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CHROMBIO. 1250

Note

Mass spectrometric identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine

FRITS A.J. MUSKIET*, C. MARGOT STRATINGH and DINEKE C. FREMOUW-OTTE-VANGERS

Central Laboratory for Clinical Chemistry. University Hospital. Oostersingel 59. POB 30.001, 9700 RB Groningen (The Netherlands)

and

M. RUUD HALIE

Division of Haematology. Faculty of Medicine. University of Groningen. Oostersingel 59. POB 30.001, 9700 RB Groningen (The Netherlands)

(First received December 22nd, 1981; revised manuscript received February 4th, 1982)

Polyamines are functional components of living cells which, most probably by their association with nucleic acids, are indissolubly united with growth processes [1—5]. During the last decade much attention has been given to their role as general markers for neoplastic diseases. In this respect the use of determinations of polyamines in biological fluids for the early diagnosis of cancer has been disappointing. On the other hand, their value as markers for tumor cell kinetics before, during and after chemotherapy is now widely accepted. The concept of Russell [6], linking extracellular putrescine concentrations to the tumor growth fraction and those of spermidine to cell turnover, has contributed much to this picture.

Although the regulation of polyamine synthesis during growth processes has been studied in detail [1—5], in man relatively little attention has been paid to the deactivating mechanisms of these potent growth factors. From the two most frequently mentioned deactivating routes, i.e. N-conjugation with acetic acid, propionic acid, glutamic acid, peptides and pyridoxal phosphate and (amino) oxidation, possibly followed by intermediate aldehyde oxidation, only fragmentary knowledge is available about the importance of the latter route in man [7].

In this study the mass spectrometric identification of isoputreanine, a metabolite of spermidine and/or spermine, is described. The possible significance of this compound in the diagnosis and follow-up of cancer is discussed.

MATERIALS AND METHODS

Standards and reagents

1,3-Diaminopropane , putrescine, cadaverine, spermidine, spermine, 1,6-diaminohexane and 1,7-diaminoheptane were from Sigma Chemical Co. (St. Louis MO, U.S.A.), bis-(3-aminopropyl)amine and 1,2-bis-(3-aminopropyl-amino)ethane were from Fluka (Buchs, Switzerland) and N-(3-aminopropyl)-2-pyrrolidone (the γ-lactam form of isoputreanine) was from Aldrich Europe, (Beerse, Belgium). Sep-Pak silica cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.); heptafluorobutyric anhydride was from Pierce Chemical Co. (Rockford, IL, U.S.A.); all other reagents were from Merck (Darmstadt, G.F.R.).

Samples

Urine samples (24 h) from normal persons and patients were collected, acidified to pH 1—2 with hydrochloric acid and stored at —20°C until analysis.

Equipment

Gas chromatography with flame ionization detection was performed with a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automated sampler and a 30 m X 0.32 mm I.D., CP-Sil-5 coated (0.2 μm film thickness), fused silica capillary column (Chrompack, Middelburg, The Netherlands). Gas flow-rate (helium) was 1.0 ml/min, split ratio 1:12, detector temperature 300°C, and injector temperature 280°C. The oven temperature program was 110°C, 7°C/min to 240°C, 15 min at 240°C.

Gas chromatography—mass spectrometry was performed using a Varian Aerograph 1400 gas chromatograph directly coupled to a Varian MAT 112 mass spectrometer equipped with a similar capillary column. Ionization energy was 70 eV; mass spectra were taken at a scanning rate of 100 a.m.u. per sec near the top of the gas chromatographic peak. No corrections were made for background or changes in total ion current during scanning.

PROCEDURES

Acid hydrolysis

To five millilitres of acidified urine were added 400 μl of an internal standard cocktail containing 50 nmol each of 1,6-diaminohexane, 1,7-diaminoheptane, bis-(3-aminopropyl)amine and 1,2-bis-(3-aminopropylamino)ethane, dissolved in a 1:1 (v/v) mixture of methanol and 1 mol/l hydrochloric acid. The urine was evaporated to almost dryness at 120°C under a stream of air and hydrolyzed at 120°C overnight in 2 ml of 6 mol/l hydrochloric acid.

