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ISOLATION AND IDENTIFICATION OF SOME GUANIDINO COMPOUNDS IN THE URINE OF PATIENTS WITH HYPERARGININAEMIA BY LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Liquid column chromatographic studies of monosubstituted guanidino compounds, which are excreted in the urine of patients with hyperargininaemia are reported. The guanidino-positive peaks, with the highest excretion values, were isolated from urine and the isolated compounds were identified by thin-layer chromatography and gas chromatography—mass spectrometry. Guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid, arginine and α-keto-δ-guanidinovaleric acid were found to be excreted at high levels in the urine of patients with hyperargininaemia compared with controls.

INTRODUCTION

Patients with hyperargininaemia have an arginase deficiency, which leads to blockage of the urea cycle in the last step with symptoms of coma, epilepsy, spasticity and vomiting.

Owing to the arginase deficiency, patients with hyperargininaemia accumu-

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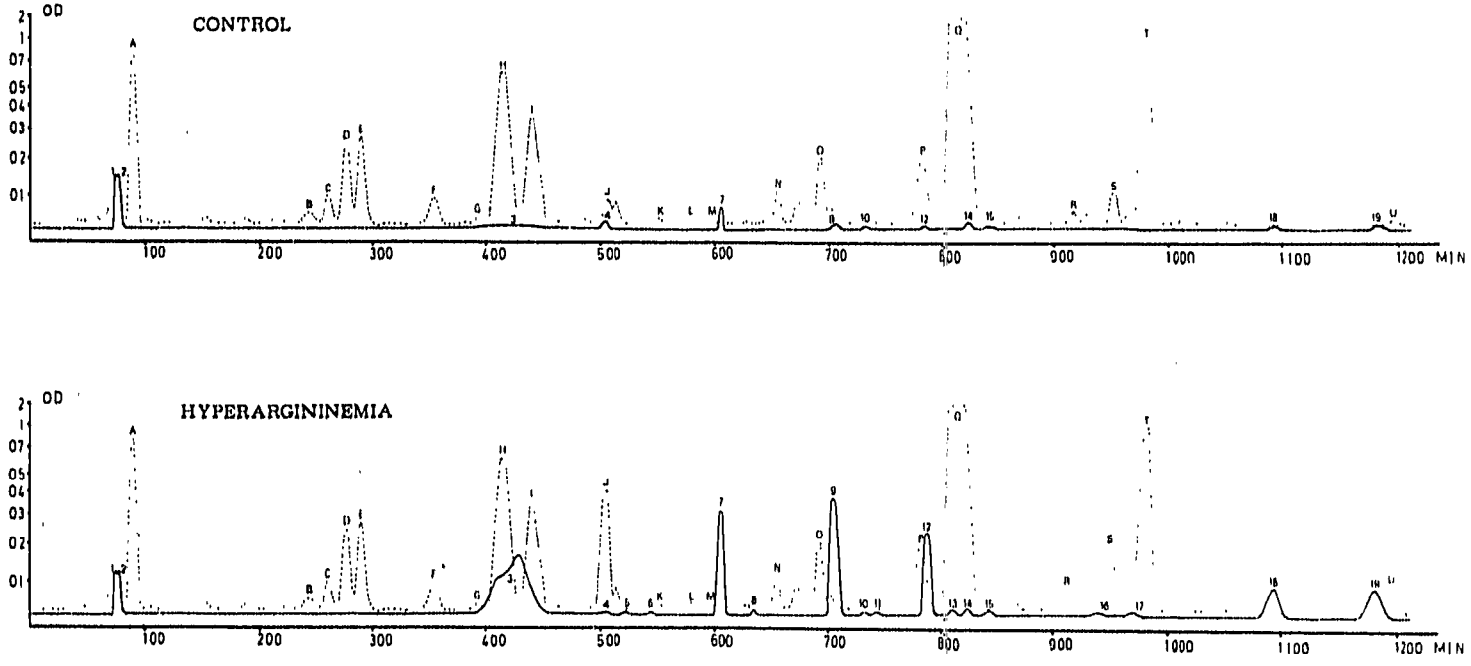

Fig. 1. Elution pattern of urinary monosubstituted guanidino compounds, in parallel with amino acids, of patients with hyperargininaemia and controls. The peak numbers correspond to those in Table I. A, Urea; B, aspartic acid; C, threonine; D, serine; E, glutamine; F, glutamic acid; G, citrulline; H, glycine; I, alanine; J, cystine; K, methionine; L, isoleucine; M, leucine; N, tyrosine; O, β-aminoisobutyric acid; P, ethanolamine; Q, ammonia; R, ornithine; S, lysine; T, histidine; U, arginine.

