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Note

Identification of 1-guanidino-2-(4-imidazole)propionic acid in human urine by high-performance liquid chromatography and field desorption mass spectrometry

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Ten or more guanidino compounds, which are involved in ammonia metabolism and are related to arginine metabolism, are known to exist in human body fluids [1]. The accumulation of guanidinosuccinic acid [2] and methylguanidine [3] has been reported in patients with renal failure. Increases in the levels of several guanidino compounds, such as 2-oxo-5-guanidinovaleric acid, N-acetylarginine, argininic acid, 4-guanidinobutyric acid and homoarginine, have been observed in body fluids of patients with hyperargininaemia, which is caused by arginase deficiency [1]. Further, some guanidino compounds, such as 4-guanidinobutyric acid [4], N-acetylarginine [5], methylguanidine [6], 2-oxo-5-guanidinovaleric acid [7] and homoarginine [8], induce convulsions in experimental animals. These observations suggest that the accumulation of guanidino compounds could be the cause of neurological symptoms in patients with uraemia and hyperargininaemia. Therefore, it is important to elucidate the structure of unknown guanidino compounds in biological fluids and their physiological role and metabolic pathway.

In this study, a new derivative of L-histidine, 1-guanidino-2-(4-imidazole)propionic acid (GIPA), was identified by high-performance liquid chromatography (HPLC) and field desorption (FD) mass spectrometry. The excretion level of GIPA in normal human urine was also determined.

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EXPERIMENTAL

Synthesis of GIPA

Authentic GIPA was synthesized from L-histidine and O-methylisourea sulphate as follows, according to Weiss and Krommer [9]. An aqueous mixture of L-histidine and O-methylisourea sulphate was adjusted to pH 10 with 2 *M* sodium hydroxide solution and then heated at 65°C for 3 h with stirring. The mixture was located on a column of Amberlite CG-120 (H^+), which was washed with water and then eluted with 1 *M* ammonia solution. The Sakaguchi-positive fractions were collected and evaporated under reduced pressure. The residue was washed with hot methanol and recrystallized from hot water.

Purification of GIPA from human urine

Urine (4.5 l) from healthy humans was loaded on a column (30 cm×5 cm I.D.) of Amberlite CG-120 (H^+). After washing with water, the column was eluted with 1 *M* ammonia solution. The effluent was neutralized with 6 *M* hydrochloric acid and passed through a column (40 cm×2.5 cm I.D.) of Dowex 1-X8 (CH_3COO^-) to remove acidic compounds. The effluent was collected and evaporated to dryness and the residue was dissolved in water and loaded on a column (40 cm×2.5 cm I.D.) of Amberlite CG-120 (NH_4^+). The column was washed with water and the retained basic amino acids were eluted with 2 *M* ammonia solution. The effluent was evaporated to dryness and the residue was dissolved in a small amount of water, passed through a column (20 cm×2.5 cm I.D.) of Amberlite CG-50 (NH_4^+) and eluted with water. The first 400 ml of effluent with water were discarded and the next 300 ml were collected and evaporated to dryness. The dried residue was dissolved in a small amount of water and analysed by thin-layer chromatography (TLC) using a cellulose plate (5552, Merck, Darmstadt, F.R.G.). The solvent system was ethanol–28% ammonia (77:23, v/v). Detection was carried out by the Sakaguchi reaction. The substance with the same R_F value as synthetic GIPA was scraped off from the plate and was extracted with water. This TLC procedure was repeated twice to remove histidine. The aqueous extract from cellulose was applied to a column (15 cm×1.5 cm I.D.) of Amberlite CG-120 (H^+) and eluted with dilute ammonia solution (pH 11). The effluent was concentrated and a small amount of water was added to the residue. The precipitate formed by addition of acetone was used for FD mass spectrometry.

Urine specimens

Human urine specimens were obtained from thirteen healthy men (26–45 years of age) and twelve healthy women (23–54 years of age). Each urine specimen was adjusted to pH 2.0 with 0.1 *M* hydrochloric acid and was ultrafiltered with a CF25 membrane cone (Amicon, Lexington, MA, U.S.A.). A 200-μl aliquot of the filtrate was analysed by HPLC.

