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Gas chromatographic determination of N-acetylisoputreanine-γ-lactam, a unique catabolite of N^1-acetylspermidine

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ABSTRACT

A capillary gas chromatographic method with nitrogen–phosphorus detection for the determination of N-acetylisoputreanine-γ-lactam (acisoga) in urine is described. The method was validated by comparing the results with those given by an isotope dilution mass fragmentographic method. Making use of specific inhibitors for copper-dependent amine oxidase and polyamine oxidase in rats, it was demonstrated that acisoga is formed by oxidative deamination of N^1-acetylspermidine by the former enzyme. Moreover, acisoga is not a substrate for pig liver polyamine oxidase. Increased concentrations of acisoga, relative to N^1-acetylspermidine, in urines of patients with non-Hodgkin's lymphoma indicated that this conversion diminishes the sensitivity of N^1-acetylspermidine as a marker for (tumour) cell death.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are essential constituents of living cells and play important roles in cell growth and differentiation [1]. Cellular polyamine levels are well controlled. This control takes place via regulation of biosynthesis, retroconversion, terminal catabolism and transport [2–5]. Augmentation of retroconversion by means of induction of cytosolic spermidine–spermine N^1-acetyltransferase (c-SAT) results in, among other things, increased production of N^1-acetylspermidine from spermidine (Fig. 1). N^1-Acetylspermidine is either excreted or converted to putrescine by flavine adenine dinucleotide (FAD)-dependent polyamine oxidase (PAO). N^1-Acetylspermidine seems to be the quantitatively most prominent unique form of spermidine excreted in the urine of healthy persons and patients with some particular neoplasia, such as non-Hodgkin's lymphoma [6,7]. Terminal catabolism takes place through oxidative deamination by copper-dependent amine oxidases (CuAO). The aldehydes formed in these reactions are subsequently oxidized to the corresponding amino acids and excreted. Putrescine, spermidine and spermine are considered to

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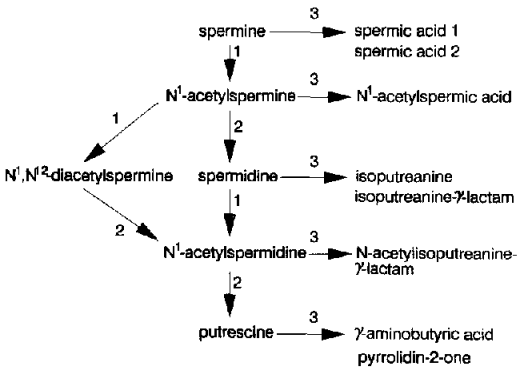

Fig. 1. Retroconversion and terminal catabolism of polyamines. Key to enzymes: 1 = cytosolic spermidine–spermine N^1-acetyltransferase (c-SAT); 2 = FAD-dependent polyamine oxidase (PAO); 3 = copper-dependent amine oxidase (CuAO).

be the natural substrates of CuAO. Oxidative deamination of spermidine finally leads to the formation of isoputreanine [8] and isoputreanine-γ-lactam [9] (Fig. 1). Van den Berg *et al.* [10] showed that about half of the total isoputreanine in urine from healthy humans is originally excreted as N-acetylisoputreanine-γ-lactam (acisoga). The existence of this compound suggests that N^1-acetylspermidine is a substrate of CuAO [9]. Patients with non-Hodgkin's lymphoma have increased urinary levels of both total isoputreanine and acisoga [10].

We have developed a simple capillary gas chromatographic method with nitrogen–phosphorus detection (GC–NPD) for the determination of acisoga in urine and compared the results with those obtained by the previously reported isotope dilution mass fragmentographic method [gas chromatography–mass spectrometry (GC–MS)] employed by Van den Berg *et al.* [10]. With the former method we measured urinary acisoga excretion levels of healthy volunteers and rats treated with the PAO inhibitor N,N′-bis(buta-2,3-dienyl)butane-1,4-diamine (MDL 72527) and/or the CuAO inhibitor aminoguanidine (AG) in an effort to obtain further insight into its origin.

EXPERIMENTAL

Chemicals

Polyamines, acetylpolyamines, homovanillic acid, dithiotreitol, AG and horseradish peroxidase type 1 (E.C. 1.11.1.7) were obtained from Sigma (St. Louis, MO, U.S.A.). Isoputreanine-γ-lactam was purchased from Janssen (Beerse, Belgium). MDL 72527 was a generous gift from the Merrell Dow Research Institute (Strasbourg, France).

