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SIMULTANEOUS DETERMINATION OF IMIDAZOLEACETIC ACID AND N^{τ}- AND N^{π}-METHYLIMIDAZOLEACETIC ACIDS IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A sensitive method for the simultaneous determination of urinary imidazoleacetic acid and N^{τ}- and N^{π}-methylimidazoleacetic acids which employs high-performance liquid chromatography with fluorescence detection is described. The compounds were converted into the corresponding fluorescent esters by reaction with 4-bromomethyl-7-methoxycoumarin. These derivatives are separated by liquid chromatography on a Radial-Pak silica column. The detection limits for imidazoleacetic acid and N^{τ}- and N^{π}-methylimidazoleacetic acids in urine are 15, 10 and 20 pmol/ml, respectively. The 24-h urinary excretion of imidazoleacetic acid and N^{τ}- and N^{π}-methylimidazoleacetic acids by healthy persons was 5.7–39.9, 4.3–24.6 and 1.5–19.3 nmol/mg of creatinine, respectively.

INTRODUCTION

N^{τ}-Methylimidazoleacetic acid (1-methylimidazole-4-acetic acid, N^{τ}-MIAA), imidazoleacetic acid (IAA) and N^{τ}-methylhistamine are metabolites of histamine and their simultaneous determination is required in the investigation of the role and turnover of histamine. Many methods have been reported for the determination of N^{τ}-MIAA [1–5] and IAA [6, 7]. A thin-layer chromatographic method [8, 9] was not very sensitive. The separation of radioactive N^{τ}-MIAA

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and IAA, derived from [^{14}C]histamine, was achieved by ion-exchange column chromatography [10].

N^{π}-Methylimidazoleacetic acid (1-methylimidazole-5-acetic acid, N^{π}-MIAA) occurs in urine but it is not a metabolite of histamine [11]. It has been assayed together with N^{τ}-MIAA [1, 4].

We have developed a sensitive method for the simultaneous determination of N^{τ}-MIAA, IAA and N^{π}-MIAA in human urine by high-performacne liquid chromatography (HPLC) with fluorescence detection. After clean-up using Bond Elut C_{18} cartridge and by AG 1-X4 anion-exchange column chromatography, these acids are converted into the corresponding fluorescent esters by reaction with 4-bromomethyl-7-methoxycoumarin (4-BrMC), a fluorescence derivatization reagent for carboxylic acids [12,13]. The fluorescent esters are separated by liquid chromatography using a Radial-Pak silica column.

EXPERIMENTAL

Materials and reagents

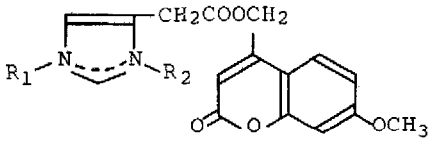
All chemicals were of analytical-reagent grade, unless stated otherwise. Water was deionized and distilled. IAA hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.) and N^{τ}-MIAA hydrochloride was from Calbiochem (La Jolla, CA, U.S.A.). N^{π}-MIAA hydrochloride was synthesized from 1-methyl-4-histidine (Sigma) according to a published method [4]. 4-BrMC was purchased from Tokyo Kasei (Tokyo, Japan) and 18-crown-6 from Aldrich (Beerse, Belgium). Bond Elut C_{18} cartidges were obtained from Analytichem (Harbor City, CA, U.S.A.) and AG 1-X4 anion-exchange resin (100–200 mesh) from Bio-Rad (Richmond, CA, U.S.A.).

Urine samples (24 h) were collected in dark-brown bottles containing 6 *M* hydrochloric acid (20 ml) and stored at 4°C for up to five days or at −20°C for longer periods. Urinary creatinine was determined by a method described previously [14].

AG 1-X4 anion-exchange resin (100–200 mesh) was washed successively with 3 *M* hydrochloric acid, water, 3 *M* sodium hydroxide solution, water, 1 *M* acetic acid and water, and then equilibrated with 50 m*M* Tris–acetate buffer (pH 7.4). The resin suspension was poured on to a glass column (150×10 mm I.D.) to a height of 30 mm; the flow-rate was 0.4–0.6 ml/min.