Clean-up

The hydrolysate was evaporated to dryness at 120°C under a stream of air

and the residue resuspended in 2 ml of 0.01 mol/l hydrochloric acid. The suspension was centrifuged and the supernatant transferred to a plastic tube containing 8 ml of 0.05 mol/l borax buffer pH 9.0. The solution was adjusted to pH 9.0 by the addition of a few drops of 4 mol/l sodium hydroxide solution, centrifuged and passed over a Sep-Pak silica column, previously washed with 0.1 mol/l hydrochloric acid in methanol (12 mol/l hydrochloric acid in water, diluted to 0.1 mol/l with methanol) and water. The Sep-Pak column was washed with 25 ml of water and eluted with 11 ml of 0.1 mol/l hydrochloric acid in methanol. Regeneration was performed by washing with 10 ml of 0.1 mol/l hydrochloric acid in methanol and 30 ml of water.

Derivatization

The eluate (11 ml) was evaporated to dryness at 80°C under a stream of air. To the residue were added 400 μl of a 5:1 (v/v) mixture of acetonitrile and heptafluorobutyric anhydride. The tube was sonicated for 5 min and stored at room temperature overnight.

Isolation of derivatives

The solutions were evaporated to dryness at room temperature under a stream of air and the residue dissolved in 1 ml of 0.5 mol/l phosphate buffer pH 7.0. The derivatives were extracted into 3 ml of dichloromethane. After drying the dichloromethane layer over a small amount of anhydrous sodium sulfate, the solution was evaporated to dryness at room temperature under a gentle stream of air. The residue was dissolved in 200 μl of ethyl acetate, and 2-μl aliquots were injected into the gas chromatograph.

RESULTS AND DISCUSSION

During the development of a new gas chromatographic method with flame ionization detection for the profiling in urine of the five naturally occurring polyamines — 1,3-diaminopropane, putrescine, cadaverine, spermidine, and spermine — we observed three relatively large peaks in the gas chromatograms of urines of a patient with a non-African, Burkitt-type, non-Hodgkin lymphoma. The elucidation of the structures and possible clinical importance of the three corresponding chemical compounds is the subject of this paper. More details about the methodology, quality control, normal values and values during various types of cancer will be described elsewhere.

Fig. 1A shows the gas chromatogram of a urine sample of the patient described above, in which three large peaks, assigned to 1,3-diaminopropane and two other substances named X_1 and X_2, can be seen. Their mass spectra are shown in Fig. 2. The mass spectrum of urinary 1,3-diaminopropane was found to be similar to that of the derivatized authentic compound.

