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# Isolation and Identification of 2-Oxo-5-guanidinovaleric Acid in Urine of Patients with Hyperargininaemia by Chromatography and Gas Chromatography/Mass Spectrometry

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**Summary**: An unknown guanidino-positive compound peak has been identified in urines of three sisters affected with hyperargininaemia. Identification was made on the basis of its similarity with the liquid and thin-layer chromatographic characteristics of enzymatically synthesized 2-oxo-5-guanidinovaleric acid. Identification was also made by combined gas chromatography – mass spectrometry of the unknown compound peak. The synthesis of enzymatically formed 2-oxo-5-guanidinovaleric acid was controlled by nuclear magnetic resonance and combined gas chromatography – mass spectrometry.

*Isolierung und Identifizierung von 2-Oxo-5-guanidinovaleriansäure im Harn von Patienten mit Hyperargininämie durch Chromatographie und Gaschromatographie/Massenspektrometrie*

**Zusammenfassung**: Eine unbekannte Guanidin-positive Verbindung im Harn dreier Schwestern mit Hyperargininämie wurde identifiziert. Die Identifizierung erfolgte aufgrund der Ähnlichkeit des flüssig- und dünnschicht-chromatographischen Verhaltens von enzymatisch synthetisierter 2-Oxo-5-guanidinovaleriansäure sowie kombinierter Gaschromatographie/Massenspektrometrie der unbekannten Substanz. Die Synthese der enzymatisch hergestellten 2-Oxo-5-guanidinovaleriansäure wurde mit Kernresonanzspektrometrie und kombinierter Gaschromatographie/Massenspektrometrie kontrolliert.

## Introduction

An unknown guanidino-positive compound peak was previously shown by liquid chromatography in the urine of three sisters affected with hyperargininaemia. This compound peak was named compound X as shown in the previous publications (1–3). This peak, having more than one shoulder, appears complex and is *Sakaguchi* and α-naphthol positive. This peak is unstable and can degrade to 4-guanidinobutyric acid. By using the same color factor as the nearest eluted guanidino compounds, concentration values up to 5000 μmol/g creatinine were found. Only traces are found in the urines of controls. In our preliminary study, this unknown compound peak was identified as 2-oxo-5-guanidinovaleric acid (4).

Figure 1 gives the different monosubstituted guanidino compounds in parallel with the amino acids, in urine of the patients. The concentrations of the guanidino compounds in patients with hyperargininaemia are ten to hundred times higher than in controls, except for guanidinosuccinic acid. There is much controversy about the biosynthesis of guanidinosuccinic acid (5, 6).

The neurological disturbances in the patients with hyperargininaemia could be caused by hyperammonaemia, but the high guanidino levels could also play a pathological role. Indeed, some guanidino compounds have convulsive effects on rabbits and cats when administered intracisternally (7).