HPLC

The guanidino compounds in urine were analysed by HPLC based on the reaction with 9,10-phenanthrenequinone (PQ) according to Higashidate et al. [10],

with some modifications. The column used was Guanidinopak-III (5 cm × 6.0 mm I.D.) (Jasco, Tokyo, Japan) packed with a strongly acidic cation-exchange resin (SO_3^-); cross-linkage (divinylbenzene), 10%; particle size, 5 μm; column temperature, 70°C. Sodium citrate buffer (0.4 *M*) and 1 *M* sodium hydroxide solution were used for elution. The column was equilibrated with a buffer of pH 3.0 for 10.2 min and eluted with a buffer of pH 3.5 for 4.0 min, followed by a buffer of pH 5.25 for 4.2 min, a buffer of pH 10.0 for 9.4 min and 1 *M* sodium hydroxide solution for the last 5.2 min. The eluent was delivered to the separation column by the pump (Jasco TWINCLE) at a constant flow-rate of 0.1 ml/min. Samples were injected via a Jasco AS-60 μD autosampler.

The fluorescence-inducing reagents used were 0.05% PQ in dimethylformamide and 2 *M* sodium hydroxide solution. Each reagent was delivered successively by the pump (Jasco SP-024) and mixed with the column effluent containing separated guanidino compounds. The flow-rates of the fluorescence-inducing reagents were 0.5 ml/min. The reaction was preformed at 70°C in a 5 m × 0.5 mm I.D. tube. The reaction product is a strongly fluorescent product, 2-amino-1-hydrophenanthro[9,10-*d*]imidazole [11]. The fluorescence was continuously monitored by a Jasco FP-110C fluorimeter at excitation wavelength of 365 and emission wavelength of >460 nm. The whole process was performed automatically by a computerized sequential programmer (Jasco UP-200).

Reference samples

The following reference substances were purchased: guanidinoacetic acid, arginine, guanidine (Wako Chemicals, Osaka, Japan), guanidinosuccinic acid, 3-guanidinopropionic acid (Sigma, St. Louis, MO, U.S.A.), N-acetylarginine (Eastman Kodak, Rochester, NY, U.S.A.), creatinine (Nakarai Chemicals, Osaka, Japan), homoarginine (Calbiochem, San Diego, CA, U.S.A.) and methylguanidine (Tokyo Kasei Organic Chemicals, Tokyo, Japan). Guanidinoethanesulphonic acid and 4-guanidinobutyric acid were donated by Ono Pharmaceutical (Osaka, Japan). 2-Guanidinoglutaric acid was synthesized from L-glutamic acid and S-methylisothiourea sulphate [12] and 2-guanidinoethanol from ethanolamine and O-methylisourea sulphate [13]. The above-mentioned chemicals were dissolved in 0.01 *M* hydrochloric acid at the concentrations indicated in Fig. 1.

FD mass spectrometry

FD mass spectra were recorded using a Shimadzu GCMS 9020-DF mass spectrometer with a Shimadzu SCAP-1123 mass data system (Shimadzu, Kyoto, Japan). Tungsten wires were used as emitters. After the synthetic GIPA and urine samples obtained as described above had been dissolved in water, a 1-μl aliquot of solution (containing about 3 μg of product) was placed on a tungsten wire and the solvent was evaporated. The emitter current was set to start at 0 mA and to stop at 45 mA; the current was increased 0.1 mA/s until the maximum current was reached. Other conditions were as follows: accelerating voltage, 3.0 kV; ionizing voltage, 70 eV; and mass scanning range, m/z 100–400.

RESULTS AND DISCUSSION

Fig. 1A shows a typical chromatogram of guanidino compounds in human urine. The unknown peak with a retention time of 15.6 min corresponded to the synthetic GIPA peak (Fig. 1B).

Fig. 2A shows the FD mass spectrum of synthetic GIPA. Typical ion peaks were observed at m/z 180, 198 and 359. The ion peak at m/z 198 corresponds to the protonated molecular ion $[MH]^+$; the others at m/z 180 and 359 could represent $[MH]^+ - H_2O$ and $2[MH]^+ - 2H_2O$, respectively. In the mass spectrum of the sample from human urine (Fig. 2B), ion peaks were observed at m/z 180, 198 and 359. By comparison of these spectra, the isolated guanidino compound from human urine was identified as GIPA. Although these spectra were recorded at an emitter current of 23–25 mA, the relative intensities of the characteristic ions for the synthetic and human GIPA were slightly different. The relative intensities