Apparatus and HPLC conditions

A Shimadzu (Kyoto, Japan) LC-4A liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (10-μl loop) and a Shimadzu RF-530 fluorescence spectromonitor fitted with a 12-μl flow cell. It was operated at an emission wavelength of 391 nm and an excitation wavelength of 336 nm. The column was a Radial-Pak silica (particle size 10 μm, 100×8 mm I.D.) (Japan Waters, Tokyo, Japan); the column temperature was 22–27°C. The mobile phase was acetone–benzene–acetic acid (70:30:0.05, v/v/v) at a flow-rate of 2.0 ml/min. Uncorrected fluorescence spectra of the eluates were measured with a Hitachi (Tokyo, Japan) 650-10S spectrofluorimeter using quartz cells



N^{τ}-MIAA : $R_1 = CH_3$
($C_{17}H_{16}N_2O_5 = 328$)

IAA : R_1 or $R_2 = H$
($C_{16}H_{14}N_2O_5 = 314$)

N^{π}-MIAA : $R_2 = CH_3$
($C_{17}H_{16}N_2O_5 = 328$)

Fig. 1. Fluorescent derivatives of N^{τ}-MIAA, IAA and N^{π}-MIAA.

(optical path lengths 10 mm). Spectral band widths of 10 nm were used both for the excitation and emission wavelengths. Electron-impact mass spectra were obtained with a Shimadzu GCMS 6020 mass spectrometer. The samples were introduced directly into the ion source.

Procedure

Quantitative evaluation was based on the standard additions method. Urine (0.5 ml) and urine (0.5 ml) plus 20 μl of a standard mixture of N^{τ}-MIAA, IAA and N^{π}-MIAA (1.5 μmol/ml each) were worked up simultaneously as follows.

Samples were mixed with 1.5 ml of 50 m*M* Tris–acetate buffer (pH 7.4) and the pH was adjusted to 7.4 with 1 *M* sodium hydroxide solution or 1 *M* acetic acid. The mixtures were filtered through a Bond Elut C_{18} cartridge, then washed with 3 ml of water. The combined mixtures of filtrates and washings were applied to an AG 1-X4 (CH_3COO^-) column. After washing the column successively with 3 ml of water and 2 ml of 50 m*M* acetic acid, N^{τ}-MIAA, IAA and N^{π}-MIAA were eluted with 1.5 ml of 50 m*M* acetic acid. The eluate was evaporated to dryness in vacuo. The residue was dissolved in 50 μl of dimethylformamide containing 10 m*M* 18-crown-6 and 5 m*M* triethylamine. The solution was well mixed with 0.4 g of anhydrous potassium carbonate. To the mixture were added 50 μl of 10 m*M* 4-BrMC in dimethylformamide and it was vortexed again and then allowed to stand at room temperature (22–27°C) for 20 min. Aliquots of 10 μl of the reaction mixture were separated. Peak heights were used for the quantitative evaluation of the chromatograms.

RESULTS AND DISCUSSION

N^{τ}-MIAA, IAA and N^{π}-MIAA reacted with 4-BrMC in the presence of potassium carbonate, 18-crown-6 and triethylamine in dimethylformamide to form the corresponding fluorescent esters (Fig. 1).

Fig. 2 shows a typical chromatogram of (A) a standard mixture of N^{τ}-MIAA, IAA and N^{π}-MIAA and (B) the reagent blank. The retention times of N^{τ}-MIAA, IAA and N^{π}-MIAA (peaks 1, 2 and 3) were 7.5, 10.0 and 15.0 min, respectively.

