Journal of Chromatography, 382 (1986) 237—241
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3256

Note

Gas chromatography of amino acids in urine: identification and removal of creatinine as the major interfering substance

D. LABADARIOS*, I.M. MOODIE and G.S. SHEPHARD

The MRC Metabolic Research Group, Tygerberg Hospital and Department of Internal Medicine, University of Stellenbosch, P.O. Box 63, Tygerberg 7505 (South Africa)

(First received March 13th, 1986; revised manuscript received May 7th, 1986)

Gas chromatography (GC) is now widely regarded as an excellent tool in the analysis of amino acids in biological fluids and tissues. It offers advantages over other chromatographic techniques particularly in regard to its speed, sensitivity, versatility and low initial capital cost.

Sample purification, prior to chromatography, has in the past most frequently been attempted by using short ion-exchange columns. More recently, however, reports have appeared [1—3] which cast considerable doubt on recoveries and reproducibility of results obtained for certain amino acids, if such factors as the strength of eluting solutions [2] or the type of ion-exchange resin used [3] are not taken into account, as is very often the case [4]. Furthermore, ion-exchange techniques do not always remove interfering substances as is demonstrated by the presence of an unidentified major interference in urine samples previously cleaned-up by ion-exchange [5, 6]. Labadarios et al. [7], in a more recent paper, have demonstrated the advantage of adopting an approach directed at removing a specifically identified interfering compound, rather than using non-specific procedures.

In this preliminary communication, the major interference in the GC determination of amino acids in urine is identified as creatinine and a method for its removal is described.

EXPERIMENTAL

Reagents

Picric acid was obtained from Merck (Darmstadt, F.R.G.). Creatinine was obtained from Riedel de Haen (Seelze, F.R.G.). Heptafluorobutyric anhydride (HFBA) was obtained from Fluka (Buchs, Switzerland).

0378-4347/86/$03.50

Procedure

Removal of phosphate, previously shown to be necessary [8], was achieved in freshly voided urine (500 μl) by adjusting the pH to 7.5 with 0.5 *M* sodium hydroxide, treating the urine with 1.9 *M* barium acetate (16 μl) and again adjusting to pH 7.5. Precipitated barium phosphate was removed by centrifuging and the supernatant solution evaporated to dryness in vacuo. The residue, resuspended in distilled water (150 μl), was treated with hot saturated picric acid solution (200 μl), followed by 2.5 *M* sodium hydroxide solution (40 μl) yielding a deep red suspension. After chilling in ice-water, the mixture was further treated with cold concentrated hydrochloric acid (40 μl), vigorously stirred and allowed to stand in an ice-water bath for a further 15 min. The mixture was then centrifuged and the supernatant evaporated to dryness in vacuo. The residue was redissolved in 0.25 *M* hydrochloric acid (150 μl) and extracted by washing with aliquots (5 × 1 ml) of ether saturated with hydrochloric acid. Remaining traces of ether were removed using a current of nitrogen and the aqueous phase was evaporated to dryness in vacuo. Subsequent derivatisation and chromatography of the residual material was carried out as described previously [9].

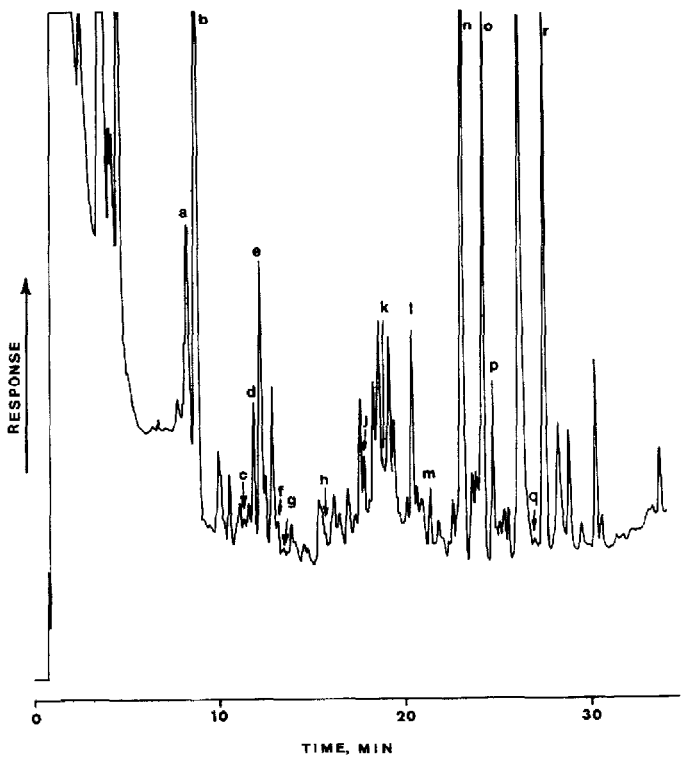

Fig 1 Chromatogram of HBB derivatives of amino acids in a sample of urine (0.5-μl injection representing 5 μl of original urine sample). Peaks: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = proline; j = hydroxyproline; k = methinone; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine.

