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Plasma and blood assay of xanthine and hypoxanthine by gas chromatography–mass spectrometry: physiological variations in humans

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ABSTRACT

Plasma and blood xanthine and hypoxanthine levels were assayed using a sensitive and specific method involving gas chromatography–mass spectrometry, associated with an optimized sample preparation procedure. Physiological variation was studied in 224 subjects with no purine metabolism disorders. An age dependency for both compounds was found, comparable with that known for uric acid. The mean plasma levels for the 224 subjects were 0.65 ± 0.24 μM for xanthine and 1.65 ± 0.78 μM for hypoxanthine. Corresponding mean blood levels were 0.59 ± 0.21 μM for xanthine and 1.72 ± 0.74 μM for hypoxanthine. Plasma and blood levels were significantly different, by ca. 10%. Rapid in vitro release of hypoxanthine from erythrocytes and continuation of intra-erythrocytal metabolism lead to overestimation exceeding 10% within half an hour after sample blood collection. Hence samples must be deproteinized promptly. Blood can therefore be conveniently used for oxypurine assay instead of plasma when prompt spinning of samples is difficult to manage, as is usually encountered in clinical practice.

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INTRODUCTION

Assay of circulating xanthine (xan) and hypoxanthine (hyp) is of particular interest because these two hydroxylated purine bases, besides being precursors of uric acid, are also essential intermediates in purine metabolism. Hypoxanthine in particular would appear to be the extracellular compound most directly linked to the intracellular ATP concentration [1]. Almost all the work published to date has used high-performance liquid chromatography and involved a small number of subjects. We report here the results of a study of inter-individual variation in plasma and blood levels of xan and hyp in a large number (224) of subjects. The assay was performed using a specific analytical method described previously [2], in which gas chromatography (GC) is coupled with mass spectrometry (MS). The preparation procedure of the blood samples which was subsequently developed [3], showed the prime importance of the anticoagulant. In order to increase the sensitivity, the GC–MS analysis conditions [2] were modified, and we used the chemical ionization (CI) mode rather than the electron impact (EI) mode.

EXPERIMENTAL

Chemicals and reagents

Stock solutions of xan and hyp (Merck, Darmstadt, F.R.G.) and the internal standard [1,3-$^{15}N_2$]xanthine (^{15}N-xan) (CEA, Saclay, France) were prepared by dissolution in a dilute ammonia solution (0.05%) to 5000 μM and were stored at 4°C in polypropylene tubes. Trichloroacetic acid (10%, w/v) which was used to deproteinate blood samples, was made up by diluting a 19.4% aqueous solution (Boehringer, Mannheim, F.R.G.). Organic extraction solvents were diethyl ether (SDS, Peypin, France) and *n*-butanol (Carlo Erba, Milan, Italy). Reagents for derivatization were N,N-dimethylacetamide (Fluka, Buchs, Switzerland), 0.1 *M* tetrabutylammonium hydroxide in methanol (Merck, Darmstadt, F.R.G.) and pentyl iodide (Merck).

GC–MS system

The apparatus used was a Hewlett-Packard 5985 B quadrupolar GC–MS system fitted with a combined EI–CI source and driven by an HP 1000 computer. Chromatographic separation of oxypurines was achieved on a Pyrex glass column (1.80 m×2 mm I.D.) filled with 0.6% OV17 on Supelcoport 80–100 mesh and silylated at 150°C with three injections of 10 μl of Silyl-8 (Pierce, Rockford, IL, U.S.A.) before each series of runs. The injector and transfer line temperatures were both set at 270°C. The column temperature was programmed to run from 190 to 270°C at 20°C/min. The carrier gas was helium at a flow-rate of 21 ml/min. In CI, the flow-rate of methane as reactant gas was set so as to obtain a pressure near 1 Torr in the source. The electron energy