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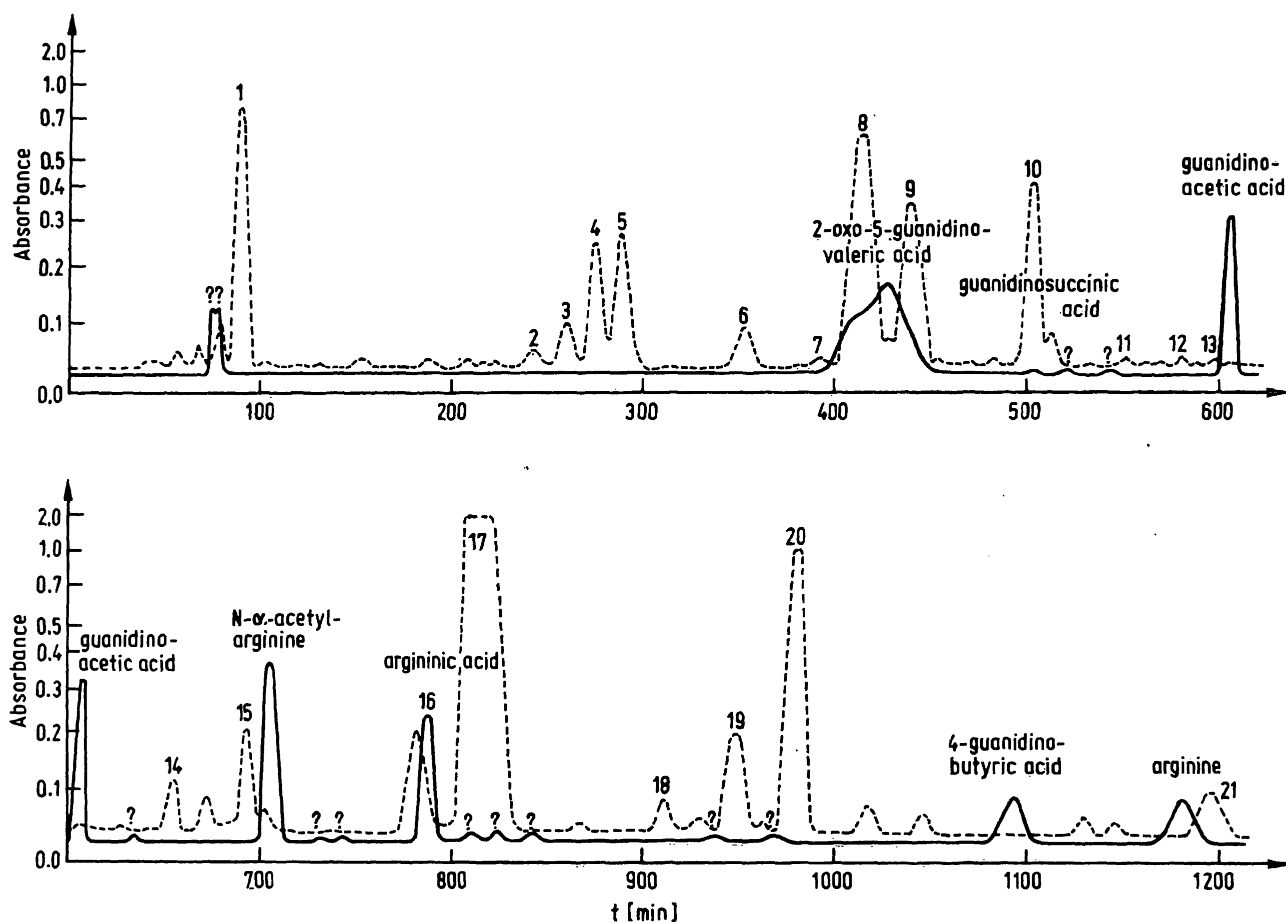


Fig. 1. Elution pattern of urinary monosubstituted guanidino compounds, in parallel with amino acids, of patients with hyperargininemia.
? = unknown monosubstituted guanidino compounds.
1. urea, 2. aspartic acid, 3. threonine, 4. serine, 5. glutamine, 6. glutamic acid, 7. citrulline, 8. glycine, 9. alanine, 10. cystine, 11. methionine, 12. isoleucine, 13. leucine, 14. tyrosine, 15. 3-amino*iso*butyric acid, 16. ethanolamine, 17. ammonia, 18. ornithine, 19. lysine, 20. histidine, 21. arginine.

## Materials and Methods

### *Materials*

#### *Apparatus*

Liquid chromatography was performed on a Technicon AAI amino acid analyzer (Technicon Instruments, Tarrytown NY 10591). The column, length 140 cm, internal diameter 6.2 mm, was filled to 130 cm with Dowex 50 X 8 (resin Technicon Chromobeads® type A, particle size 21 μm). The operating flow rate was 0.5 ml/min.

The GC-MS apparatus was a Ribermag R10-10, fitted with a 2.5 m 3% SE 30 on chromosorb W column, (NERMAG, 92502, Rueil-Malmaison, Paris, France). Nuclear magnetic resonance spectra were determined with a Jeol JNM-PS 100 spectrometer operating at 100 MHz for protons (Jeol LTD 1418 Nakagami Akishima, Tokyo 196)

For desalting the unknown peak a jacketed Econo-column with nylon 3-way Luer Stopcocks, measuring 50 X 1.5 cm was used, (Bio-Rad, Richmond, California).

#### *Reagents*

For liquid chromatography, all the reagents were pro analysis. The resin used for desalting was Dowex 50W-X8 ($H^+$) (50 to 100 mesh) and obtained from Fluka (Fluka AG, CH-9470 Buchs, Switzerland). The silylating agents were obtained from Pierce (Pierce Chemical Company, Rockford IL 61105). The dimethylpyrimidyl forming agent was acetylacetone, Merck (E. Merck, Darmstadt, West-Germany).

#### *Specimens*

The urines of the three patients, received through the care of one of us (Prof. Dr. *H. G. Terheggen*) were studied.

### Methods

#### *Enzymatic synthesis of 2-oxo-5-guanidinovaleric acid*

2-Oxo-5-guanidinovaleric acid was synthesized according to the method described by *Cooper* et al. (8) with minor modifications. The enzymatic synthesis of 2-oxo-5-guanidinovaleric acid was controlled by nuclear magnetic resonance and GC-MS studies.

