also in agreement with Kawai and Tamura's result [6]. However, our normal level of 3-methoxytyramine, 1.35 ± 0.89 μmol/24 h (1.12 ± 0.66 μmol/g creatinine), was 2 to 3 times higher than that of Nelson et al. [5].

The detection limit of these amines was about 0.2 nmol/ml in urine. Epinephrine, norepinephrine, metanephrine, normetanephrine, and synephrine could not be detected.

Scaro et al. [19] quantitated urinary free tyramine by using high-performance liquid chromatography with a fluorescence detector. They reported that patients with pheochromocytoma, neuroblastoma, and Parkinson's disease have elevated levels of urinary tyramine. In patients with pheochromocytoma or neuroblastoma, metabolic disorders of catecholamine have been reported by many investigators [21—24]. The present procedure for simultaneous quantitation of polyamines and phenolic amines such as 3-methoxytyramine and dopamine may be useful to study these diseases.

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