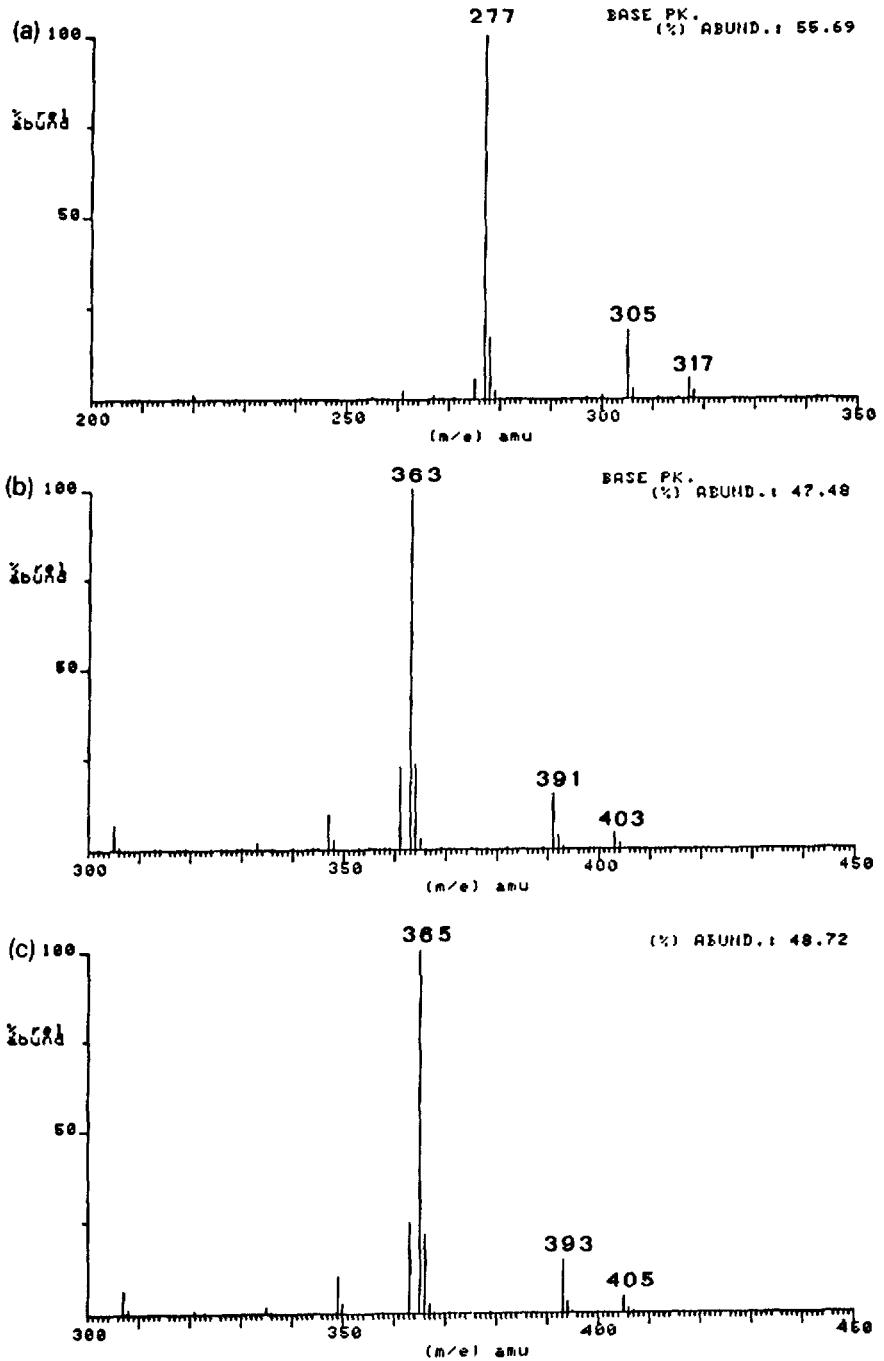

Fig. 1. Mass spectra of pentyl derivatives of hypoxanthine (a), xanthine (b) and [1,3-^{15}N]xanthine (c) in the CI mode.

was 200 eV and the emission current 300 μA. The mass spectra in CI mode of the pentyl derivatives of hyp, xan and ^{15}N-xan are shown in Fig. 1. Quantitative analysis was carried out on the basis of quasi-molecular $(M+1)^+$ ions, i.e. *m/z* 277.2 for hyp, *m/z* 363.3 for xan and *m/z* 365.3 for ^{15}N-xan. Pentylated solutions of xan and ^{15}N-xan were injected before each series of analyses to determine the percentage of ion *m/z* $363 = [M-1]^+$ from ^{15}N-xan in ion *m/z* $363 = [M+1]^+$ from xan, and that of isotopic ion *m/z* 365 from xan in the ion *m/z* $365 = [M+1]^+$ from ^{15}N-xan. These components were then automatically subtracted out of the area of the peaks due to xan and ^{15}N-xan.

Plasma and blood samples

Blood (10 ml) was collected on EDTA and separated into two fractions. One was immediately spun for 10 min at 1000 *g* to obtain plasma. Aliquots (0.5 ml) of blood and plasma samples were transferred to polypropylene tubes and spiked with the internal standard (10 μl of a 165 μM solution of ^{15}N-xan. The blood samples were haemolysed (0.5 ml of distilled water at 4°C, vortex-mixed for 1 min). All fractions were deproteinated with trichloroacetic acid (1 ml, vortex-mixed for 1 min) and spun for 15 min at 1000 *g*. Supernatants were separated, neutralized by washing three times with 1.5 ml of diethyl ether and then stored at −20°C until analysis.

