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## Note

# Purification procedure for some urinary guanidino compounds

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Guanidino compounds are accumulated in the biological fluids of hyperargininaemic [1] and uraemic [2, 3] patients. These compounds are proven toxins.

Identification of unknown guanidino compounds by nuclear magnetic resonance (NMR) or mass spectrometric (MS) techniques usually requires clean-up procedures of the samples and chromatographic techniques for isolation of the unknown compounds [4, 5]. One of the inconveniences during purification by cation-exchange chromatography is that many guanidino compounds are eluted together with amino acids, owing to their chemical similarities [5, 6].

In this paper, we present a clean-up procedure for urine samples and a method to separate and purify some guanidino compounds from amino acids using an anion-exchange resin in an alkaline medium.

## EXPERIMENTAL

### *Elimination of acidic compounds*

A 10-ml aliquot of filtered hyperargininaemic urine, acidified to pH 2.0, was passed through a column (100 × 9 mm I.D.) filled with 5 ml of Dowex 50W-X8 (Fluka, Buchs, Switzerland; 50—100 mesh, $H^+$ form). The resin was washed with 250 ml of water. This step eliminated the acidic compounds such as organic acids and carbohydrates. More basic compounds, such as amino acids and guanidino compunds, were eluted by passing 150 ml of 0.5 *M* ammonium hydroxide through the column. The effluent was concentrated by evaporation on a rotary evaporator to ca. 10 ml. A 0.5-ml aliquot was used to determine the concentrations of amino acids and guanidino compounds.

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*Purification of guanidino compounds*

The cation-exchange effluent was made alkaline (pH 12) with a 25% ammonia solution. An anion-exchange column (100 × 9 mm I.D.) containing 3 ml of Dowex 1-X8 (50—100 mesh, $OH^-$ form) was equilibrated with 250 ml of 8% ammonia solution (pH 12). The alkaline effluent from the cation-exchange column was passed through the anion-exchange column, which was then washed with 250 ml of 8% ammonia solution. This effluent was again evaporated to ca. 10 ml and analysed for amino acids and guanidino compounds.

*Determination of amino acids and guanidino compounds*

The concentrations of amino acids and guanidino compounds were determined in the filtered hyperargininaemic urine and the effluents from the cation-exchange and anion-exchange columns. Amino acids were determined using a TSM (Technicon Sequential Multi-Sample) amino acid analyser (Technicon Instruments, Tarrytown, NY, U.S.A.). Guanidino compounds were determined using a Biotronik LC 6001 amino acid analyser (Biotronik, Puchheim, F.R.G.) adapted for guanidino compound determination as described earlier

