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Note

Quantitation of the urinary methylhistidine isomers by a combination of thin-layer and fluorometric techniques

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In the course of research devoted to the separation of the hydroxyproline isomers by thin-layer chromatography after derivatization with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), we found that one of the rare interfering substances was 3-methylhistidine (3-MeHis) [1]. We investigated the suitability of this technique for the evaluation of the two methylhistidine isomers, 1-methylhistidine and 3-methylhistidine, and found it as valuable as ion-exchange column chromatography and more versatile.

The interest for the determination of the methylhistidine isomers has been increasing recently. 3-MeHis is a derivative of histidine found in actin and myosin [2]. In this respect it is particularly abundant in muscle. 1-MeHis, on the other hand, is formed in a peptide of the neuromuscular junction, the function of which is still unkown. Tallan et al. [3] found 3-MeHis as a normal component of human urine and pointed out its relationship to the muscular condition. The two MeHis isomers are not reused in protein synthesis and seem to be excreted through the urine without modification or degradation, thus they are useful as urinary markers of the muscular turnover.

The usual analysis techniques are based on ion-exchange chromatography either with the amino acid analyzer [4,5] or with a colorimetric method using ninhydrin and *o*-phthalaldehyde [6—8]. The evaluation may also be performed by high-performance liquid chromatography [9] or gas chromatography [10].

The technique that we propose comprises a preliminary fast step of ion-exchange chromatography on Dowex 50-X2, permitting the separation of the basic amino acids which are then combined with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The fluorescent NBD derivatives of His, 1-MeHis and 3-MeHis are separated by thin-layer chromatography on silica gel

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and quantitated by the use of a spectrofluorometer equipped with a thin-layer recording device.

MATERIAL AND METHODS

Chemicals (analytical grade) are purchased from Prolabo (Paris, France) or Merck (Darmstadt, G.F.R.); NBD-Cl is bought from Aldrich-Europe (Beerse, Belgium). The standard amino acids are obtained from Calbiochem (Los Angeles, CA, U.S.A.), except 3-hydroxyproline which was prepared in the laboratory [11]. Resin Dowex 50-X2 (50—100 mesh) is purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). The chromatographic plates are Merck silica gel plates without fluorescent indicator (ref. 5721).

Prior to analysis, the urine samples are stored in the freezer at −80°C. They are deproteinized by heating at 100°C for 2 min, then centrifuged at 1500 *g* for 10 min. It was verified on several proteinuric urines that the precipitate does not contain any MeHis. One milliliter of clear supernatant is mixed with 1 ml of 12 *M* hydrochloric acid and hydrolysed in sealed tubes for 18 h at 105°C. The hydrolysates are evaporated under a stream of nitrogen and the residue is taken up in a few drops of water and evaporated again. This operation is repeated twice. The residue is dissolved in 1 ml of 0.1 *M* sodium phosphate buffer, pH 6.4. This solution is centrifuged at 1500 *g* for 5 min and 100 μl of clear supernatant are layered on the top of the Dowex 50-X2 column.

Ion-exchange chromatography on Dowex 50-X2

A 3 × 0.7 cm column of Dowex 50-X2, 50—100 mesh (total capacity 1.2 mequiv.) is equilibrated in the triethylammonium form by passing 10 ml of a solution of triethylamine—ethanol—water (25: 25: 50, v/v) and washing with distilled water until the pH of the effluent is 6.6.

The sample, prepared as previously described, is deposited and the resin washed with 20 ml of distilled water. Most of the mineral salts and all the neutral and acidic amino acids are not bound and leave the column in the first 6 ml of effluent. The column is then eluted with 15 ml of 2 *M* ammonium hydroxide solution. The eluate is evaporated to dryness under a stream of nitrogen at room temperature and the residue dissolved in 0.1 ml of ethanol—water (50:50, v/v).

Derivatization and thin-layer chromatography

To 0.1 ml of the alcoholic solution are added 0.1 ml of a 3.0 *M* solution of triethylamine in ethanol and 0.1 ml of a 0.03 *M* solution of NBD-Cl in ethanol. The mixture is incubated at 65°C for 30 min. The NBD derivatives are stable for weeks if stored at 4°C in the dark. They are separated by thin-layer chromatography according to the following procedure. Samples of 5 μl of the derivatized solution are deposited in triplicate on a starting line which is 1.5 cm from the lower edge of a 20 cm × 20 cm silica gel plate. Standards of 1-MeHis and 3-MeHis ranging from 200 to 500 pmoles are spotted on both sides of the unknown sample. The spots are dried under a cold stream of air and predeveloped in methanol put in the bottom of a thin-layer chromatography glass jar. The predevelopment is stopped when the spots have travelled 5 mm. This step pro-

duces very thin spots all deposited on the same starting line at 2 cm from the lower edge.