Quantitative data, obtained from the determination of polyamines and the estimation of X_1 and X_2 in 56 24-h urines during a three-month follow-up of the patient, were collected. These data clearly show that the excretion of 1,3-diaminopropane, X_1 and X_2 run almost parallel to that of spermidine and spermine.
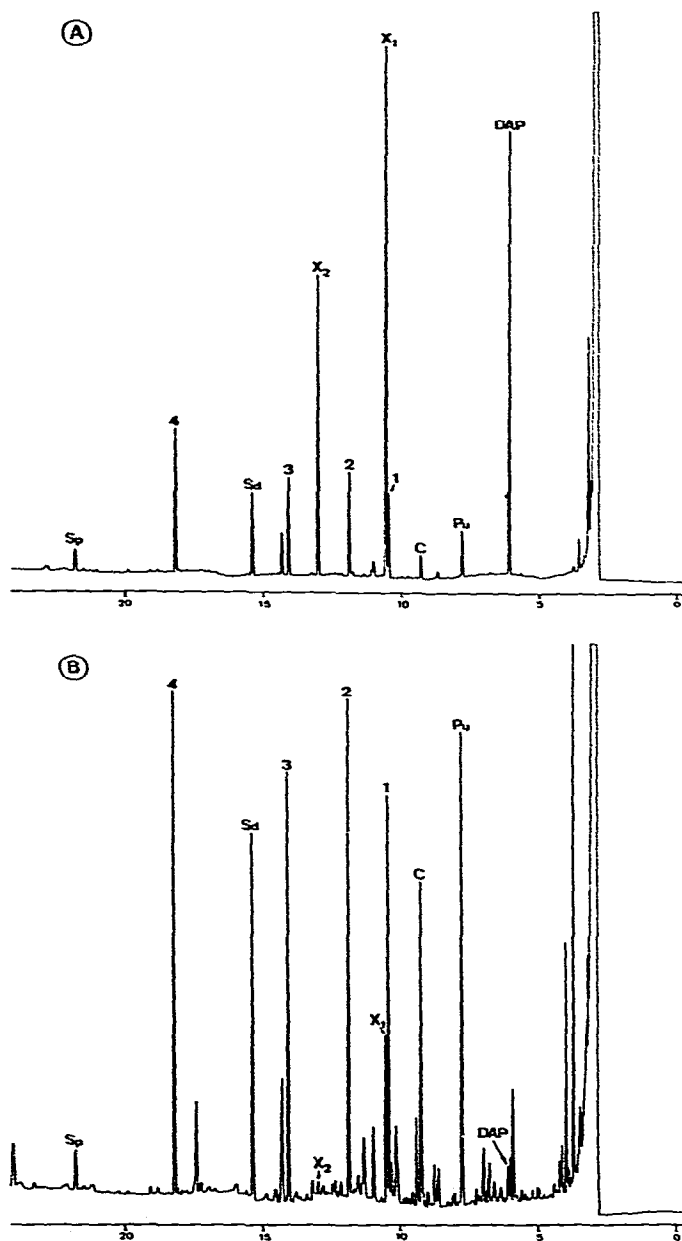
Fig. 1. Capillary gas chromatograms of the heptafluorobutyryl derivatives of polyamines extracted from the urine of a six-year-old patient with a non-African, non-Hodgkin, Burkitt-type lymphoma (A), and of a normal, healthy, 31-year-old adult (B). DAP = 1,3-diaminopropane; Pu = putrescine; C = cadaverine; 1 = 1,6-diaminohexane; X_1 = N-heptafluorobutyryl-γ-lactam form of isoputreanine; 2 = 1,7-diaminoheptane; X_2 = N,N'-diheptafluorobutyrylmethylester of isoputreanine; 3 = bis-(3-aminopropyl)amine; Sd = spermidine; 4 = 1,2-bis-(3-aminopropylamino)ethane; Sp = spermine; 1—4 are added internal standards. Time axis in minutes.
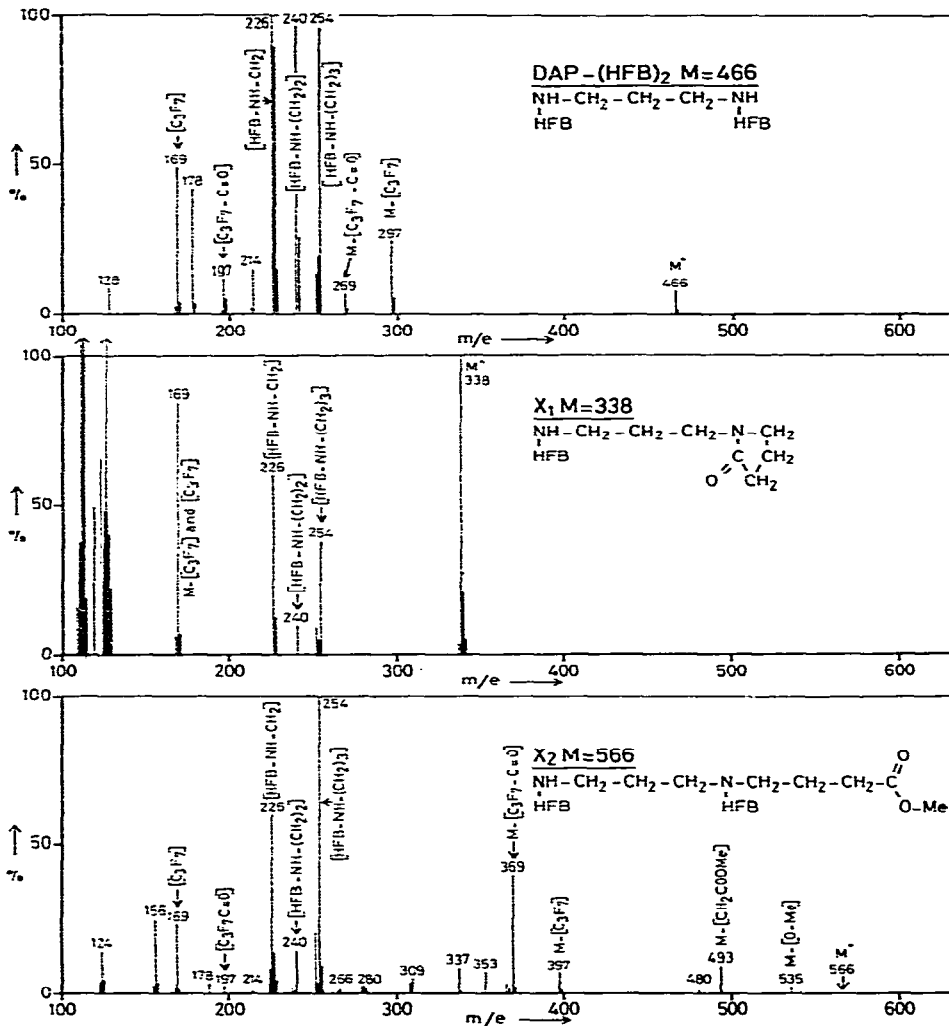
Fig. 2. Mass spectra of 1,3-diaminopropane (DAP), X_1 and X_2, obtained from a derivatized urinary extract of a patient with a Burkitt-type lymphoma. For gas chromatogram and abbreviations used see Fig. 1A. HFB, heptafluorobutyryl; Me, methyl.