RESULTS AND DISCUSSION

GC analysis of urine N-heptafluorobutyryl isobutyl ester (HBB) amino acid derivatives (Fig. 1) reveals a major interference in the chromatogram, obstructing the peaks of hydroxyproline and methionine. Following ion-exchange clean-up, a similar unidentified interference has been reported [5, 6] in chromatograms of the same urine derivatives separated on a capillary column coated with the same apolar liquid phase used in our analysis. It is also significant that this interference becomes more pronounced when using an alkali flame ionisation detector [6], indicating a nitrogen-rich material. Creatinine, a major urine component, which displays a high nitrogen content, will not be removed from amino acids by the usual ion-exchange clean-up on account of the similarity between its pK_a values (4.8 and 9.2) and those of the basic amino acids, particularly histidine (6.04 and 9.33).

As further evidence towards establishing the identity of this interference, the product formed from exposing creatinine to the esterification and acylation processes gave rise to a chromatogram (Fig. 2) showing a large broad-based peak similar to that observed following the same treatment of a urine sample (Fig. 1).

Creatinine is spectrophotometrically assayed using the Jaffe reaction [10], in which treatment with alkaline picrate solution leads to the formation of a

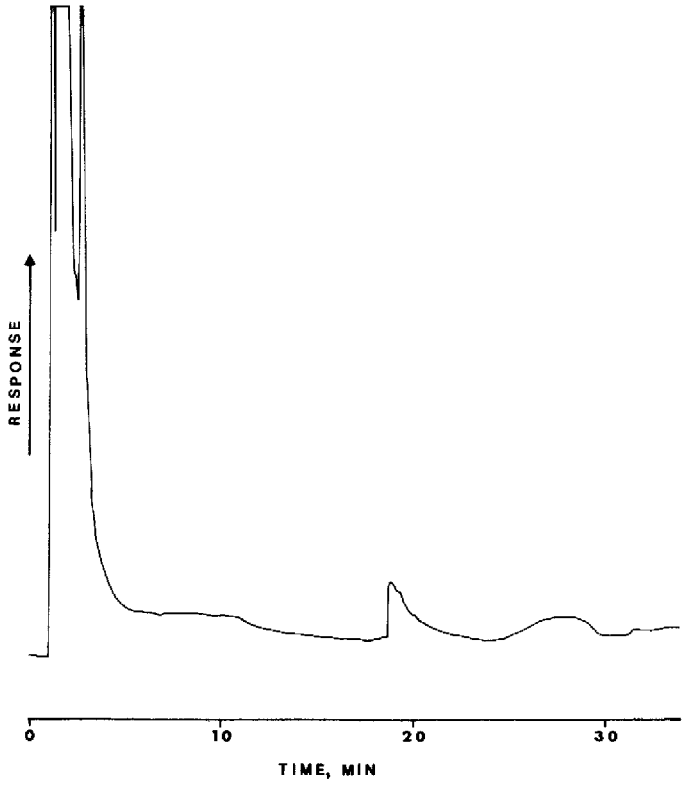

Fig. 2. Chromatogram of derivatised creatinine (1-μl injection, representing 2 μg creatinine).