#### *Nuclear magnetic resonance control of the enzymatically synthesized 2-oxo-5-guanidinovaleric acid*

An aliquot was dissolved in $D_2O$ and the spectra were run at 100 MHz.

#### *Desalting of the urine fraction corresponding with the unknown guanidino-positive compound peak*

The eluent from the AAI analyzer, containing the unknown guanidino positive peak, was collected and desalted on the jacketed Econo-column of Dowex 50 W-X8 ($H^+$) (50 to 100 mesh). The jacket temperature was 3 °C. After applying the salt

solution, the column was washed with distilled water. The elution was carried out with $NH_4OH$ (0.3 mol/l) long enough for the eluent to become basic. After this, the column was washed with a final volume of water. For a resin height of 25 cm, the first washing volume was 250 ml, the elution volume 250 ml and the final volume 75 ml. The used flow rate was 0.5 ml/min. Ammonia was removed as soon as possible from the eluent with a rotavapor. Then the eluent was lyophilised and an aliquot was used for thin-layer chromatography and for GC-MS studies.

*Liquid ion-exchange chromatography*

Free monosubstituted guanidino compounds were analysed in parallel with amino acids as described by *Durzan* (9). The elution procedure for liquid ion-excange chromatography was according to *Efron* (10).

*Thin-layer chromatography*

One dimensional thin-layer chromatography was performed in a chamber saturated with solvent by means of filter paper lining. Different solvents were used, e.g. *n*-propanol/acetic acid 1 mol/l (150ml + 50 ml).

After chromatography, the plates were dried at room temperature. To locate the guanidino compounds, the plates were sprayed with *Sakaguchi* reagent prepared according to *Jepson* et al. (11). Orange to red spots appeared.

*Derivatization and GC-MS conditions*

For GC-MS studies the guanidino compounds were converted to dimethylpyrimidyl derivatives by a method described by *Mori* et al. (12): aliquots of about 5 mg of enzymatically synthesized 2-oxo-5-guanidinovaleric acid and the desalted urine fraction were used. The dimethylpyrimidyl derivatives were silylated with 0.5 ml pyridine, 0.2 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane. The silylation occurred at room temperature. A helium flow of 20 ml/min was used. After injection of an aliquot of 1 $\mu$l, the oven was programmed from 80 °C up to 220 °C at 8 °C/min.

The mass spectral conditions were: source temperature 140 °C; ionisation voltage: 80 eV; emission current : 200 $\mu$A; integration time : 6 ms.

## Results

The proton nuclear magnetic resonance studies have confirmed the findings of *Cooper* et al. (8) that 2-oxo-5-guanidinovaleric acid exists in equilibrium with its cyclic form. We have found 23% open and 77% closed form. Figure 2 gives the structure, and table 1 gives the chemical shift $\delta$-values in ppm relative to internal DSS.

The shape and elution time of the unknown guanidino-positive compound peak were the same as those of enzymatically synthesized 2-oxo-5-guanidinovaleric acid. One and the same compound peak was observed by chromatography of hyperargininaemia urine together with the enzymatically synthesized 2-oxo-5-guanidinovaleric acid. The presence of more shoulders could be caused by modifications of the equilibrium during the elution. The elution time was the same as those of citrulline, glycine and alanine.

The $R_F$ value of enzymatically synthesized 2-oxo-5-guanidinovaleric acid was the same as those of the unknown guanidino positive urine peak. One and the same spot was observed by chromatography of a mixture of both fractions. Figure 3 gives the TLC results in *n*-propanol/

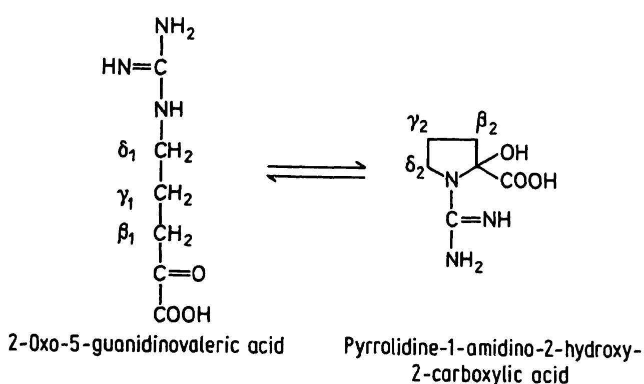


Fig. 2. Structures of 2-oxo-5-guanidinovaleric acid and its cyclic form.