The excretion of putrescine and of cadaverine was not related to spermidine, nor were they related to each other. This suggested that 1,3-diaminopropane, X_1 and X_2 are metabolically linked to spermidine and/or spermine and reflect cell turnover according to the concept of Russell [6].

On the basis of these findings, the mass spectral data and retention times, we were able to identify X_2 as the methylester—N,N'-diheptafluorobutyryl derivative of isoputreanine and X_1 as its N-monoheptafluorobutyryl-γ-lactam derivative (for structures see Fig. 2). The methylation of part of the isoputreanine was found to be an artefact introduced by evaporating the Sep-Pak eluate at 80°C, whereas γ-lactamization of isoputreanine occurs readily by dehydration during derivatization with heptafluorobutyric anhydride. In urine,

isoputreanine is most probably predominantly present as a conjugate since the analysis of unhydrolyzed urines did not show peaks with the correct retention times.

Isoputreanine has previously been identified in rat urine [8] and normal human urine [9]. On the basis of tracer experiments in rats, Asatoor [9] considered N^8-amino oxidation of (N^1-conjugated) spermidine, followed by oxidation of the intermediate aldehyde, to be a possible explanation for the formation of isoputreanine. Another possibility may be that, analogous to the spermidine dehydrogenase found in *Serratia marcescens* [7], spermidine and spermine are degraded by dehydrogenases, giving rise to 1,3-diaminopropane/Δ^1-pyrroline and 1,3-diaminopropane/isoputreanine, respectively. However, until now, neither enzymatic pathway leading to the formation of isoputreanine has been shown to exist in man.

Isoputreanine was excreted in relatively low amounts by normal persons (Fig. 1B). In the urine of an adult patient with non-Hodgkin lymphoma we observed a highly significant increase of isoputreanine during successful chemotherapy but not of spermidine. These data, together with the (in comparison to spermidine) much higher excretion of isoputreanine and 1,3-diaminopropane in the urine of the patient with Burkitt-type lymphoma, suggest that at least some cases of cancer are characterized by an increased degradative metabolic pressure on polyamines. The impact of this phenomenon on the diagnosis and follow-up of such patients using measurements of polyamines and/or their acetyl conjugates remains to be established. However, the ignorance of the polyamine degradative routes in health and disease may have considerably contributed to the scepticism concerning the use of determinations of polyamines as general markers for neoplastic diseases.

REFERENCES

1 D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973.
2 D.H. Russell and B.G.M. Durrie, (Editors), Progress in Cancer Research and Therapy, Vol. 8, Raven Press, New York, 1978.
3 R.A. Campbell, D.R. Morris, D. Bartos, G.D. Daves and F. Bartos (Editors), Advances in Polyamine Research, Vol. 2, Raven Press, New York, 1978.
4 J. Jänne, H. Pösö and A. Raina, Biochim. Biophys. Acta, 473 (1978) 241—293.
5 G. Milano, M. Schneider, J.P. Cassuto, E. Viguier, P. Pastorini, J.L. Boublil, M. Namer, P. Cambon and C.M. Lalanne, Tumor Diagn., 3 (1980) 121—125.
6 D.H. Russell, Clin. Chem., 23 (1977) 22—27.
7 R. Aigner-Held and G.D. Daves, Physiol. Chem. Phys., 12 (1980) 389—400.
8 T. Noto, T. Tanaka and T. Nakajima, J. Biochem., 83 (1978) 453—552.
9 A.M. Asatoor, Biochim. Biophys. Acta, 586 (1979) 55—62.