TABLE I

ABSOLUTE RETENTION TIMES OF MONOSUBSTITUTED GUANIDINO COMPOUNDS ON THE AAI AMINO ACID ANALYSER

Standard*	Hyperargininaemic urine peak number	Retention time (min)	Concentration in urine** (μmole/g creatinine)			
			Patient A.W.***	Patient M.W.***	Patient I.W.***	Controls§
Opheline	—	68	—	—	—	—
§§	1	76	138	N.D.	59	106–180
Urea	2	78	229	N.D.	98	269–469
Taurocyamine	—	86	—	—	—	—
α-Keto-δ-GVA	3	422	5650	6410	904	Trace
Guanidinosuccinic acid	4	505	Trace	—	—	14–97
§§	5	522	Trace	13	—	—
Octopine	—	538	—	—	—	—
§§	6	545	Trace	32	—	—
α-Guanidinoglutaric acid	—	577	—	—	—	—
Guanidinoacetic acid	7	605	1764	2764	1182	182–1213
α-Guanidinopropionic acid	8	634	—	19	—	—
α-Guanidinobutyric acid	—	677	—	—	—	—
N-α-Acetylarginine, α-G-β(CH_3)-BA	9	706	1037	4271	649	19–50
§§	10	730	—	6	10	Trace–19
§§	11	745	21	16	—	—
Argininic acid, α-G-β(CH_3)-VA	12	789	909	1069	61	3–36
§§	13	810	Trace	Trace	Trace	—
§§	14	823	50	280	Trace	28–78
γ-Guanidino-β-OH-butyric acid	—	827	—	—	—	—
§§	15	843	26	75	Trace	Trace–9
β-Guanidinopropionic acid	—	882	—	—	—	—
β-Guanidinoisobutyric acid	—	920	—	—	—	—
L-Arginylaspartic acid	16	940	Trace	Trace	Trace	—
α-Guanidino-β-phenylpropionic acid	—	950	—	—	—	—
§§	17	968	13	52	Trace	—
α-NH_2-β-guanidinopropionic acid	—	1057	—	—	—	—
γ-GB.$CONH_2$, γ-OH-Arg, γ-GBA	18	1093	167	233	19	Trace–31
α-NH_2-γ-guanidinobutyric acid	—	1118	—	—	—	—
Arginine	19	1181	86	127	497	10–73

*α-Keto-δ-GVA = α-keto-δ-guanidinovaleric acid; α-G-β(CH_3)-BA = α-guanidino-β-methylbutyric acid; α-G-β(CH_3)-VA = α-guanidino-β-methylvaleric acid; γ-GB.$CONH_2$ = γ-guanidinobutyramide; γ-OH-Arg = γ-hydroxyarginine; γ-GBA = γ-guanidinobutyric acid.

**N.D. = not determined.

***Excretion values on low-protein diet.

§Range for eight control children.

§§Unknown monosubstituted guanidino compound.

late arginine [1,2], which leads to excretion of high levels of guanidino compounds, catabolites of arginine, in the urine [3,4]. Fig. 1 shows the elution patterns of the different monosubstituted guanidino compounds in parallel with the amino acids in the urine of patients and controls. As can be seen in Table I, some guanidino compounds are excreted in 10—100-fold greater amounts than in controls. In addition, some monosubstituted guanidino compounds are excreted at much lower levels but always in higher concentration than in controls. The guanidino compound that is present in the greatest amount has been identified as α-keto-δ-guanidinovaleric acid [5] by liquid, thin-layer (TLC) and gas chromatography—mass spectrometry (GC—MS).

In this work the structures of other guanidino-positive urine peaks were elucidated by using the above-mentioned techniques. These identification techniques were necessary as different guanidino-positive compounds can have identical retention times in liquid column chromatography (Table I). The isolated compounds were identified by TLC and GC—MS.