Tab. 1. Chemical shift $\delta$-values in ppm relative to internal DSS of 2-oxo-5-guanidinovaleric acid and its cyclic form.

| | $\delta$-values |
|---|---|
| $\beta_1$ | 2.75 (triplet) |
| $\gamma_1$ | 1.79 (pentuplet) |
| $\delta_1$ | 3.13 (triplet) |
| $\beta_2 + \gamma_2$ | 1.55–2.6 (broad band) |
| $\delta_2$ | 3.2 –3.8 (multiplet) |

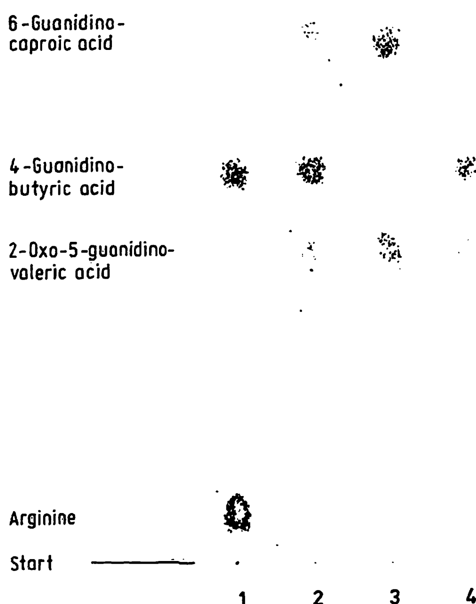


Fig. 3. Thin layer chromatography of
1 standard of arginine and 4-guanidinobutyric acid,
2 unknown peak and 6-guanidinocaproic acid as internal standard,
3 standard of 2-oxo-5-guanidinovaleric acid and 6-guanidinocaproic acid as internal standard,
4 mixture of unknown peak and standard of 2-oxo-5-guanidinovaleric acid;
solvent: *n*-propanol/ acetic acid (1 mol/l) (150 ml + 50 ml).

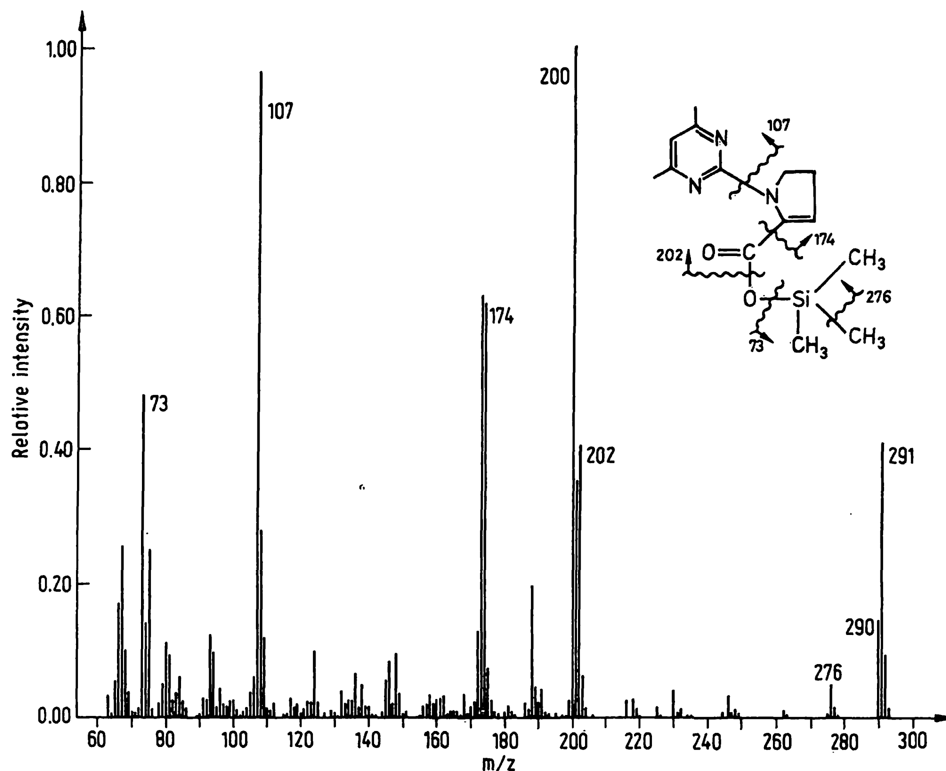


Fig. 4. Mass spectrum of the silylated dimethylpyrimidyl derivative of 2-oxo-5-guanidinovaleric acid.

acetic acid 1 mol/l (150 ml + 50 ml). The first spot is a standard of 4-guanidinobutyric acid and arginine. The second spot is the unknown guanidino-positive compound peak, with 6-guanidinocaproic acid added as internal standard. The third spot is enzymatically synthesized 2-oxo-5-guanidinovaleric acid, with 6-guanidinocaproic acid added as internal standard. The fourth spot is a mixture of the urine fraction and enzymatically synthesized 2-oxo-5-guanidinovaleric acid. 2-Oxo-5-guanidinovaleric acid degrades to 4-guanidinobutyric acid (fig. 3) in an aqueous medium enriched with oxygen. This is a chemical degradation (13).