Before analysis, deproteination supernatants were adjusted to pH 5.5 by addition of 125 μl of 1 *M* phosphate buffer and extracted twice with 2 ml of *n*-butanol. The extracts were evaporated in a stream of dry nitrogen (50°C), and the pentyl derivatives were prepared by the method of Greeley [4] by addition of 125 μl of N,N-dimethylacetamide, 50 μl of tetrabutylammonium hydroxide and then treatment with 35 μl of pentyl iodide for 20 min at room temperature.

Plasma (or haemolysed blood) depleted of nucleosides and bases was prepared by the technique of Wung and Howell [5] by stirring 10 ml of sample with 0.5 g of activated charcoal for 30 min and then spinning. These controls were used to set up calibration scales by adding xan and hyp at six concentrations: 0.4, 0.6, 1, 2, 5 and 10 μM.

Subjects

Study of physiological variations. Two groups of subjects were studied: (i) control subjects ($n=139$), male and female, from the Occupational Medicine Department, of various socioeconomic groups, aged 40 and 50 years, and (ii) patients from the Rheumatology Department, male and female, aged 14–90 years, showing no pathology associated with any purine metabolic disorder ($n=85$). The oxypurine assay was performed in the course of a biological and biochemical investigation. When sampled, the subjects had fasted overnight.

In vitro kinetics. The time-course of the plasma and blood concentrations of xan and hyp as a function of duration of contact of the plasma with the other blood components was monitored in thirteen healthy volunteers, male and female, aged from 23 to 53 years (37.5 ± 9.5), after fasting overnight. Blood and

plasma samples were prepared at various intervals after collection: 0, 0.5, 1, 2, 6 and 24 h. A 10-ml volume of blood was collected at each time and maintained at room temperature during these periods.

Statistical analysis

The different subject groups were compared using the *t*-test for comparison of paired means. Plasma and blood levels were compared using the *t*-test by difference for both xan and hyp. Correlations were established by one-way linear regression.

RESULTS

Validation of the method

Over the range of concentrations studied, 0.4–10 μM, the linearity was satisfactory and the correlation coefficients were over 0.998. The equations of the mean plots ($n=10$ ranges) were: $y=0.305x+0.048$ ($r=0.9997$) for xan and $y=0.136x-0.029$ ($r=0.9990$) for hyp.

Precision and accuracy were evaluated from plasma samples depleted of nucleosides and bases to which xan and hyp had been added and which were analysed either the same day ($n=10$) at 1 and 10 μM or over several days ($n=10$) at 1 and 5 μM. The coefficients of variation (C.V.) are listed in Table I.

The theoretical limit of quantitation was found to be 0.095 μM for xan and 0.161 μM for hyp. However, repeated analysis of these theoretical limits showed inadequate reproducibility with C.V. above 20%. The acceptable limit of quantitation is thus 0.16 μM for xan (0.17 ± 0.03, $n=10$, C.V. = 15.3%) and 0.20 μM for hyp (0.21 ± 0.04, $n=10$, C.V. = 19.0%).

TABLE I

PRECISION AND ACCURACY OF THE PLASMA ASSAY

Compound	Spiked concentration (μM)	Calculated concentration, (mean ± S.D.) (μM)	Precision, C.V. (%)	Accuracy, C.V. (%)
Within-day ($n=10$)				
Xan	1	1.01 ± 0.11	10.9	+1.0
	10	9.95 ± 0.11	1.1	−0.5
Hyp	1	0.98 ± 0.17	17.3	−2.0
	10	9.88 ± 0.38	3.9	−1.2
Between-day ($n=10$)				
Xan	1	1.01 ± 0.09	8.9	+1.0
	5	5.12 ± 0.24	5.7	+2.4
Hyp	1	0.97 ± 0.18	18 4	−3.0
	5	4.84 ± 0.55	11.5	−3.2

The specificity of the method depends on the specificity of the ions selected for the quantitative analysis. In the CI mode, under the operating conditions described, oxypurines undergo practically no fragmentation, and the ions formed are essentially quasi-molecular. Hence the method is highly specific.

Applications

Physiological variations. The mean concentrations and their standard deviations obtained in controls from each group are given in Table II.

Plasma levels in the two groups were homogeneous as regards sex distribution but not age distribution: the mean age of subjects from the Occupational Medicine group was 45.7 ± 6.7 for the females and 48.8 ± 5.9 for the males, and that of the Rheumatology group was 57.5 ± 16.7 for the females and 55.6 ± 14.4

TABLE II

MEAN BLOOD AND PLASMA LEVELS OF XANTHINE AND HYPOXANTHINE

The investigation used 139 control subjects from the Occupational Medicine Department and 85 patients from the Rheumatology Department.

