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Note

Fluorometric evaluation of sarcosine in urine and serum

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Sarcosine (N-methylglycine) is a metabolic derivative of choline. Sarcosine is normally demethylated into glycine by an oxidative process necessitating tetrahydrofolate and catalysed by the flavoenzyme sarcosine dehydrogenase [1—3]. This enzyme is present in the human liver but not in fibroblasts [4—6]. Some rare examples of inactive sarcosine dehydrogenase have been described, characterized by hypersarcosinuria and hypersarcosinaemia [5, 7—13]. If one excludes some cases that were corrected by injections of folic acid [14], a genetic origin appears probable, as an autosomal recessive character [13].

The evaluation of sarcosine has, up to now, been realized by several adaptations of the amino acid ion-exchange chromatographic technique initially described by Spackman et al. [15]. Separation and evaluation are satisfactory as regards urinary sarcosine, but do not permit the evaluation of blood sarcosine except in cases of very large increases. The normal concentration of sarcosine in blood is below the limit of detection and remains undetermined.

In our laboratory, we have been engaged for several years in the evaluation of picomole amounts of proline and hydroxyproline by a technique of derivatization with a fluorogenic compound, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), followed by thin-layer chromatography of the fluorescent derivative and scanning fluorometry [16—18].

Adapting the thin-layer fluorometric recording technique used for hydroxyproline, we set up the following evaluation methods for sarcosine in urine and blood.

MATERIALS AND METHODS

Reagents

When not specifically mentioned, chemicals were purchased from Prolabo,

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Paris, and were of reagent grade. Chloroform, triethylamine, 2-mercaptoethanol, methanol and formic acid were bought from E. Merck (Darmstadt, F.R.G.). *o*-Phthaldialdehyde was obtained from Sigma (St. Louis, MO, U.S.A.) and NBD-Cl from Aldrich (Beerse, Belgium).

Standard sarcosine and 4-hydroxyproline were bought from Sigma. A mixture of eighteen reference amino acids was purchased from Beckman (Gagny, France) for the purpose of amino acid analyser standardization. A standard of 3-hydroxyproline was prepared in our laboratory [19].

Preparation of urine samples

Deproteinization was performed by addition of an equal volume of absolute ethanol at 4°C. After several minutes of stirring, the mixture was centrifuged and 4 ml of supernatant were evaporated to dryness under a stream of nitrogen at ordinary temperature.

The urine residue was dissolved in 0.4 ml of a 0.2 mol/l sodium citrate buffer pH 2.2 and clarified by centrifugation at 1000 *g* for 10 min. An aliquot of 0.2 ml of supernatant was chromatographed as described previously [18].

Derivatization reaction

The eluate, after concentration to dryness, was dissolved in 0.1 ml of water. To this solution was added 0.2 ml of a 0.06 mol/l *o*-phthaldialdehyde solution [18]. After 1 min standing at room temperature, 0.2 ml of a 0.03 mol/l solution of NBD-Cl in ethanol was added. The preparation of the NBD-Cl solution has been previously described [16]. The mixture of amino acids, *o*-phthaldialdehyde and NBD-Cl was then incubated for 30 min at 65°C in a stoppered test tube. The NBD-amino acid derivatives were ready for thin-layer chromatography.

Preparation of blood serum samples

To 2 ml of serum was added 1 ml of a 20 g per 100 ml sulphosalicylic acid solution. After stirring, the mixture was centrifuged at 1000 *g*. A 1-ml volume of supernatant was diluted with 0.5 ml of 0.2 mol/l sodium citrate buffer, pH 2.2; 1 ml of this mixture was layered at the top of a 52 × 0.9 cm column of M 82 resin in a Multichrom B Beckman amino acid analyser connected to a fraction collector. The amino acids were eluted by 0.2 mol/l sodium citrate buffer pH 3.2 at 39°C for 40 min and then at 65°C for a further 40 min period at a flow-rate of 70 ml/h. Fractions of 7 ml were collected. Sarcosine was eluted between glutamine and glutamic acid with a retention time of 66 min and was obtained quantitatively in fraction 11.

This fraction was deionized on a 6.4 × 1.1 cm column of Dowex 50W-X2 and processed as described in the case of urinary sarcosine for the derivatization reaction.

Thin-layer chromatography

The system has been fully described in several previous papers [16–18]. In the case of sarcosine, two solvents were alternatively used: solvent I, chloroform–methyl ethyl ketone–formic acid (75:20:5, v/v), and solvent II, chloroform–acetone–toluene–methanol–triethylamine (40:20:20:15:5, v/v).