EXPERIMENTAL

Apparatus

A Technicon AAI amino acid analyser (Technicon Instruments, Tarrytown, NY, U.S.A.) was used. The liquid chromatograph was equipped with a column (140 cm × 6.2 mm I.D.) packed with Dowex 50-X8 resin (Technicon Chromobeads, Type A, particle size 21 μm). The flow-rate was 0.5 ml/min.

GC—MS analyses were carried out on a Nermag R 10-10 quadrupole mass spectrometer. The mass spectrometer was connected with a Girdel gas chromatograph, and a PDP/8a computer system (Nermag, Rueil-Malmaison, France).

TLC was carried out on glass plates coated with cellulose, layer thickness 0.1 mm (E. Merck, Darmstadt, G.F.R.).

Reagents

For liquid and thin-layer chromatography, all reagents were of analytical-reagent grade. The silylating agents hexamethyldisilazane and trimethylchlorosilane were obtained from Pierce (Rockford, IL, U.S.) and the acetylating agent trifluoroacetic anhydride from Aldrich (Milwaukee, WI, U.S.A.). The dimethylpyrimidyl-forming agent was acetylacetone (Merck).

Specimens

The patients were hyperargininaemia were three sisters.

Monosubstituted guanidino standard compounds

Guanidinosuccinic acid, urea, octopine, guanidinoacetic acid, N-α-acetylarginine, argininic acid, β-guanidinopropionic acid, L-arginylaspartic acid, γ-guanidinobutyric acid and arginine were purchased from Sigma (St. Louis, MO, U.S.A.) and α-amino-β-guanidinopropionic acid and α-amino-γ-guanidinobutyric acid from Calbiochem (Lucerne, Switzerland).

Taurocyamine, α-guanidinopropionic acid, α-guanidinobutyric acid, α-guanidino-β-methylbutyric acid, α-guanidino-β-methylvaleric acid and α-guanidino-β-phenylpropionic acid were prepared starting from the corresponding

amino derivative and S-methylisothiourea, according to the method described by Schütte [6]. α-Keto-δ-guanidinovaleric acid was prepared enzymatically as described by Cooper and Meister [7].

γ-Guanidino-β-hydroxybutyric acid, γ-guanidinobutyramide and γ-hydroxyarginine were gifts from Prof. D.J. Durzan (Dept. of Fisheries and Forestry, Petawawa Forest Experiment Station, Chalk River, Ontario, Canada). Opheline and β-guanidinoisobutyric acid were kindly supplied by Prof. Y. Robin (Biochimie Marine, Collège de France, Paris, France). α-Guanidinoglutaric acid was a gift from Prof. A. Mori (Institute for Neurobiology, Okayama, Japan).

Liquid column ion-exchange chromatography

Urine of patients with hyperargininaemia and the standard products were applied either separately or together on the amino acid analyser. The free monosubstituted guanidino compounds were analysed in parallel with amino acids according to the method of Durzan [8]. Liquid ion-exchange chromatography was performed according to the procedure described by Efron [9].

The monosubstituted guanidino compounds were detected with Sakaguchi reagent, prepared as described by Durzan [8]. This reagent is specific for these compounds.

Desalting of the fractions corresponding to the guanidino-positive compounds

The eluent from the amino acid analyser, containing the guanidino-positive peaks 3, 7, 9, 12, 18 and 19 (Table I), was collected and desalted on Dowex 50W-X8 (H^+) ion-exchange resin (50—100 mesh). The guanidino compounds were eluted with 0.5 mol/l ammonia solution, except for arginine, for which 0.75 mol/l ammonia solution was used. Ammonia was removed immediately from the eluent by means of a rotary evaporator. The eluent was then lyophilized and aliquots were used for TLC and GC—MS studies.

Thin-layer chromatography

One-dimensional TLC was performed in a saturated chamber. In order to obtain identical saturation conditions, a device in the lid was made for holding the loaded plates before lowering them in the solvent.

The solvents used were *n*-butanol—glacial acetic acid—water (BuA) (120:30:50) and *n*-butanol—pyridine—water (BuP) (65:65:65). After chromatography the plates were dried at room temperature and the guanidino compounds were located spraying the plates with Sakaguchi reagent prepared according to Robin [10].