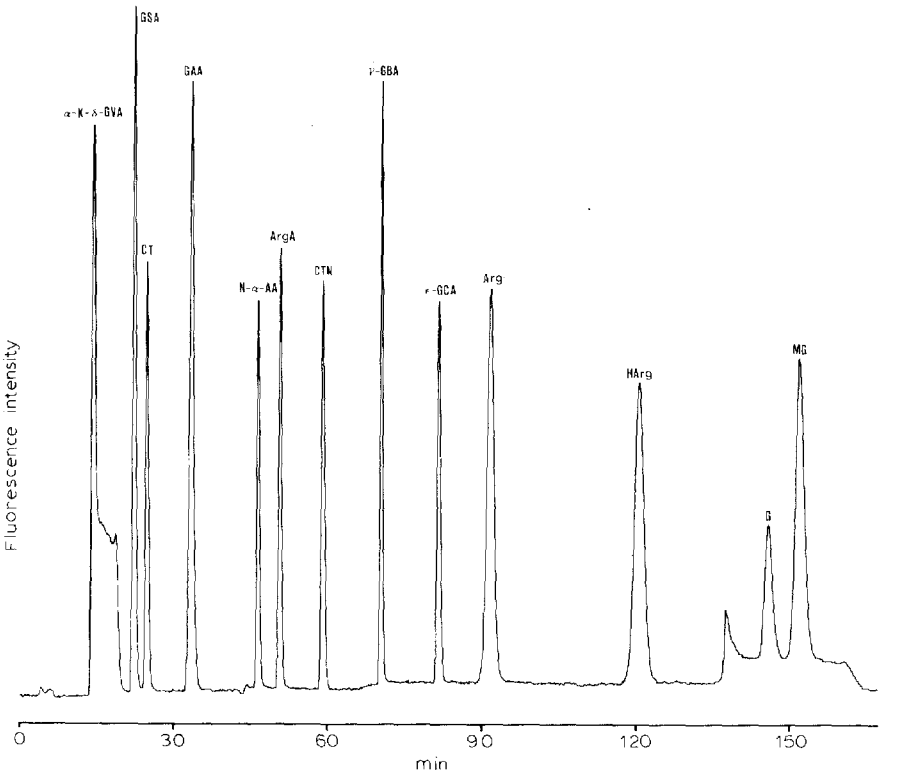


Fig. 1. Chromatogram of a standard mixture of guanidino compounds. Peaks: α-K-δ-GVA = α-keto-δ-guanidinovaleric acid (2.5 nmol); GSA = guanidinosuccinic acid (1 nmol); CT = creatine (0.5 nmol); GAA = guanidinoacetic acid (1 nmol); N-α-AA = N-α-acetylarginine (1 nmol); ArgA = argininic acid (1 nmol); CTN = creatinine (100 nmol); γ-GBA = γ-guanidinobutyric acid (1 nmol); ε-GCA = ε-guanidinocaproic acid (1 nmol); Arg = arginine (2 nmol); HArg = homoarginine (2 nmol); G = guanidine (2 nmol); MG = methylguanidine (2 nmol).

[1]. A typical separation of a standard mixture of guanidino compounds is given in Fig. 1.

*Validation of the chromatographic procedures*

The recoveries (%) were determined using human urine spiked with known quantities of the standard compounds. They ranged from 95.6 to 98% for amino acids at a concentration of 10 nmol per 20 μl. For guanidino compounds, the recoveries varied from 97.3 to 98.4% at a concentration of 1 nmol per 100 μl (for creatinine 20 nmol per 100 μl), except for α-keto-δ-guanidinovaleric acid (94.2%). The lower recovery of the latter compound might be due to the poor precision of the area measurement (planimeter). The coefficients of variation, determined from ten measurements, were obtained from day-to-day analyses. They ranged from 0.3 to 2.4% for all amino acids, except for taurine (3.7%) and cystine (4.1%), and from 0.8 to 1.5% for the guanidino compounds, except for γ-guanidinobutyric acid (2.3%) and α-keto-δ-guanidinovaleric acid (6.2%). The detection limits for amino acids determined at a signal-to-noise ratio of 2 were ca. 150 pmol, except ethanolamine (400 pmol). For the guanidino compounds, the detection limits were ca. 2.5 pmol, except for α-keto-δ-guanidinovaleric acid (5 pmol), arginine, homoarginine and methylguanidine (10 pmol), guanidine (20 pmol) and creatinine (270 pmol). Calibration plots established for amino acids and the guanidino compounds were linear over the concentration range of interest. For glycine and alanine, for instance, the plot was linear between 1 and 200 nmol, and for phenylalanine and aspartic acid between 1 and 500 nmol. For the guanidino compounds, the fluorescence responses were linear up to at least 20 nmol. The lower limit of the linearity test was 20 pmol for creatine and guanidinosuccinic acid and 80 pmol for guanidine.

## RESULTS

The amino acid levels in filtered hyperargininaemic urine and cation-exchange and anion-exchange effluents are given in Table I. The results for the guanidino compounds in the same samples are given in Table II.

## DISCUSSION

The aim of this study was to develop a procedure for the isolation and purification of urinary guanidino compounds. Knowing the $pK_a$ values of carboxyl and amino groups of amino acids, we decided to use an anion-exchange resin in the $OH^-$ form and to work at pH 12 to retain as much as possible the acidic, neutral and basic amino acids and no guanidino compounds. Using ammonia solution as eluent, purified guanidino compounds could easily be obtained after evaporation.