The development time was 70 min with solvent I and 45 min with solvent II at 20°C. Both solvents gave satisfactory separations of sarcosine. The plates were dried for 10 min at 65°C. They can be stored in the dark up to three days prior to spectrofluorometric evaluation [16].

RESULTS

The various steps of the evaluation were checked for recovery and reproducibility with known concentrations of control sarcosine. After the preliminary column chromatography, the recovery, measured in four separate experiments, was 99 ± 2% of the amount deposited. The reproducibility of the complete method was checked by eight separate measurements of the same sample of urine supplemented with sarcosine in order to reach a concentration of 100 pmol in every final deposit on thin layer. Recovery was 95.5 ± 2%. For assessing the reproducibility, a sample of urine was evaluated eight times and gave the results (mean ± 1 S.D.) 7.88 ± 0.22 μmol per 24 h.

A photo of a thin-layer chromatogram of urine samples viewed under ultraviolet light is shown in Fig. 1. The clearing effect of *o*-phthaldialdehyde is demonstrated by the differences between lanes 7 and 8. A fluorometric record of a urine sample is shown in Fig. 2.

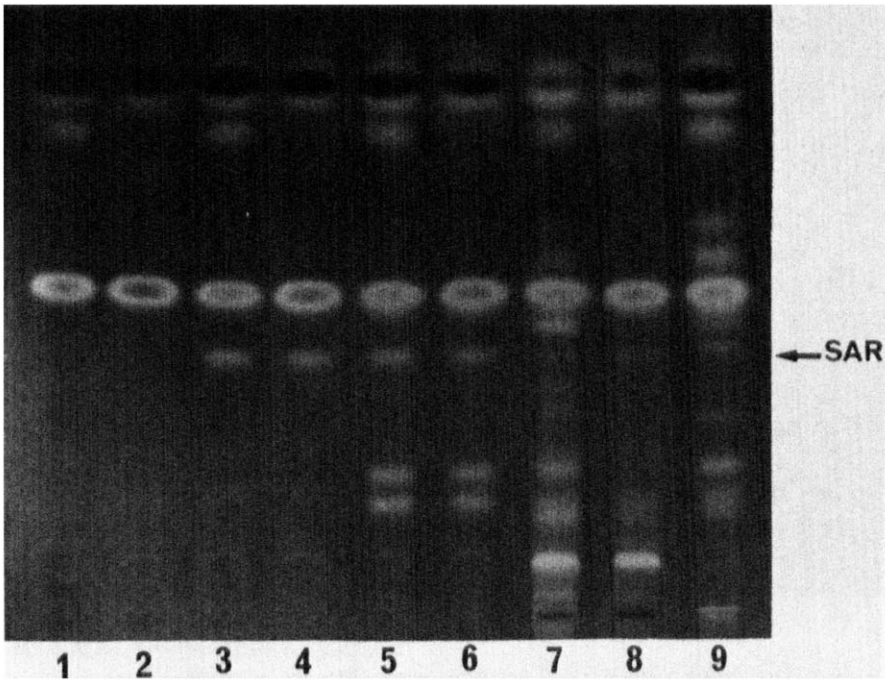

Fig. 1. Photo under ultraviolet light of a thin-layer chromatogram of NBD-Cl derivatives from control and urinary amino acids. Lanes 1, 3, 5, 7, 9: direct reaction with NBD-Cl. Lanes 2, 4, 6, 8: reaction with *o*-phthaldialdehyde prior to derivatization with NBD-Cl. Lanes 1, 2: blank reagents. Lanes 3, 4: control sarcosine. Lanes 5, 6: control sarcosine, 3-hydroxyproline and 4-hydroxyproline. Lanes 7, 8: urine samples. Lane 9: mixture of control amino acids. Solvent: formic acid—ethyl methyl ketone—chloroform (5:20:75, v/v). SAR = sarcosine.