Derivatization and GC—MS conditions

As compounds containing a guanidino function are not suitable for GC—MS analysis, the isolated urine fractions were converted into dimethylpyrimidyl derivatives using the method described by Mori et al. [17]. The dimethylpyrimidyl derivatives were then silylated or acylated.

The silylation procedure applied to the dimethylpyrimidyl derivatives of urine peaks (9 and 19) was not satisfactory. Therefore, these dimethylpyrimidyl derivatives were acylated: the dimethylpyrimidyl derivatives were dissolved in 10 ml of *n*-butanol, saturated with dry hydrogen chloride gas and refluxed for 3 h in a water-bath.

The syrup-like substance obtained after drying in vacuo was trifluoroacetylated with 10% trifluoroacetic anhydride in ethyl acetate and applied to the gas chromatograph.

The dimethylpyrimidyl derivatives of peaks 3, 7, 9 and 12 (Table I) were silylated: the dimethylpyrimidyl derivatives were treated with a mixture of 0.5 ml of pyridine, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. The silylation occurred at room temperature.

The gas chromatograph was fitted a 2.5-m column of 3% SE-30 on Chromosorb W and a helium flow-rate of 20 ml/min was used. After injection of an aliquot of 1 μl, the oven was programmed from 80 to 220°C at 8°C/min. The mass spectral conditions were as follows: source temperature, 150°C; ionization voltage, 70 eV; emission current, 200 μA; and integration time, 6 msec/peak.

RESULTS

The absolute retention times for monosubstituted guanidino compounds, standards and urine compounds on the amino acid analyser are given in Table I.

Comparison of the retention times of a set of guanidino standards with those of the products eluted from the urine sample showed that peaks 3, 7, 9, 12, 18 and 19 could be attributed to α-keto-δ-guanidinovaleric acid, guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid and arginine, respectively. These designations were confirmed by spiking the urine sample with the standard guanidino compounds, identical peaks being observed.

The identities of peaks 3, 7, 9, 12, 18 and 19 were first investigated by TLC. The hR_F values of the standard products are the same as those of the corresponding isolated urine fraction. Using BuA as solvent, the hR_F values for α-keto-δ-guanidinovaleric acid, guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid and arginine were 45, 39, 51, 46, 58 and 18, respectively. Using BuP as solvent, the hR_F values were 43, 32, 41, 38, 41 and 5, respectively.

Fig. 2 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 3. The molecular ion $M^{+\cdot}$ at m/z = 291 was due to the dehydrated cyclic silylated dimethylpyrimidyl derivative of α-keto-δ-guanidinovaleric acid. Loss of water occurs during the derivatization procedure. Typical fragment ions are at m/z = 276, 202, 174, 107 and 73, the origin of which is shown in Fig. 2. The ion at m/z = 200 (base peak) can be explained by the elimination of $(CH_3)_3SiOH$ from the $(M - H)^+$ fragment ion (m/z = 290). Loss of a $(CH_3)_3$-SiOCO radical from the $(M - H)^+$ ion leads to m/z = 173.

Fig. 3 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 7. The molecular ion $M^{+\cdot}$ at m/z = 253 corresponds to the silylated dimethylpyrimidyl derivative of guanidinoacetic acid. The typical fragment ions are at m/z = 238, 180, 163, 136, 107—108 and 73, the origin of which is shown in Fig. 3.

Fig. 4 shows the mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 9. The molecular ion $M^{+\cdot}$ at m/z = 336 corresponds to the dimethylpyrimidyl derivative of N-α-acetylarginine. The typical fragment ions are at m/z = 263, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 4.