Comparison of Tables I and II clearly demonstrates that most of the acidic, neutral and basic amino acids are retained by the anion-exchange resin under these conditions. Ethanolamine, having no carboxyl group, is not retained by the resin. $N^G,N^G$-dimethylarginine and $N^G,N'^G$-dimethylarginine have an α-amino group, but they also possess a guanidino group and can be considered,

TABLE I

CONCENTRATIONS OF AMINO ACIDS IN FILTERED HYPERARGININAEMIC URINE, THE CATION-EXCHANGE AND THE ANION-EXCHANGE EFFLUENT

| Amino acid* | Concentration (mean ± S.D., $n$ = 3) ($\mu$mol per 10 ml) | | | |
|---|---|---|---|---|
| | Filtered urine | Cation-exchange effluent | | Anion-exchange effluent |
| Tau | 1.56 ± 0.73 | — | — | — |
| Asp | 5.77 ± 0.45 | 3.94 ± 0.55 | 3.72 ± 0.49** | — |
| Thr | 4.53 ± 0.35 | 3.52 ± 0.20 | 3.32 ± 0.17 | — |
| Ser | 21.68 ± 3.21 | 16.67 ± 0.97 | 15.73 ± 0.74 | 0.21 ± 0.10 |
| Glu | 32.61 ± 12.45 | 19.41 ± 4.61 | 18.36 ± 4.21 | 0.10 ± 0.02 |
| Gly | 56.88 ± 5.45 | 44.25 ± 2.72 | 41.86 ± 2.94 | 0.17 ± 0.03 |
| Ala | 16.83 ± 0.78 | 12.49 ± 0.77 | 11.80 ± 0.67 | 0.18 ± 0.02 |
| Cit | 1.91 ± 0.45 | 1.69 ± 0.31 | 1.60 ± 0.29 | — |
| α-ABA | 0.82 ± 0.19 | 0.63 ± 0.10 | 0.59 ± 0.09 | — |
| Val | 0.56 ± 0.07 | 0.47 ± 0.03 | 0.45 ± 0.03 | — |
| Cys | 1.12 ± 0.09 | 0.89 ± 0.07 | 0.84 ± 0.07 | — |
| Ile | 0.68 ± 0.09 | 0.56 ± 0.03 | 0.53 ± 0.03 | — |
| Leu | 1.00 ± 0.14 | 0.76 ± 0.03 | 0.72 ± 0.03 | — |
| Tyr | 3.10 ± 0.39 | 2.29 ± 0.12 | 2.17 ± 0.13 | — |
| Phe | 0.81 ± 0.05 | 0.61 ± 0.04 | 0.58 ± 0.04 | — |
| $EtNH_2$ | 10.90 ± 1.67 | 8.24 ± 2.14 | 7.80 ± 2.10 | 7.44 ± 1.49 |
| Orn | 0.50 ± 0.08 | 0.45 ± 0.04 | 0.42 ± 0.04 | — |
| Lys | 1.78 ± 0.19 | 1.55 ± 0.34 | 1.47 ± 0.32 | — |
| His | 30.44 ± 3.94 | 27.77 ± 3.34 | 26.28 ± 3.43 | 0.56 ± 0.21 |
| 1-$CH_3$-His | 1.45 ± 0.18 | 0.93 ± 0.27 | 0.88 ± 0.25 | — |
| 3-$CH_3$-His | 2.38 ± 0.54 | 2.14 ± 0.41 | 2.02 ± 0.38 | — |
| Car | 1.42 ± 0.27 | 1.25 ± 0.14 | 1.18 ± 0.15 | — |
| $N^G,N^G(CH_3)_2$Arg | 1.41 ± 0.17 | 1.10 ± 0.02 | 1.04 ± 0.03 | 1.01 ± 0.01 |
| $N^G,N^{G'}(CH_3)_2$Arg | 1.17 ± 0.28 | 0.99 ± 0.13 | 0.94 ± 0.13 | 0.78 ± 0.15 |
| Arg | 0.88 ± 0.11 | 0.76 ± 0.10 | 0.72 ± 0.08 | 0.66 ± 0.05 |