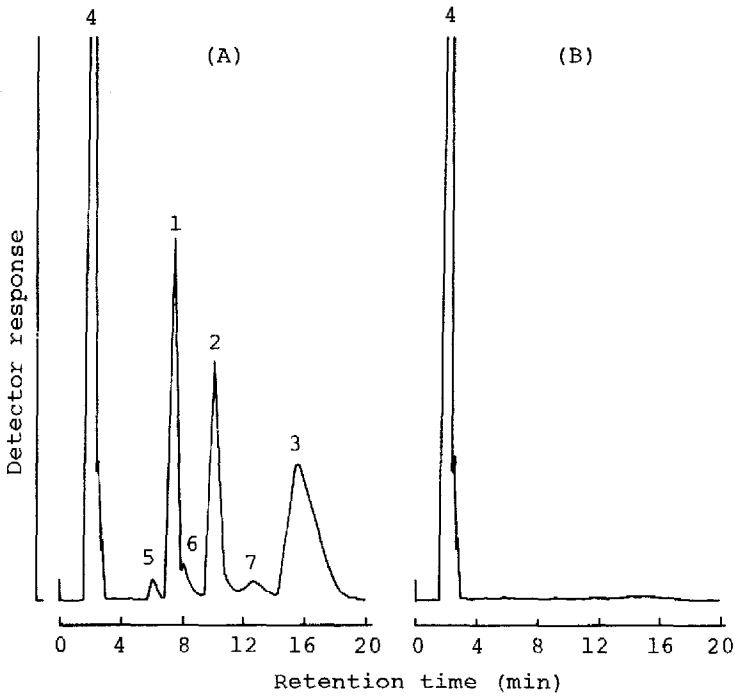


Fig. 2. Chromatograms of (A) 4-BrMC derivatives of N^{τ}-MIAA, IAA and N^{π}-MIAA and (B) the reagent blank. A portion (0.5 ml) of a standard mixture of N^{τ}-MIAA, IAA and N^{π}-MIAA (30 pmol/ml each) (or 0.5 ml of water for the reagent blank) was treated according to the procedure. Peaks: 1 = N^{τ}-MIAA; 2 = IAA; 3 = N^{π}-MIAA; 4 = 4-BrMC; 5, 6 and 7 = unidentified compounds, probably impurities of 4-BrMC and/or its decomposition products.

The eluates corresponding to peaks 1, 2 and 3 showed identical fluorescence excitation and emission spectra with an excitation maximum at 336 nm and an emission maximum at 391 nm. These were used for monitoring the fluorescence intensity of the eluates. Mass spectra of the eluates of peaks 1, 2 and 3 showed molecular ions at *m/z* 328, 314 and 328, respectively.

The derivatization reaction of the imidazoleacetic acids with 4-BrMC proceeded successfully in dimethylformamide and dimethyl sulphoxide, but no reaction occurred when using methanol and water as solvents. The fluorescence intensities of the imidazoleacetic acids obtained in dimethylformamide were 20% higher than those in dimethyl sulphoxide. The reaction was complete within 10 min at 25–50 °C. The fluorescent derivatives were more stable in the presence of triethylamine; the fluorescence intensities did not change for at least 120 min.

Fig. 3 shows typical chromatograms of (A) normal urine and (B) the urine with standard compounds added. The retention times of peaks 1, 2 and 3 in Fig. 3A and B were the same as those in Fig. 2A, as were the fluorecence excitation and emission spectra and mass spectra. These results suggest that peaks 1, 2 and 3 correspond to N^{τ}-MIAA, IAA and N^{π}-MIAA, respectively.

Acetic acid in the mobile phase affected the separation of the peaks. When its concentration was higher than 0.03%, an unknown substance (peak 4 in Fig. 3A

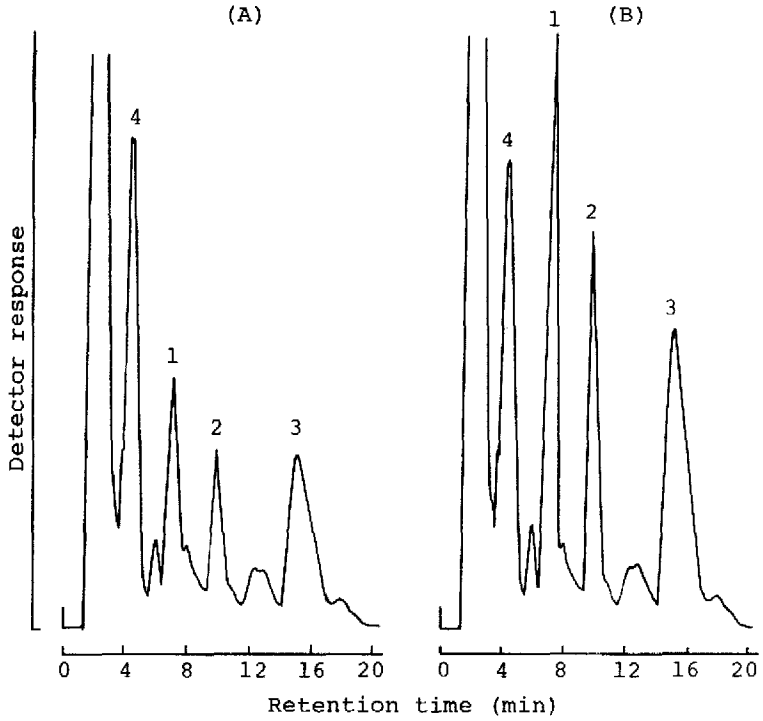


Fig. 3. Chromatograms of (A) normal urine and (B) urine with added standard compounds. Peaks: 1, 2 and 3 as in Fig. 2; 4 = unknown. Concentrations of N^{τ}-MIAA, IAA and N^{π}-MIAA: 18.8, 20.6 and 36.9 nmol/ml of urine, respectively.

and B), which could not be removed completely by a Bond Elut C_{18} cartridge and AG 1-X4 anion-exchange column chromatography, overlapped with the peak of N^{τ}-MIAA. At concentrations of acetic acid above 0.1%, the column was damaged.