Since compounds containing a guanidino function are not suitable for GC-MS analysis, both enzymatically synthesized 2-oxo-5-guanidinovaleric acid and the unknown urine peak were converted into the silylated dimethylpyrimidyl derivative.

The mass spectrum of this derivative is depicted in figure 4. The same fragment ions were observed for both the enzymatically synthesized product and the unknown urine peak.

No signal at m/z = 309 was observed because of the ease of water elimination from the cyclic form during the derivatisation procedure. The molecular ion $(M)^+$ at m/z = 291 was due to the dehydrated cyclic silylated dimethylpyrimidyl compound. Typical fragment ions are m/z = 276, 202, 174, 107 and 73 of which the origin is shown in figure 4.

Ion m/z = 200 (base peak) can be explained by the elimination of $(CH_3)_3$ SiOH from the $(M\text{-}H)^+$-fragment ion (m/z = 290). Loss of a $(CH_3)_3$ SiOCO-radical from the $(M\text{-}H)^+$-ion leads to m/z = 173.

## Discussion

Only eight patients with hyperargininaemia, from five different families, have been described (14–18). The first clinical symptoms such as irritability, coma and convulsions were the same as those of the other hyperammonaemias. The development of the patients is characterised by neurological degradation, resulting in spasticity, athetosis and ataxia. They are also mentally retarded. Hepatomegaly and an increase of the activity of transaminases and aldolases was also seen in our patients. Different problems arise in these patients:

a) In view of the neurological picture of the patients, the main question is: is there a correlation between the neurological symptoms and the elevated arginine and guanidino compounds in the biological fluids?

b) In the biochemistry of these patients, there are several interesting points like the normal or low normal values of blood urea, in contrast with arginase deficiency. Three possibilities for this anomaly have been suggested:

1. that the enzyme defect is only partial (19);

2. that the urea-cycle enzymes in different organs are under different genetic controls and that the biochemical defect involves one or more, but not all, organs (20);
3. that there is an alternative pathway for urea production (21).

c) Metabolism of guanidino compounds:

1. The patients with hyperargininaemia try to metabolize their high arginine load. Therefore elevated levels of guanidino compounds were to be expected. As can be seen in table 2, the excretion values of the monosubstituted guanidino compounds are ten to hundred times higher than those of controls, except for guanidinosuccinic acid. The excretion value of guanidinosuccinic acid is even lower than in controls. Guanidinosuccinic acid does not seem to be synthesized from arginine.
2. Little is known about the synthesis of guanidino compounds in vivo. Guanidinoacetic acid is formed by a transamidination reaction: the amidino group of arginine is transferred to glycine (22). Guanidinoacetic acid is an intermediate in the biosynthesis of creatine and creatinine. 4-Guanidinobutyric acid can also be formed by transamidination from arginine to 4-aminobutyric acid (23). N-α-acetylarginine could be formed by an acetylation of arginine. And deamination of arginine, followed by an hydrogenation could give argininic acid.

Patients with hyperargininaemia are characterised by an abnormal metabolism of the guanidino compounds. In this work we have demonstrated that the liquid and thin-layer chromographic characteristics and the mass spectral data of the unknown guanidino positive peak in the urines of the three patients with hyperargininaemia were identical with the results obtained with the enzymatically synthesized 2-oxo-5-guanidinovaleric acid. Therefore we can conclude that 2-oxo-5-guanidinovaleric acid is present in the urine of the three patients with hyperargininaemia.

Tab. 2. Excretion values of some monosubstituted guanidino compounds in urine.

| | Controls | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|---|
| | µmol/g cratinine | | | |
| Guanidinoacetic acid | 418 | 745 | 2764 | 1182 |
| Guanidinosuccinic acid | 63 | traces | traces | traces |
| N-α-acetylarginine | 33 | 734 | 4271 | 649 |
| Arginic acid | 14 | 355 | 1069 | 61 |
| 4-Guanidinobutyric acid | 9 | 123 | 233 | 19 |
| 2-Oxo-5-guanidino-valeric acid | traces | 2228 | 6410 | 904 |

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