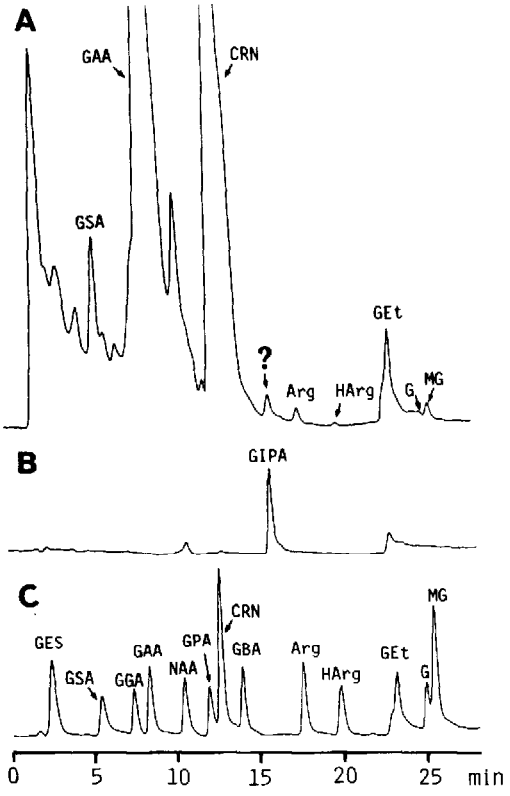

Fig. 1. High-performance liquid chromatogram of (A) guanidino compounds in human urine, (B) synthetic GIPA (3.5 μM) and (C) an authentic sample of other guanidino compounds. Abbreviations and concentrations in the authentic sample: GES = guanidinoethanesulphonic acid, 10 μM; GSA = guanidinosuccinic acid, 5 μM; GGA = 2-guanidinoglutaric acid, 20 μM; GAA = guanidinoacetic acid, 3 μM; NAA = N-acetylarginine, 3 μM; GPA = 3-guanidinopropionic acid, 3 μM; CRN = creatinine, 50 μM; GBA = 4-guanidinobutyric acid, 20 μM; Arg = arginine, 20 μM; HArg = homoarginine, 10 μM; GEt = 2-guanidinoethanol, 1 μM; G = guanidine, 20 μM; MG = methylguanidine, 2 μM. The chromatographic conditions are indicated in the text.

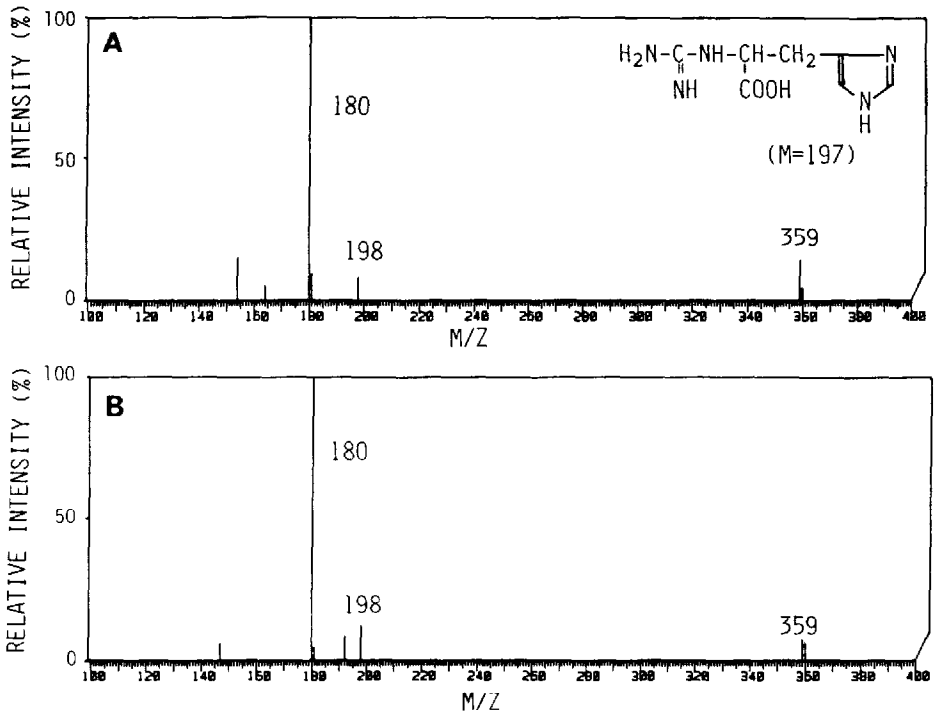

Fig. 2. FD mass spectra of (A) synthetic GIPA and (B) human urinary GIPA.