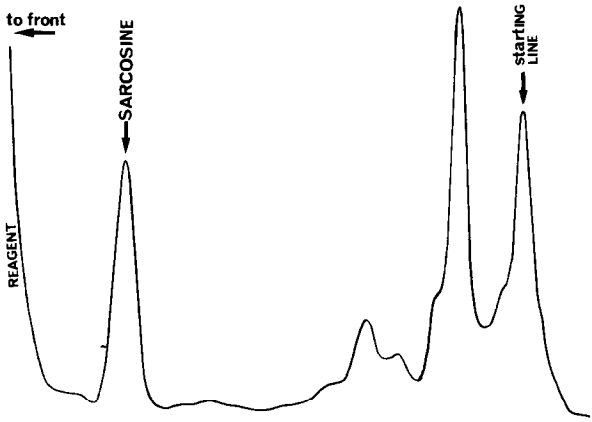

Fig. 2. Scan of a thin-layer chromatogram showing the peak of NBD-sarcosine (114 pmol) from a sample of urine. Solvent: formic acid—ethyl methyl ketone—chloroform (5:20:75, v/v). Spectrofluorometer Farrand Mark I, sensitivity 0.3, scan speed 150 mm/min.

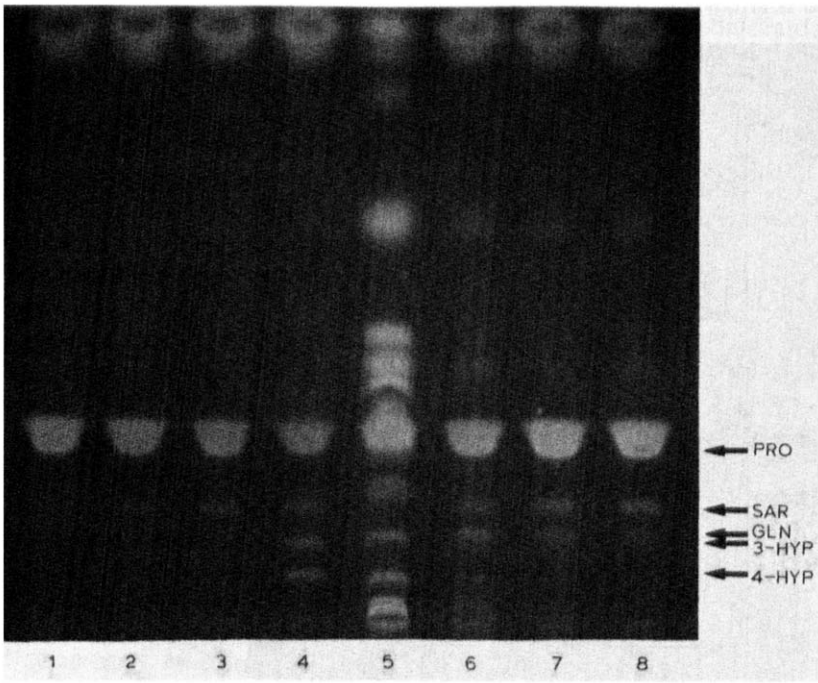

Fig. 3. Photo under ultraviolet light of a thin-layer chromatogram of NBD-Cl derivatives from control and serum amino acids. Lanes 1, 2, 3: control sarcosine in 50, 100 and 200 pmol amounts. Lane 4: control 4-hydroxyproline, 3-hydroxyproline and sarcosine. Lane 5: mixture of amino acids. Lane 6: fraction 11 eluted from a Beckman amino acid analyser and fraction collector, from a sample of serum containing 200 pmol of sarcosine in the deposit. Lanes 7, 8: same fraction 11 obtained from two other samples of serum. Solvent: chloroform—acetone—toluene—methanol—triethylamine (40:20:20:15:5, v/v). PRO = proline, SAR = sarcosine, GLN = glutamine, 3-HYP and 4-HYP = 3- and 4-hydroxyproline.

Serum sarcosine

The elution of sarcosine from the Beckman amino acid analyser was checked in four separate experiments using samples of the same serum each supplemented with 200 pmol of control sarcosine. The recovery was 97.1 ± 5.3% of the amount deposited. All the sarcosine was found in fraction 11, between glutamine and glutamic acid, as shown in Fig. 3.

On the thin-layer chromatograms there is a linear relationship between the surface of the NBD-sarcosine peaks and the amount of NBD-sarcosine deposited on the plates at concentrations ranging from 0 to 400 pmol. The fluorescence spectrum of NBD-sarcosine is the same as that of NBD-hydroxyproline [16].

Results of evaluation of sarcosine in urine and serum

This technique was used for the evaluation of sarcosine in the urines of 54 apparently normal subjects. The normal range, defined within the limits of two standard deviations from the mean, was 1.75—20 μmol per 24 h. No sex-related difference was found.

We measured serum sarcosine in fifteen normal subjects and found levels of 1.59 ± 1.08 μmol/l (mean ± 2 S.D.).