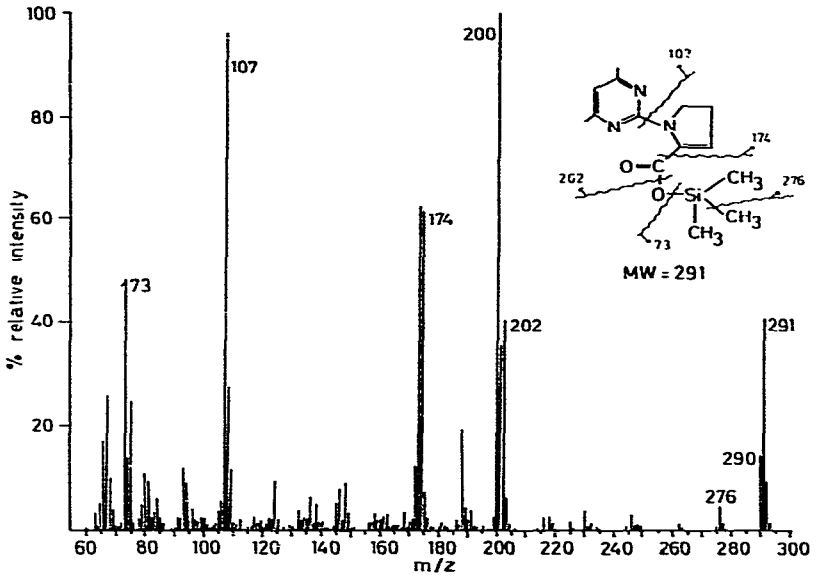

Fig. 2. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 3 (dimethylpyrimidyl derivative of α-keto-δ-guanidinovaleric acid).

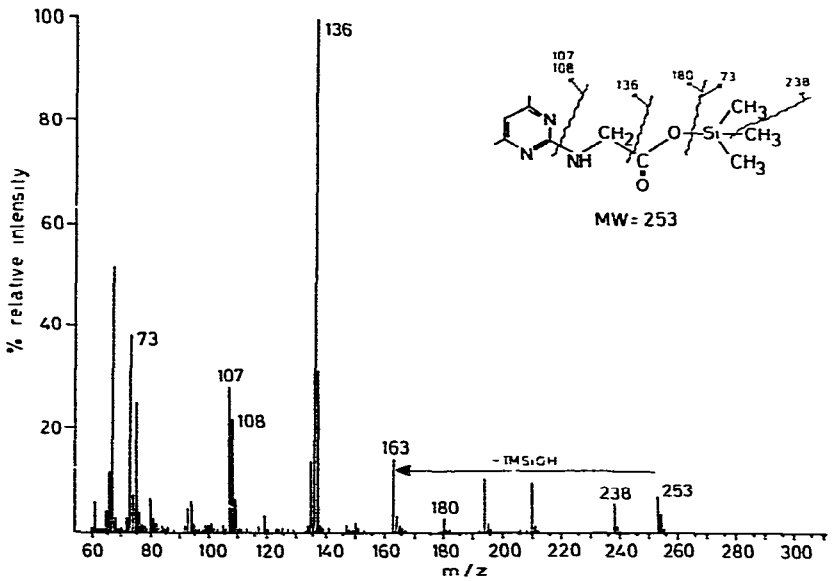

Fig. 3. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 7 (silylated dimethylpyrimidyl derivative of guanidinoacetic acid).

Fig. 5 illustrates the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 12. The molecular ion $M^{+\bullet}$ at $m/z = 383$ corresponds to the silylated dimethylpyrimidyl derivative of argininic acid. The typical fragment ions are at $m/z = 368$, 278, 266, 176, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 5.