*Abbreviations: Tau = taurine; Asp = aspartic acid; Thr = threonine; Ser = serine; Glu = glutamic acid; Gly = glycine; Ala = alanine; Cit = citrulline; α-ABA = α-amino-$n$-butyric acid; Val = valine; Cys = cystine; Ile = isoleucine; Leu = leucine; Tyr = tyrosine; Phe = phenylalanine; Orn = ornithine; Lys = lysine; His = histidine; 1-$CH_3$-His = 1-methylhistidine; 3-$CH_3$-His = 3-methylhistidine; Car = carnosine; $N^G,N^G(CH_3)_2$Arg = $N^G,N^G$-dimethylarginine; $N^G,N^{G'}(CH_3)_2$Arg = $N^G,N^{G'}$-dimethylarginine; Arg = arginine.

**Concentrations after subtraction of a 0.5-ml aliquot needed for the amino acid and guanidino compound determinations.

like arginine, as guanidino compounds. Most of the studied guanidino compounds are either not retained or only partially retained by the anion-exchange resin. However, guanidinosuccinic acid is ca. 80% bound, probably due to its two carboxyl groups.

For the purification of basic guanidino compounds such as guanidine and methylguanidine, it is necessary to use a more basic eluent than 0.5 $M$ ammonium hydroxide for the elution from the cation-exchange resin. The increase of methylguanidine after elution from the anion-exchange resin is probably due to the degradation of other guanidino compounds [7].

In conclusion, using a strongly basic, quaternary ammonium anion-exchange resin in the $OH^-$ form, the guanidino compounds of major importance can be

TABLE II

CONCENTRATIONS OF GUANIDINO COMPOUNDS IN FILTERED HYPERARGININAEMIC URINE, THE CATION-EXCHANGE AND THE ANION-EXCHANGE EFFLUENT

| Guanidino compound* | Concentration (mean ± S.D., $n$ = 3) ($\mu$mol per 10 ml) | | | |
|---|---|---|---|---|
| | Filtered urine | Cation-exchange effluent | | Anion-exchange effluent |
| α-K-δ-GVA | 5.03 ± 0.45 | 3.6 ± 0.96 | 3.40 ± 0.89** | 2.43 ± 0.29 |
| GSA | 0.09 ± 0.02 | 0.06 ± 0.03 | 0.05 ± 0.03 | 0.01 ± 0.01 |
| CT | 201 ± 18 | 188 ± 37 | 179 ± 34 | 150 ± 9 |
| GAA | 28 ± 4.62 | 25 ± 2.89 | 23 ± 2.40 | 21 ± 4.58 |
| N-α-AA | 4.93 ± 0.40 | 3.97 ± 0.15 | 3.80 ± 0.20 | 3.66 ± 0.15 |
| ArgA | 5.67 ± 1.02 | 4.90 ± 0.72 | 4.67 ± 0.71 | 4.20 ± 0.36 |
| CTN | 145 ± 45 | 130 ± 34 | 124 ± 33 | 106 ± 35 |
| γ-GBA | 2.73 ± 0.42 | 3.80 ± 0.89 | 3.60 ± 0.89 | 3.97 ± 0.51 |
| Arg | 1.00 ± 0.35 | 0.80 ± 0.18 | 0.77 ± 0.15 | 1.00 ± 0.53 |
| HArg | 0.07 ± 0.02 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| G | 0.27 ± 0.03 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.03 ± 0.01 |
| MG | 0.22 ± 0.11 | 0.03 ± 0.02 | 0.03 ± 0.01 | 0.47 ± 0.38 |

*Abbreviations: α-K-δ-GVA = α-keto-δ-guanidinovaleric acid; GSA = guanidinosuccinic acid; CT = creatine; GAA = guanidinoacetic acid; N-α-AA = N-α-acetylarginine; ArgA = argininic acid; CTN = creatinine; γ-GBA = γ-guanidinobutyric acid; Arg = arginine; HArg = homoarginine; G = guanidine; MG = methylguanidine.

**Concentrations after subtraction of a 0.5-ml aliquot needed for the amino acid and guanidino compound determinations.

separated and purified from amino acids in urine. This purification procedure is a useful step prior to identification of these compounds by spectroscopic techniques.

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