DISCUSSION

The method that we propose for sarcosine has been in use in our laboratory for the evaluation of the hydroxyproline isomers for several years. It has proved reliable and very sensitive. We have now extended it to sarcosine because we found that this methylated amino acid reacted with NBD-Cl as well as hydroxyproline. Two preliminary steps of ion-exchange chromatography are necessary because the NBD-Cl derivatization is impaired by mineral ions and the thin-layer separation suffers from interferences by other amino acids when they are in large amounts.

In the case of serum, the use of an amino acid analyser is justified by the fact that it concentrates sarcosine within a few millilitres of effluent and separates it from most of the other amino acids. Direct detection of sarcosine by the ninhydrin reaction in the amino acid analyser is not possible because the amounts involved are 100 times smaller than the lower limit of detection. Direct fluorometric detection of sarcosine in the effluent would probably not work because the concentration would still not be high enough. The advantage of thin-layer chromatography is to concentrate the fluorescent substance in a very restricted area. In our system, picomole amounts are easily quantitated. The NBD-sarcosine spots are perfectly separated from any other fluorescent substance in the proposed system.

Our method gives as reference values for blood serum 0.50 to 2.70 μmol/l. Reference values for serum sarcosine have not previously been given. In the case of urine, the reference values that we found are 1.75—20 μmol per 24 h. The reference values given in the literature [7, 8] are inacurate: "normal range inferior to 22.5 μmol/l". The method does not permit the evaluation of sarcosine conjugates, if they exist in urine. We found that creatine, hydrolysed for 110 h at 105°C in 6 mol/l hydrochloric acid, liberates sarcosine. When

urine is hydrolysed in the same conditions, significant amounts of sarcosine are liberated. It is not certain whether this originates from sarcosine conjugates or from creatine.

Until now, the medical usefulness of sarcosine evaluations has been restricted to the exceptional cases of hypersarcosinaemia of genetic origin (some twenty cases described), in which the amounts of sarcosine in blood and urine exceeded several hundred times the normal range [7, 8, 10, 12, 13]. The more sensitive method that we propose in this paper may help still unknown limited defects of sarcosine metabolism to be discovered.

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REFERENCES

1 C.G. Mackenzie and W.R. Frisell, J. Biol. Chem., 232 (1958) 417.
2 D.D. Hoskins and C.G. Mackenzie, J. Biol. Chem., 236 (1961) 177.
3 W.R. Frisell and C.G. Mackenzie, J. Biol. Chem., 237 (1962) 237.
4 D.D. Hoskins and R.A. Bjur, J. Biol. Chem., 239 (1964) 1856.
5 M.L. Rehberg and T. Gerritsen, Arch. Biochem. Biophys., 127 (1968) 661.
6 C.R. Scott, S.H. Clark, C. Chiang Teng and K.R. Swedberg, J. Pediatr., 77 (1970) 805.
7 T. Gerritsen and H.A. Waisman, N. Engl. J. Med., 275 (1966) 66.
8 H.A. Waisman and T. Gerritsen, Amer. J. Dis. Child., 113 (1967) 134.
9 W. Hagge, J. Brodehl and K. Gellissen, Pediatr. Res., 1 (1967) 409.
10 F.H. Glorieux, F. Mohyuddin, D.T. Whelan and C.R. Scriver, Pediatr. Res., 4 (1970) 450.
11 F.H. Glorieux, C.R. Scriver, E. Delvin and F. Mohyuddin, J. Clin. Invest., 50 (1971) 2313.
12 C. Willens, A. Heusden, A. Hainaut and P. Chapelle, J. Genet. Hum., 19 (1971) 101.
13 J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 4th ed., 1978, p. 514.
14 E.S. Kang, J. Seyer, T.A. Todd and C. Herrera, Hum. Genet., 64 (1983) 80.
15 D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem., 30 (1958) 1190.
16 M. Bisker, V. Pailler, A. Randoux and J.P. Borel, Anal. Biochem., 122 (1982) 52.
17 G. Bellon, A. Bisker, F.X. Maquart, H. Thoanes and J.P. Borel, J. Chromatogr., 230 (1982) 420.
18 G. Bellon, A. Malgras, A. Randoux and J.P. Borel, J. Chromatogr., 278 (1983) 167.
19 A. Szymanowicz, O. Mercier, A. Randoux and J.P. Borel, Biochimie, 60 (1978) 499.