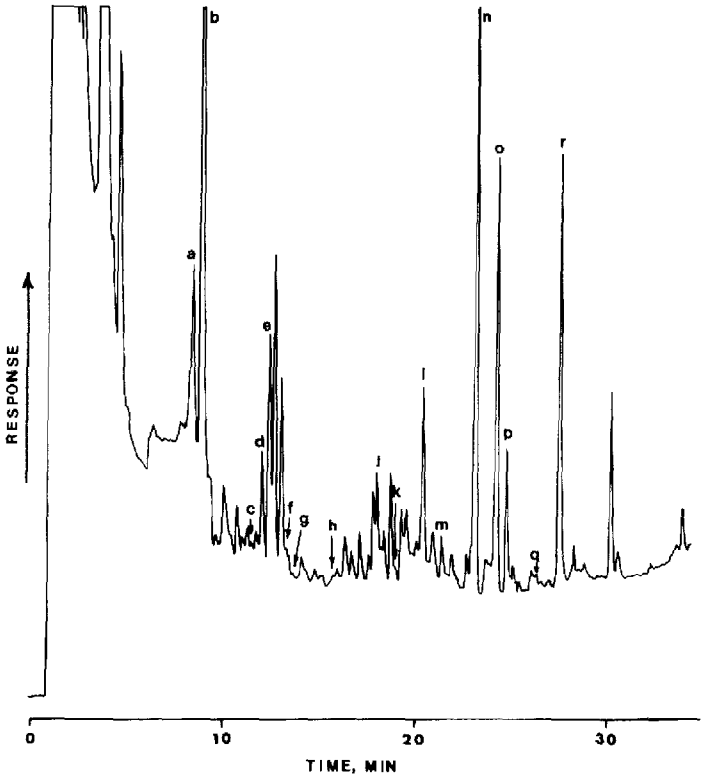

Fig. 3. Chromatogram of HBB derivatives of amino acids in a sample of urine subjected to creatinine removal treatment (0.5-μl injection, representing 5 μl of original urine sample). Peak identification as in Fig 1.

yellow-orange product. We have modified the procedure of Blass et al. [11] and removed creatinine from urine using excess picric acid in alkaline medium followed by precipitation with concentrated hydrochloric acid. Prior to derivatisation, unreacted picric acid was removed by diethyl ether extraction which may also serve to remove organic acids thereby contributing further to the clean-up. Use of the new procedure, followed by derivatisation and chromatography, results in a chromatogram (Fig. 3) in which the central portion is now free from major interference.

In addition, a chromatogram (Fig. 4) derived from analysis of a similar sample on a fused-silica capillary column clearly reveals the absence of this interference.

CONCLUSION

Creatinine has been shown to be responsible for major interference in the GC of HBB derivatives of urine amino acids. By adopting the established principle of using a clean-up procedure designed to remove a specifically identified compound, creatinine was removed by precipitation using alkaline picrate.

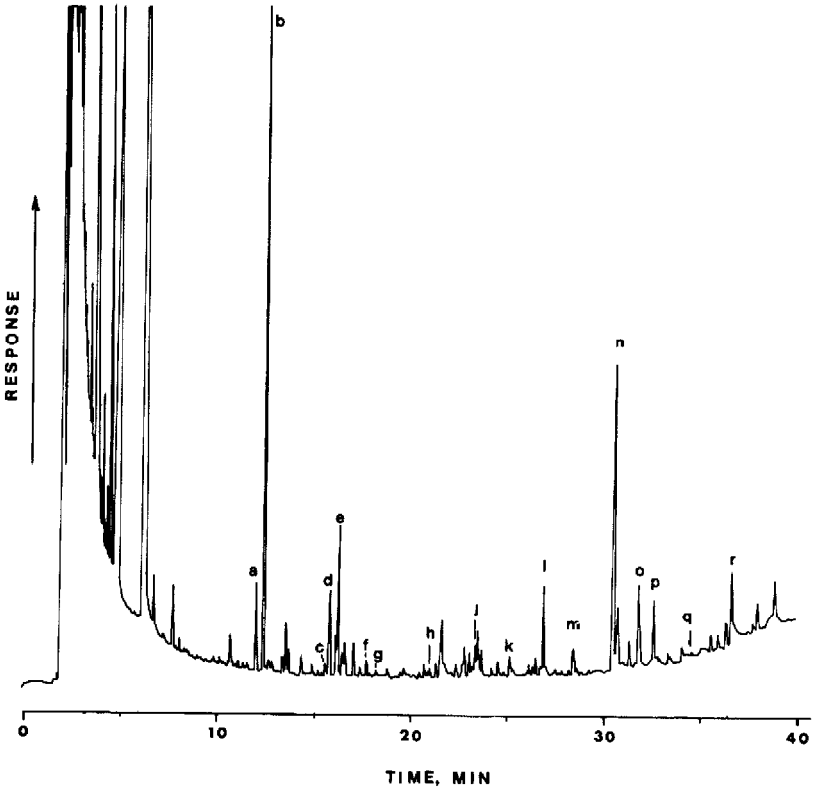

Fig. 4. Capillary chromatogram of HBB derivatives of amino acids in a sample of urine subjected to creatinine removal treatment (0.4-μl injection of 1:4 dilution of derivative solution in ethyl acetate, representing 0.08 μl of original urine sample). Column details: DB-1 fused-silica column (30 m × 0.32 mm I.D.; 1 μm film thickness) obtained from J & W Scientific, Rancho Cordova, CA, U.S.A. Peak identification as in Fig. 1.

ACKNOWLEDGEMENTS

We thank SABAX SA for the donation of the gas chromatograph and Miss J.A. Burger for the diagrams.

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