The plate is dried in an oven at 65°C for 5 min and transferred to a second chromatography jar previously saturated with the solvent chloroform—methanol—ethyl acetate—acetone—triethylamine (70:15:10:10:5, v/v). The development takes 75 min and the solvent front is 16 cm from the bottom of the plate. The plate is dried at 65°C for 5 min and stored in the dark.

The fluorescent spots are recorded with a Farrand Model Mark I spectrofluorometer equipped with a thin-layer recording device. The excitation light is set at 340 nm with an additional violet filter absorbing light over 500 nm and a slot of 1 cm × 0.5 mm. The emitted light is measured at 525 nm with an additional yellow filter to absorb radiation under 450 nm.

The concentrations of the unknown samples are obtained from a standard curve drawn every day by plotting the values of the area of the peaks corresponding to the spots of known amounts of standards 1-MeHis and 3-MeHis run in parallel. In practice, the heights of the peaks may be used because they are highly correlated to the area under the chromatographic conditions described.

RESULTS AND DISCUSSION

This paper describes a new technique for the evaluation of the urinary excretion of 1-MeHis and 3-MeHis. Five unknown samples may be deposited on every plate, together with four standards containing known amounts of 1-MeHis and 3-MeHis. One technician can easily perform ten urine analyses in a day.

The chromatographic solvent furnishes an excellent separation of histidine and the methylhistidine isomers (Fig. 1). No interference was found by any of

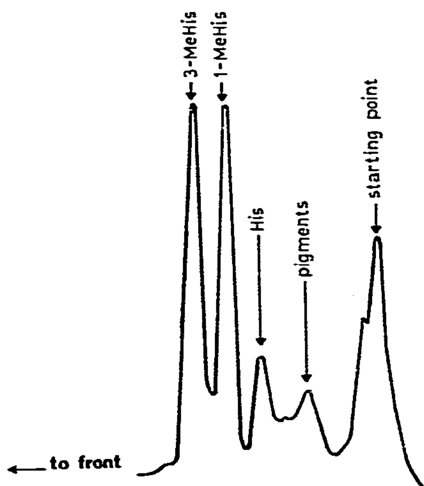

Fig. 1. Scanning of a thin-layer chromatogram showing the peak of NBD-3-MeHis and NBD-1-MeHis from a hydrolysate of urine. Mobile phase: chloroform—methanol—ethyl acetate—acetone—triethylamine (75: 15: 10: 10: 5, v/v). Development: 75 min at room temperature. Spectrofluorometer: Farrand Mark I, sensitivity 0.3, scan speed 120 mm/min.

the usual basic amino acids (Table I). The preliminary ion-exchange step permits the elimination of 3-hydroxyproline which has the same mobility as 3-MeHis on silica gel thin layers.

TABLE I

MOBILITY ON SILICA GEL PLATES OF DIFFERENT BASIC AMINO ACIDS AFTER DERIVATIZATION WITH NBD-Cl

Mobile phase: chloroform—methanol—ethyl acetate—acetone—triethylamine (75: 15: 10: 10: 5, v/v). Development: 75 min at room temperature.

Amino acid	R_F
Histidine	0.12
1-Methylhistidine	0.16
3-Methylhistidine	0.18
Arginine	0.04
Ornithine	0.01
Lysine	0.02
Hydroxylysine	0.01
γ-Aminobutyric acid	0.32

The lower limit of quantitation is 10 pmoles for 3-MeHis and 30 pmoles for 1-MeHis, which is more than sufficient for analysis in urine. The fluorescence intensity is highly dependent on the amino acid bound to NBD. If the fluorescence of 4-hydroxyproline is used as a reference [1] with an arbitrary fluorescence of 100, His gives a fluorescence equal to 23.1, 3-MeHis 54.8 and 1-MeHis 21.3.

The fluorescence of 1-MeHis and 3-MeHis is linear from 10 to 400 pmoles. The measurements are usually performed with the sensitivity of the apparatus set at 0.3, which permits a simple evaluation of the usual urinary concentrations corresponding to deposition ranging from 200 to 400 pmoles.

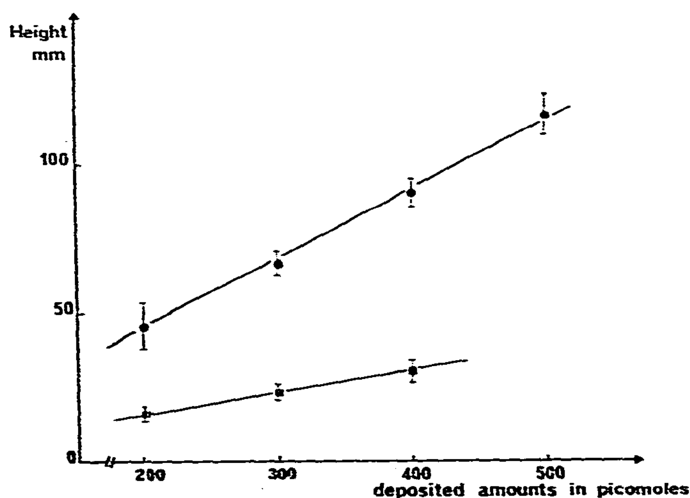

Fig. 2. Linearity of fluorescence with: (•) 3-MeHis, range 200—500 pmoles; (▪) 1-MeHis, range 200—400 pmoles. Bar: mean ± S.D. (ten determinations).