Acisoga was synthesized by acetylation of isoputreanine-γ-lactam, as previously described [10]. Trideuteroacisoga and N-propionylisoputreanine-γ-lactam

were prepared similarly, using trideuteroacetyl chloride and propionyl chloride (Sigma) as reagents, respectively.

Urine collection from humans

Urine samples (24 h) from seventeen apparently healthy persons were collected and adjusted to pH 1.0 with hydrochloric acid. Samples were stored at −20°C until analysis.

Animals and animal experiments

Eight female Wistar rats, four months old and weighing 220–250 g, were divided into four groups. The rats were housed individually in metabolic cages under standard laboratory conditions (20–22°C, 50–60% relative humidity, 12 h light–12 h dark cycle, acidified drinking water of pH 3–4 and standard rat chow *ad libitum*). After two days of adaptation, 24-h urines on the next two days were collected in containers with 1 ml of 2 *M* hydrochloric acid. On the following five days the control group received daily intraperitoneal injections of 0.5 ml of phosphate-buffered saline (PBS) (140 m*M* NaCl, 9.0 m*M* Na_2HPO_4, 1.3 m*M* NaH_2PO_4; pH 7.4). The other three groups received daily intraperitoneal injections of AG (25 mg/kg), MDL 72527 (20 mg/kg) or their combination in 0.5 ml of PBS. The collected urines were stored at −20°C until analysis.

Analytical methods

Acetylpolyamines were measured using GC–NPD [6]. For the determination of acisoga, 10 nmol of N-propionylisoputreanine-γ-lactam (internal standard) in 100 μl of dichloromethane were evaporated to dryness, after which 1 ml of urine was added. The sample was adjusted to pH 9.0 with 4 *M* sodium hydroxide solution and extracted twice with 3 ml of methanol–chloroform (1:3, v/v). The organic layers were collected after centrifugation, pooled and dehydrated with anhydrous sodium sulphate. This solution was evaporated to dryness at room temperature under a stream of nitrogen and the residue was dissolved in 200 μl of dichloromethane containing 2 g/l Carbowax 1000M. An aliquot of 1.5 μl was injected into a Model 5880 gas chromatograph equipped with a nitrogen–phosphorus detector and a Model 7672A automatic sampler (all from Hewlett-Packard, Amstelveen, The Netherlands). For separation we used a bonded-phase HP-17 fused-silica capillary column (25 m × 0.20 mm I.D.; film thickness 0.17 μm). The splitting ratio was 1:10, detector temperature 300°C and injector temperature 260°C. The oven temperature was programmed from 180 to 270°C at 3°C/min.

The GC–NPD method for urinary acisoga was validated by comparing the results with those obtained by a GC–MS method [10]. In the latter instance 10 nmol of the internal standard trideuteroacisoga were added to 1 ml of urine. Analyses were performed with a Model 5890 gas chromatograph (Hewlett-Packard) directly coupled to a Trio-2 quadrupole mass spectrometer (VG Masslab,

Manchester, U.K.) equipped with the same column and operated with the same oven temperature programme as described above. The ion source temperature was 150°C, ionization energy 70 eV and interface temperature 250°C. The instrument was operated in the ammonia chemical ionization mode and focused at the positive ions with m/z 185 and 188, corresponding to the $[M+H]^+$ ions of acisoga and its trideuterated analogue, respectively. Concentrations were calculated by means of a calibration graph, which was prepared from data of the corresponding peak-area ratios of various amounts of acisoga (0 25 nmol) added to 10 nmol of internal standard. Urinary concentrations were expressed in terms of creatinine, measured with a picric acid method [11].

Enzyme kinetics of polyamine oxidase

PAO was isolated from fresh pig liver as described by Bolkenius and Seiler [12]. Protein was measured by the biuret method [13]. The specific activity of this partially purified preparation was determined by incubating the equivalent of 0.9 mg of protein with 1.8 m*M* N^1-acetylspermine in 1 ml of 50 m*M* borate buffer (pH 9.0) (containing 0.2 m*M* dithiothreitol) at 37°C for 1 h. One unit is defined as the oxidation of 1 nmol of N^1-acetylspermine per hour.

Hydrogen peroxide production was used as a measure of PAO activity, employing a fluorescence-based method [14]. All incubations were carried out in the presence of 14 μg of horseradish peroxidase (102 U/mg protein) and 84 μg of homovanillic acid. After incubation for 1 h, the fluorescence intensity was measured immediately using an FS 970 L.C. fluorimeter (Kratos, Manchester, U.K.) equipped with a 10-μl flow cell. Fluorescence was activated at 315 nm. The optimum fluorescence emission occurred at 425 nm and was measured using a 405-nm cut-off filter. Blanks contained enzyme but no substrate.