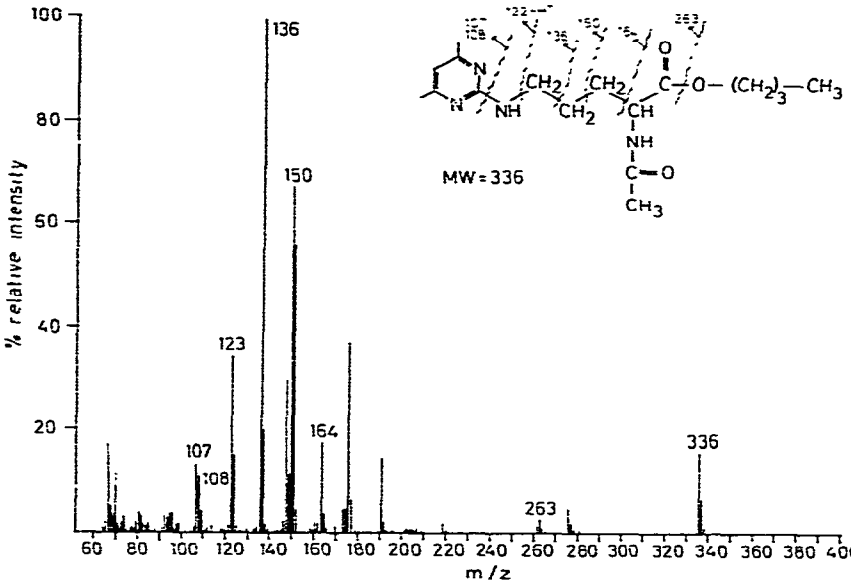

Fig. 4. Mass spectrum of the dimethylpyrimidyl derivative of peak 9 (dimethylpyrimidyl derivative of N-α-acetylarginine).

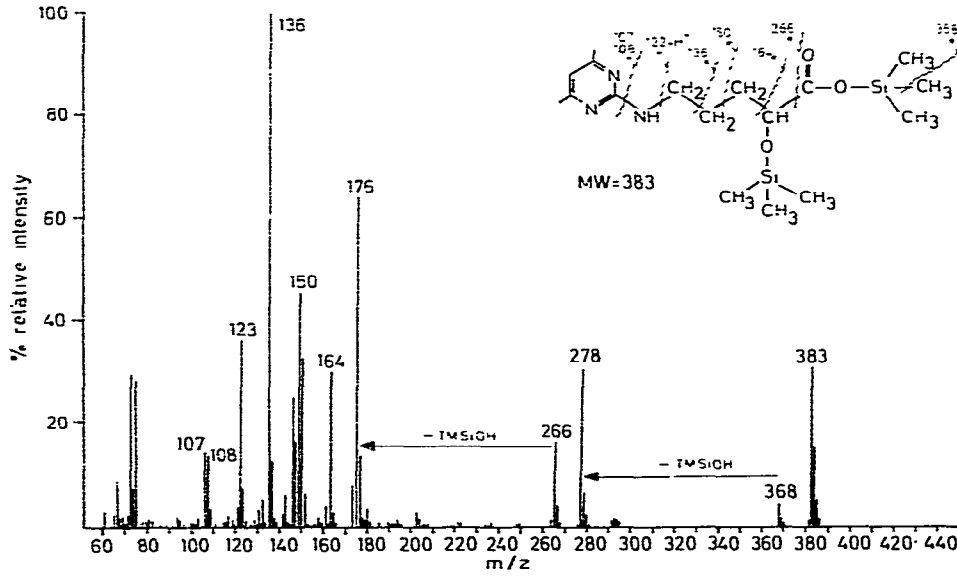

Fig. 5. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 12 (silylated dimethylpyrimidyl derivative of argininic acid).

Fig. 6 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 18. The molecular ion $M^{+\cdot}$ at m/z = 281 corresponds to the silylated dimethylpyrimidyl derivative of γ-guanidinobutyric acid. The typical fragment ions are at m/z = 266, 192, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 6.

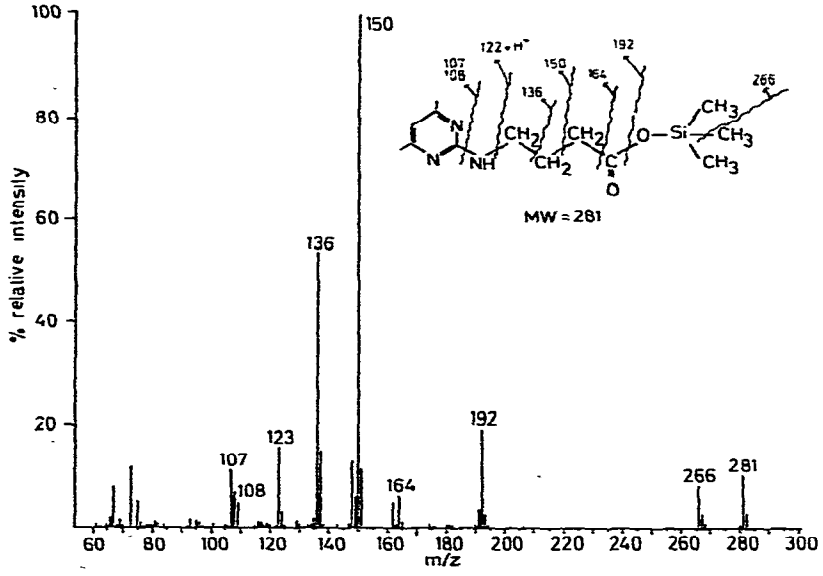

Fig. 6. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 18 (silylated dimethylpyrimidyl derivative of γ-guanidinobutyric acid).