The separation of the 4-BrMC derivatives of N^{τ}-MIAA, IAA and N^{π}-MIAA from other urinary constituents was examined using various column packings. The peaks of N^{τ}-MIAA, IAA and N^{π}-MIAA broadened on a Radial-Pak ODS column and the peak of N^{τ}-MIAA overlapped with an unknown peak on a Radial-Pak NH_2 column. The Radial-Pak silica column, if used under the described conditions, gave the most satisfactory separations, as is shown in Fig. 3.

4-BrMC derivatives of some other urinary carboxylic acids that elute near to those of the derivatives of imidazoleacetic acids may interfere in the determination of the imidazoleacetic acids if they are present in the sample solution. These interfering substances could be removed by the clean-up step with the Bond Elut C_{18} cartridge and by AG 1-X4 anion-exchange column chromatography. The imidazoleacetic acids that passed through the Bond Elut C_{18} cartridge were adsorbed by the anion-exchange column. After washing with water and 50 m*M* acetic acid, the imidazoleacetic acids were eluted quantitatively with 50 m*M* acetic acid. The used Bond Elut C_{18} cartridges could be regenerated by washing with methanol and water. They were used at least five times.

Peak heights varied linearly with the amounts of N^{τ}-MIAA, IAA and N^{π}-MIAA

TABLE I

URINARY EXCRETION OF N^{τ}-MIAA, IAA AND N^{π}-MIAA BY NORMAL INDIVIDUALS

Values are expressed in nmol/mg of creatinine; values in parentheses are μmol per 24-h urine.

Age (years)	Sex*	N^{τ}-MIAA	IAA	N^{π}-MIAA
48	M	9.9 (29.0)	5.8 (17.1)	1.5 (4.4)
35	M	6.3 (9.8)	9.3 (14.6)	1.6 (2.5)
30	M	4.3 (5.1)	5.7 (6.7)	8.7 (10.2)
28	M	15.1 (28.5)	12.4 (23.3)	19.3 (36.4)
26	M	7.6 (15.0)	9.2 (18.2)	13.1 (26.0)
20	M	8.3 (14.1)	6.1 (10.3)	18.3 (31.0)
25	F	15.2 (12.8)	11.1 (9.3)	7.9 (6.6)
23	F	20.1 (21.0)	39.9 (41.6)	6.3 (6.6)
21	F	9.4 (13.5)	7.0 (10.1)	4.4 (6.3)
21	F	24.6 (27.8)	9.5 (10.7)	15.2 (17.2)
Mean		12.1 (17.7)	11.6 (16.2)	9.6 (14.7)
S.D.		6.2 (8.0)	9.7 (9.7)	6.2 (11.6)

*M = male; F = female.

added to urine in the range 2.5–100 nmol. The limits of detection (signal-to-noise ratio = 2) for N^{τ}-MIAA, IAA and N^{π}-MIAA in urine were 10, 15 and 20 pmol/ml, respectively.

The precision of the method was determined from repeated injections ($n = 8$) of the same sample of urine. The coefficients of variation for N^{τ}-MIAA, IAA and N^{π}-MIAA were 7.6, 8.5 and 8.0% at mean concentrations of 15.0, 23.3 and 35.2 nmol/ml of urine, respectively. The recoveries of N^{τ}-MIAA, IAA and N^{π}-MIAA (30 nmol/ml each) added to a normal urine were 99.9, 100.5 and 105.2% ($n = 5$), respectively.

The amounts of N^{τ}-MIAA, IAA and N^{π}-MIAA in 24-h urines from ten healthy persons (six male, 20–48 years old; four female, 21–25 years old) were determined by the proposed method (Table I). The values for N^{τ}-MIAA, IAA and N^{π}-MIAA (nmol/mg of creatinine; μmol per 24 h in parentheses) were 4.3–24.6 (5.1–28.5), 5.7–39.9 (6.7–41.6) and 1.5–19.3 (2.5–36.4), respectively. These values are not greatly different from the reported data (N^{τ}-MIAA, 20.8 ± 1.3 nmol/mg of creatinine [4], 8.3–18.5 μmol per 24 h [3], 8.5–16.7 μmol per 24 h [5]; IAA, 2.4–27.0 μmol per 24 h [6]; N^{π}-MIAA, 19.2 ± 8.5 nmol/mg of creatinine [4], 0–81 μmol per 24 h [1]).

This study provides the first HPLC method with fluorescence detection for the simultaneous determination of N^{τ}-MIAA, IAA and N^{π}-MIAA. The method is sensitive and should be useful in investigations of the turnover of histamine.

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