For the determination of Michaelis constants (K_M) the enzyme preparation was incubated with various amounts of N^1-acetylspermidine, N^1-acetylspermine and acisoga. Maximum oxidation rates were measured by incubation of 1.8 m*M* of these substrates with 30 U of enzyme. With acisoga the change in substrate concentration was also monitored by GC–NPD.

RESULTS

Fig. 2 shows representative examples of the GC–NPD of acisoga in urine. In addition to standard (A) it shows chromatograms from rat urines collected during the basal period (B) and after two days of treatment with MDL 72527 (C). Fig. 3 shows a comparison of results of nine acisoga determinations by both GC–MS (abscissa) and GC–NPD (ordinate). The orthogonal regression line, using standard deviations of 0.16 and 0.09 μmol/l for the GC–NPD and GC–MS [10] methods, respectively, was $y = 1.03x - 0.32$ ($r = 0.9859$). The residual standard deviations were 0.39 μmol/l (S_{yx}) and 0.22 μmol/l (S_{xy}). The within-series precision and recovery for the GC–NPD method were determined by mea-

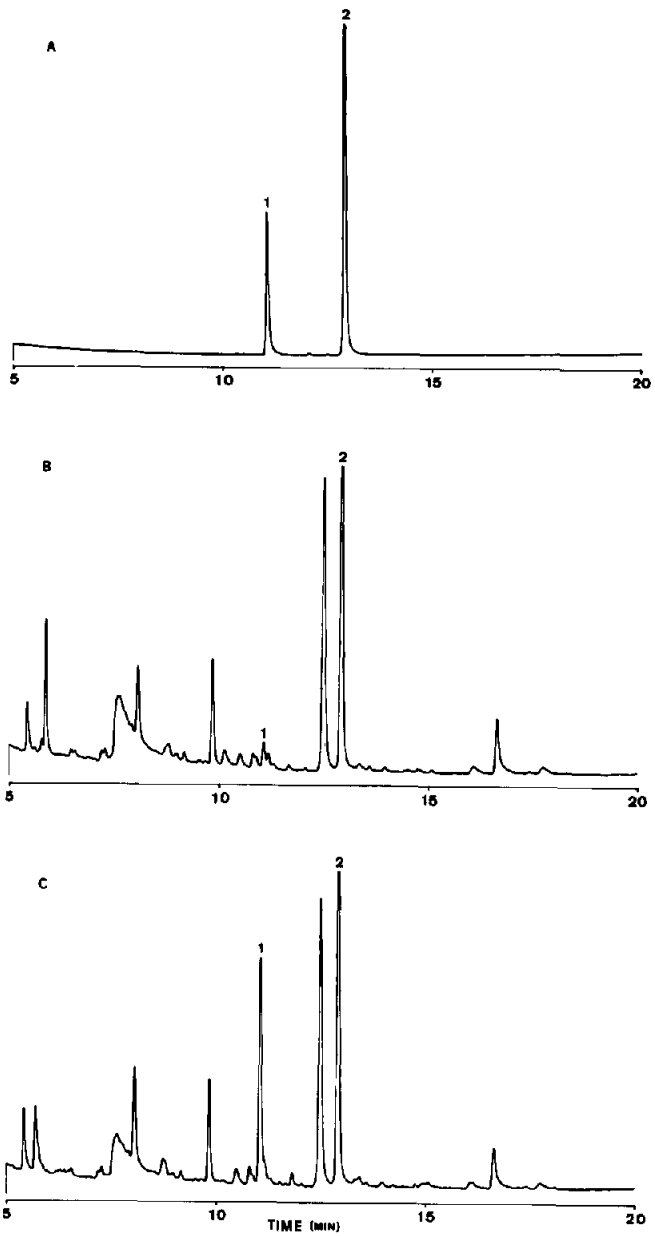

Fig. 2. Representative gas chromatograms of (A) a standard, (B) a rat urine and (C) urine of the same rat after two days of treatment with polyamine oxidase inhibitor MDL 72527 (20 mg/kg). Peak 1 is acisoga (10 nmol in standard) and peak 2 is the internal standard N-propionylisoputreanine-γ-lactam (10 nmol in standard and 10 nmol/ml in urine samples).