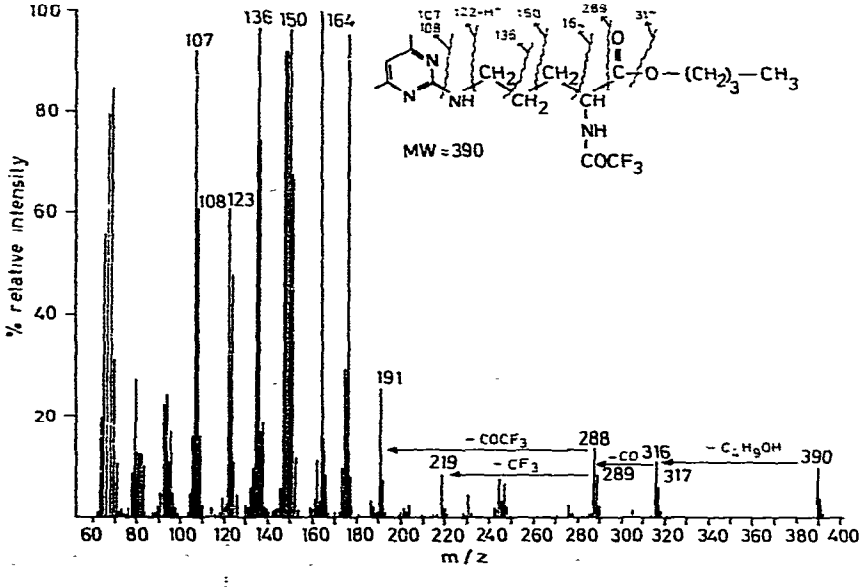

Fig. 7. Mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 19 (trifluoroacetylated dimethylpyrimidyl derivative of arginine).

Fig. 7 shows the mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 19. The molecular ion $M^{+\cdot}$ at $m/z = 390$ corresponds to the trifluoroacetylated dimethylpyrimidyl derivative of arginine. The typical fragment ions are at $m/z = 316$, 288, 219, 191, 164, 150, 136, 123 and 107–108, the origin of which is shown in Fig. 7.

DISCUSSION

The neurological complaints of hyperargininaemia patients raise the question of whether the neurological symptoms are caused by hyperammonaemia, by high arginine levels or by high levels of guanidino compounds. Perhaps all of these anomalies could influence the clinical picture.

Little is known about the neurotoxicity of guanidino compounds in man, although γ-guanidinobutyric acid [11], taurocyamine [12] and methylguanidine [13] have epileptogenic effects on rabbits and cats when administered intracisternally. When more knowledge is acquired about the neurotoxicity of the guanidino compounds, it might then be said that the guanidino compounds are more beneficial than harmful to the patients. In patients with hyperargininaemia the urea cycle is blocked at the last step. If arginine were catabolized by enzymes other than arginase, nitrogen excretion could occur as guanidino compounds instead of urea. Guanidinoacetic acid can be formed by a transamidination reaction: the amidino group of arginine is transferred to glycine [14]. Guanidinoacetic acid is an intermediate in the biosynthesis of creatine and creatinine. γ-Guanidinobutyric acid can also be formed by transamidination of arginine to γ-aminobutyric acid [15]. N-α-Acetylarginine could be formed by acetylation of arginine, just like the formation of N-α-acetylglycine [16]. Deamination of arginine, followed by hydrogenation, could give argininic acid. α-Keto-δ-guanidinovaleric acid could be formed by transamination or deamination of arginine. The other monosubstituted guanidino compounds, in minor concentrations, are also important as nitrogen waste products, and we shall therefore try to identify them all.

We have given here liquid chromatographic data for the mono-substituted guanidino compounds in the urine of three sisters with hyperargininaemia. TLC and GC—MS data for the isolated urinary guanidino compounds have confirmed the identities of guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid, arginine and α-keto-δ-guanidinovaleric acid.

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