TABLE I

URINARY EXCRETION LEVELS OF GUANIDINO COMPOUNDS IN HEALTHY HUMANS

The values are means ± S.D. ($n=25$); the figures in parentheses indicate the range.

Compound	Concentration (μmol/g of creatinine)	
1-Guanidino-2-(4-imidazole)propionic acid	1.9 ± 0.9	(0.7–3.9)
Guanidinosuccinic acid	27.5 ± 11.5	(5.7–58.9)
Guanidinoacetic acid	608.1 ± 262.9	(254.6–1258.7)
3-Guanidinopropionic acid	< 2.8	
4-Guanidinobutyric acid	< 4.5	
Arginine	25.3 ± 14.9	(10.2–66.8)
Homoarginine	< 53	
2-Guanidinoethanol	5.6 ± 1.7	(3.1–10.0)
Guanidine	10.9 ± 3.2	(4.7–19.1)
Methylguanidine	2.0 ± 1.7	(0.6–9.0)

are highly dependent on the temperature of emitter during the desorption process [14], and slight temperature differences between the two recordings could explain this difference.

Recently, mass spectrometric studies of guanidino compounds have been performed by gas chromatography–mass spectrometry (GC–MS) [15]. In this GC–MS method, guanidino compounds were converted into dimethylpyrimidyl

derivatives by reaction with acetylacetone, as guanidino compounds are not volatile; GC–MS studies by this method provide information on the structure of guanidino compounds [15–17]. GIPA was tested by this GC–MS method, but a mass spectrum was not recorded. The large basic imidazole moiety of GIPA might interrupt the conversion of the amidine group to the dimethylpyrimidyl group.

The urine concentration of GIPA in healthy humans was 1.9 ± 0.9 μmol/g of creatinine (mean $\pm$ S.D. $n = 25$) (Table I). The physiological function and biosynthetic pathway of GIPA is not known. Guanidino compounds are commonly considered to be related to ammonia metabolism. Levels of guanidino compounds are reported to increase in serum and urine in pathological conditions, such as renal failure and inborn errors of the urea cycle [1–3]. However, the contribution of GIPA to nitrogen excretion seems to be small in healthy subjects, as their urinary excretion level of GIPA was found to be very low.

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REFERENCES

1 B. Marescau, I.A. Qureshi, P. De Deyn, J. Letarte, R. Ryba and A. Lowenthal, Clin. Chim. Acta, 146 (1985) 21.
2 B.D. Cohen, I.M. Stein and J.E. Bonas, Am. J. Med., 45 (1968) 63.
3 S. Giovannetti, P.L. Balestri and G. Barsotti, Arch. Intern. Med., 131 (1973) 709.
4 D. Jinnai, A. Sawai and A. Mori, Nature (London), 212 (1966) 617.
5 A. Mori and H. Ohkusu, Adv. Neurol. Sci., 15 (1971) 303.
6 M. Matsumoto, K. Kobayashi, H. Kishikawa and A. Mori, IRCS Med. Sci., 4 (1976) 65.
7 B. Marescau, M. Hiramatsu and A. Mori, Neurochem. Pathol., 1 (1983) 203.
8 I. Yokoi, J. Toma and A. Mori, Neurochem. Pathol., 2 (1984) 295.
9 V.S. Weiss and H. Krommer, Chem. Z., 98 (1974) 617.
10 S. Higashidate, T. Maekubo, M. Saito, M. Senda and T. Hoshino, in A. Mori, B.D. Cohen and A. Lowenthal (Editors), Guanidines, Plenum Press, New York, 1985, p. 3.
11 H.A. Itano and S. Yamada, Anal. Biochem., 48 (1972) 483.
12 A. Mori, M. Akagi, Y. Katayama and Y. Watanabe, J. Neurochem., 35 (1980) 603.
13 Y. Watanabe, S. Shindo and A. Mori, Eur. J. Biochem., 147 (1985) 465.
14 E.L. Esmans, F.C. Alderweireldt, B.A. Marescau and A.A. Lowenthal, Anal. Chem., 56 (1984) 693.
15 A. Mori, T. Ichimura and H. Matsumoto, Anal. Biochem., 89 (1978) 393.
16 B. Marescau, A. Lowenthal, E. Esmans, Y. Luyten, F. Alderweireldt and H.G. Terheggen, J. Chromatogr., 224 (1981) 185.
17 I. Yokoi, Y. Watanabe, A. Edaki and A. Mori, Life Sci., 41 (1987) 1305.