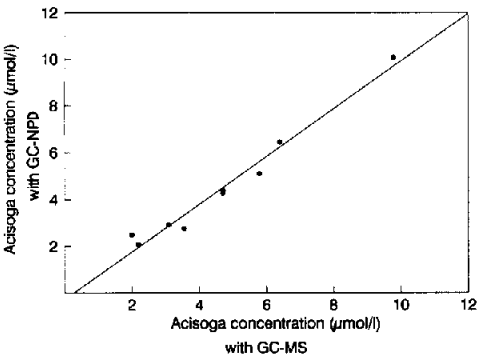

Fig. 3. Comparison of urinary acisoga concentrations determined using isotope dilution mass fragmentography (GC–MS; abscissa) and GC–NPD (ordinate). The orthogonal regression line represents $y = 1.03x - 0.32$; $r = 0.9859$; $S_{yx} = 0.39$ μmol/l and $S_{xy} = 0.22$ μmol/l.

suring ten samples of pooled urine from untreated rats, together with ten corresponding samples enriched with 20 μmol/l acisoga. The mean endogenous concentration was 4.58 μmol/l [relative standard deviation (R.S.D.) 3.5%, range 4.30–4.72 μmol/l] and the mean recovery was 98.5% (R.S.D. 3.2%, range 93.0–102.5%). The day-to-day recovery of the method was calculated from data for five different rat urines fortified with and without 10 μmol/l acisoga and determined in five series. The mean recovery was 102.4% (R.S.D. 8.6%, range 89.0–116.0%).

Table I shows that, relative to basal levels, AG treatment of rats results in almost undetectable urinary concentrations of acisoga, whereas MDL 72527 treatment causes highly increased levels. In addition, CuAO inhibition caused an increase of N^1-acetylspermidine. During PAO inhibition, N^1-acetylspermidine excretion increased in the same proportion as acisoga, as indicated by their unaltered ratio. Inhibition of both CuAO and PAO led to a decreased excretion level of acisoga, whereas that of N^1-acetylspermidine increased. Table II shows that the acisoga levels in urines of healthy controls are about twice that of N^1-acetylspermidine. From the three patients with stage IV non-Hodgkin's lymphoma, two (A and C) showed clearly increased urinary excretion levels of both components. Patients B and C had increased urinary acisoga/N^1-acetylspermidine ratios.

The isolation method employed for PAO resulted in a white powder with a specific activity for N^1-acetylspermine of 22 nmol/mg protein · h. The calculated K_M values for PAO were 0.7 and 12 μmol/l with N^1-acetylspermine and N^1-acetylspermidine as substrates, respectively. Incubations of acisoga with PAO did not lead to detectable hydrogen peroxide production, nor did monitoring of the acisoga concentration by GC–NPD show any detectable decrease. On the basis of the detection limit, the maximum oxidation rate of acisoga was calculated to be less than 1% of that of N^1-acetylspermine.

TABLE I

LEVELS OF N-ACETYLISOPUTREANINE-γ-LACTAM (ACISOGA) AND N^1-ACETYLSPERMIDINE (N^1-AcSd) IN URINES OF RATS TREATED WITH PBS, AMINOGUANIDINE (AG), MDL 72527 OR A COMBINATION OF THE INHIBITORS

Two rats were intraperitoneally injected daily from day 2 with PBS, AG (25 mg/kg), MDL 72527 (20 mg/kg) or a combination of AG and MDL 72527. Urinary excretion levels from days 1 and 2 were averaged and designated as basal values (n = 2 for each rat). The means of days 5, 6 and 7, at which steady-state levels were reached, are designated as values during treatment (n = 3 for each rat). Acisoga was measured with the GC–NPD method.

Inhibitor	Rat	Urinary excretion level (mmol/mol of creatinine)					
		Acisoga (*a*)		N^1-AcSd (*b*)		ratio *a*/*b*	
		Basal	Treatment	Basal	Treatment	Basal	Treatment
PBS	1	0.17	0.15	1.36	1.37	0.13	0.11
	2	0.19	0.16	1.43	1.47	0.13	0.11
AG	1	0.09	0.01	1.25	3.26	0.08	0.00
	2	0.12	b.d.l.[a]	1.28	3.75	0.09	b.d.l.
MDL 72527	1	0.20	2.30	1.76	20.1	0.11	0.11
	2	0.15	2.01	1.60	19.7	0.09	0.10
AG +	1	0.20	0.06	1.93	28.0	0.10	0.00
MDL 72527	2	0.17	0.06	1.82	28.9	0.09	0.00

[a] Below the detection limit of the method.