The reproducibility was tested by measuring the concentration of 3-MeHis on ten different samples of the same urine and was found to be 220.4 ± 4.6 μmoles/l (mean ± S.D.). The same urine was supplemented with 120 nmoles/ml of standards 3-MeHis and the results of five evaluations were 335.6 ± 4.4 μmoles/l, corresponding to a recovery of 97.3 ± 1.3%.

The results obtained for the urines of twenty normal adult subjects (11 males and 9 females) in the case of 3-MeHis correspond well with the reference values published by other authors using different methods (Table II). In the case of 1-MeHis, the value obtained in the urine of the same normal adult subjects was 71.4 ± 45.4 μmoles/day. Supplementation with 120 nmoles/ml of standard 1-MeHis gives a final recovery of 95.6 ± 3.7%. There are no statistically significant differences between male and female values.

TABLE II
RESULTS OBTAINED FOR 3-METHYLHISTIDINE AND 1-METHYLHISTIDINE IN URINE FROM TWENTY NORMAL ADULTS AND COMPARISON WITH THE REFERENCE VALUES PUBLISHED BY OTHER AUTHORS

Reference	3-MeHis (μmoles/day)	1-MeHis (μmoles/day)	Subjects
Yates et al. [4]	176 ± 45.1	—	20 {17 females, 3 males
Ward and Cooksley [6]	151—154.6	—	3 {2 females, 1 male
Bilmazes et al. [12]	167—252	—	4 males
Neuhaüser and Fürst [13]	299.4 ± 23.8	—	12 females
	545.4 ± 35.2	—	12 males
Bigwood et al. [14]	180—520	130—930	15 {9 females, 6 males
Mussini et al. [15]	159.34 ± 19.72	795.42 ± 99.30	4 males (7—12 years old)
This paper	242.4 ± 65.4	71.4 ± 45.4	20 {9 females, 11 males

Only two references give the normal range for this urinary metabolite [14, 15]. They both furnish a range higher than our data. This might be explained by an incomplete separation of the basic amino acids in ref. 14 published a long time ago and by the fact that the analyses of ref. 15 concerned the urines of four subjects whose ages ranged from 7 to 12 years. It is likely that the excretion of this metabolite increases during puberty.

This new method provides some advantages in comparison to the one used previously. It is faster, easily applicable to urine analysis in clinical chemistry and allows the evaluation of the second isomer 1-MeHis. In several pathological urines, we found an amount of 1-MeHis higher than that of 3-MeHis. We expect the evaluation of 1-MeHis to be of interest in the same pathological cases as 3-MeHis (for instance, in muscular diseases) and maybe to introduce some new semeiological applications.

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REFERENCES

1 G. Bellon, A. Bisker, F.X. Maquart, H. Thoannes and J.P. Borel, J. Chromatogr., 230 (1982) 420.
2 V.R. Young and H.N. Munro, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 37 (1978) 2291.
3 H. Tallan, W.H. Stein and S. Moore, J. Biol.Chem., 206 (1954) 825.
4 R.O. Yates, H. Connor and H.F. Woods, Ann. Nutr. Metab., 25 (1981) 262.
5 G. François, M. Blanc, J.C. Dumont and F. Rose, J. Med. (Marseille), 1 (1982) 27.
6 L.C. Ward and W.G. Cooksley, Clin. Chim. Acta, 91 (1979) 363.
7 H. Vielma, J. Mendez, M. Druckenmiller and H. Lukaski, J. Biochem. Biophys. Methods, 5 (1981) 75.
8 E. Radha and S.P. Bessman, Anal. Biochem., 121 (1982) 170.
9 Z. Friedman, H.W. Smith and W.S. Hancock, J. Chromatogr., 182 (1980) 414.
10 H. Vielma and J. Mendez, J. Chromatogr., 196 (1980) 166.
11 A. Szymanowicz, G. Poulin, N. Fontaine, J.P. Werquin and J.P. Borel, J. Chromatogr., 190 (1980) 457.
12 C. Bilmazes, R. Uauy, L.N. Haverberg, H.N. Munro and V.R. Young, Metab. Clin. Exp., 27 (1978) 525.
13 M. Neuhaüser and P. Fürst, Anal. Biochem., 92 (1979) 294.
14 E.J. Bigwood, E. Schram, P. Soupart and H. Vis, Expos. Annu. Biochim. Med., 23 (1961) 9.
15 E. Mussini, L. Cotellessa, L. Colombo, D. Cani, P. Sfondrini, F. Marcucci and F. Poy, J. Chromatogr., 224 (1981) 94.