TABLE II

LEVELS OF N-ACETYLISOPUTREANINE-γ-LACTAM (ACISOGA) AND N^1-ACETYLSPERMIDINE (N^1-AcSd) IN URINES OF HEALTHY PERSONS AND THREE PATIENTS WITH NON-HODGKIN'S LYMPHOMA

Reference values were determined by measuring urinary excretion levels of seventeen apparently healthy adults. Numbers in parentheses represent the range of the reference values. Acisoga was measured with the GC–NPD method. Data from patients with non-Hodgkin's lymphoma (stage IV) are the means of two consecutive 24-h urines, collected prior to chemotherapeutic treatment. These data were obtained from previous studies by Van den Berg and co-workers [6,10], in which acisoga was measured using GC–MS.

Subject	Urinary excretion level (mmol/mol of creatinine)		
	Acisoga (*a*)	N^1-AcSd (*b*)	Ratio *a*/*b*
Controls (n = 17)	0.58 ± 0.32 (0.33–1.84)	0.32 ± 0.15 (0.12–0.69)	2.00 ± 0.61 (1.00–2.94)
Patient A	4.61	4.06	1.24
Patient B	1.85	0.51	3.80
Patient C	7.14	1.72	4.21

DISCUSSION

The results demonstrate that the measurement of acisoga in urine by GC–NPD can be carried out with comparable accuracy and precision to that with GC–MS. The reference values for acisoga in human urine determined by GC–NPD (mean 0.58 mmol/mol of creatinine, range 0.33–1.84) compare favourably with those previously established by GC–MS [10] (mean 0.65 mmol/mol of creatinine, range 0.41–1.57). The advantage of the former method is that it does not need mass spectrometric equipment, making it accessible to more laboratories.

As during CuAO inhibition the peak with the retention time of acisoga almost completely disappeared (Table I), it may be concluded that this peak is solely derived from acisoga. Further, it also suggests that acisoga is a CuAO-catalysed reaction product. However, the decrease in urinary acisoga after AG treatment (about 0.1 mmol/mol of creatinine) proved incompatible with the increase in N^1-acetylspermidine (about 2.2 mmol/mol of creatinine). This apparent discrepancy may be explained by the additionally inhibited degradation of spermine, N^1-acetylspermine and spermidine by CuAO [3], leading to accumulation of spermidine, the precursor of N^1-acetylspermidine (Fig. 1). Although isoputreanine-γ-lactam, a theoretical precursor of acisoga, is also depleted during AG treatment [3,9], it is not likely that acisoga is formed from isoputreanine-γ-lactam by a c-SAT-catalysed acetylation. The minimum required substrate structure for c-SAT is considered to be an aminopropyl group attached to a nitrogen atom. In the best substrates this nitrogen is secondary, being attached to a moiety that contains a free amino group on the other end [15]. Isoputreanine-γ-lactam does not fulfil the latter requirement.

The calculated K_M values for PAO with N^1-acetylspermidine and N^1-acetylspermine as substrates are in good agreement with those in the literature [16]. From *in vitro* studies with partially purified PAO the hypothetical cleavage of acisoga into acetamidopropanal and pyrrolidin-2-one can be excluded. This finding is in agreement with observations of Bolkenius and Seiler [12], who concluded that the minimum structural requirement for a PAO substrate appears to be an aliphatic α,ω-diamine with a non-polar side-chain on one or both of the two nitrogen atoms. The increased acisoga excretion during PAO inhibition is therefore probably caused by accumulation of N^1-acetylspermidine, as a consequence of its inhibited conversion to putrescine (Fig. 1).

Rats (Table I) have a lower acisoga/N^1-acetylspermidine ratio in urine than humans (Table II). This indicates species differences in the efficacy to convert N^1-acetylspermidine to acisoga (terminal catabolism), to putrescine (retroconversion) or both. Within humans the relative efficacy seems to change under certain pathological conditions, such as the presence of non-Hodgkin's lymphoma. In two of these patients the acisoga/N^1-acetylspermidine ratios were above the reference range. This may theoretically be caused by increased activity of CuAO or PAO, or both. Induction of CuAO is a well known phenomenon [17],

whereas inducibility of PAO has not been demonstrated under conditions of increased cell turnover [18]. Moreover, PAO inhibition does not change the acisoga/N^1-acetylspermidine ratio, at least not in rats (Table I), and, based on the accumulation of N^1,N^{12}-diacetylspermine in urine, non-Hodgkin's lymphoma cells have decreased rather than increased PAO activity [6]. The most probable cause of the increased ratio in the urine of these patients is therefore induction of CuAO. Whatever the underlying mechanism, changes in metabolic pathways of polyamines have to be taken into account in patients with neoplastic diseases. Additional measurement of acisoga in urine may increase the sensitivity of polyamine conjugates as markers for (tumour) cell death.

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