Subject source	Sex	Age (years)	Sample	Xanthine (mean ± S.D.) (μM)	Hypoxanthine (mean ± S.D.) (μM)
Occupational Medicine Department	M	40	Plasma	0 61 ± 0.19	1.82 ± 1.03
			Blood	0.56 ± 0.18	1.96 ± 1.15
	M	50	Plasma	0.61 ± 0.16	1.62 ± 0.78
			Blood	0.55 ± 0.17	1.75 ± 0 71
	F	40	Plasma	0.48 ± 0.14	1.21 ± 0.57
			Blood	0.45 ± 0 16	1.34 ± 0.70
	F	50	Plasma	0.64 ± 0.21	1.86 ± 0.83
			Blood	0.60 ± 0.16	1.89 ± 0.70
	M + F		Plasma	0.58 ± 0.17	1.62 ± 0.84
			Blood	0.54 ± 0.17	1.71 ± 0.83
Rheumatology Department	M		Plasma	0.77 ± 0.30	1.70 ± 0.75
			Blood	0.73 ± 0.33	1.76 ± 0.57
	F		Plasma	0.76 ± 0.26	1.78 ± 0.65
			Blood	0.66 ± 0.20	1.67 ± 0.62
	M + F		Plasma	0.76 ± 0.26	1.75 ± 0.79
			Blood	0.67 ± 0.23	1.68 ± 0.59
Sum of both	M		Plasma	0.66 ± 0.24	1.69 ± 0.83
			Blood	0.62 ± 0.25	1.79 ± 0.78
	F		Plasma	0.63 ± 0.24	1.62 ± 0.73
			Blood	0.57 ± 0.20	1.62 ± 0.70
	M + F		Plasma	0.65 ± 0.24	1.65 ± 0.78
			Blood	0.59 ± 0.21	1.72 ± 0.74

for the males. The Student t-test showed no significant difference between the plasma levels of xan and hyp either in males versus females in the Occupational Medicine group aged 50, or in males aged 40 versus males aged 50. However, plasma levels of both xan and hyp were significantly lower in females aged 40 than in males of the same age ($p<0.01$ for xan and $p<0.02$ for hyp) or in females aged 50 ($p<0.001$ for xan and $p<0.001$ for hyp). Also, there was no significant difference in either group between plasma levels in males and females, regardless of age. Thus sex dependency was observed only in subjects aged 40, whereas age dependency was marked, particularly with xan: linear regression of plasma xan versus age, regardless of group origin or sex, showed a significant link for the whole set ($n=224$) at $p<0.01$ ($r=0.181$). The age dependency factor is clearly shown in Fig. 2. The age groups 10–20, 20–30, and 30–40 are not represented in Fig. 2, because they were statistically too small ($n<15$). Finally, the inter-individual variability observed here for hyp is higher than those observed for xan, particularly in males.

The t-test by difference applied to plasma versus blood levels showed plasma xan to be significantly higher than blood xan at $p<0.001$, and blood hyp to be higher than plasma hyp at $p<0.01$, regardless of group origin, sex or age. However, these differences were below 10%.

Kinetics. The time-course of plasma and blood xan and hyp levels in μM versus the interval of time elapsed between blood collection and sample preparation is shown in Fig. 3. The in vitro plasma kinetics of xan and hyp can be described by straight lines of which the mean equations ($n=13$) are $y=0.112x+0.587$ ($r=0.9997$) for xan, and $y=1.450x+0.779$ ($r=0.9998$) for hyp. The high value of the slope of the line for hyp shows the rapidity of the release of this oxypurine from erythrocytes and the in vitro continuation of

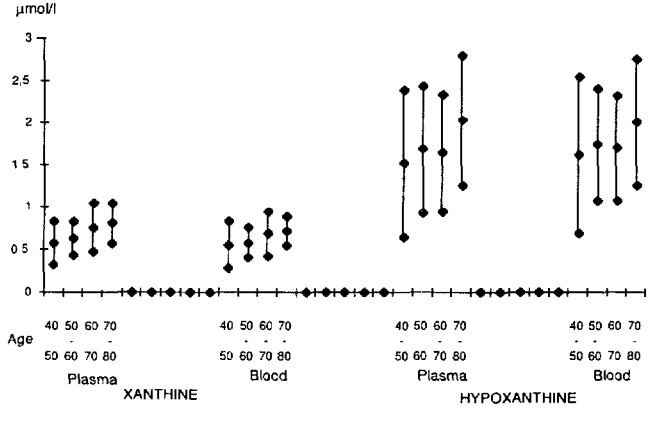

Fig. 2. Means and standard deviations of xanthine and hypoxanthine titers determined in blood and plasma of subjects in different age groups: 40-50 ($n=66$), 50-60 ($n=97$), 60-70 ($n=31$), 70-80 ($n=15$).

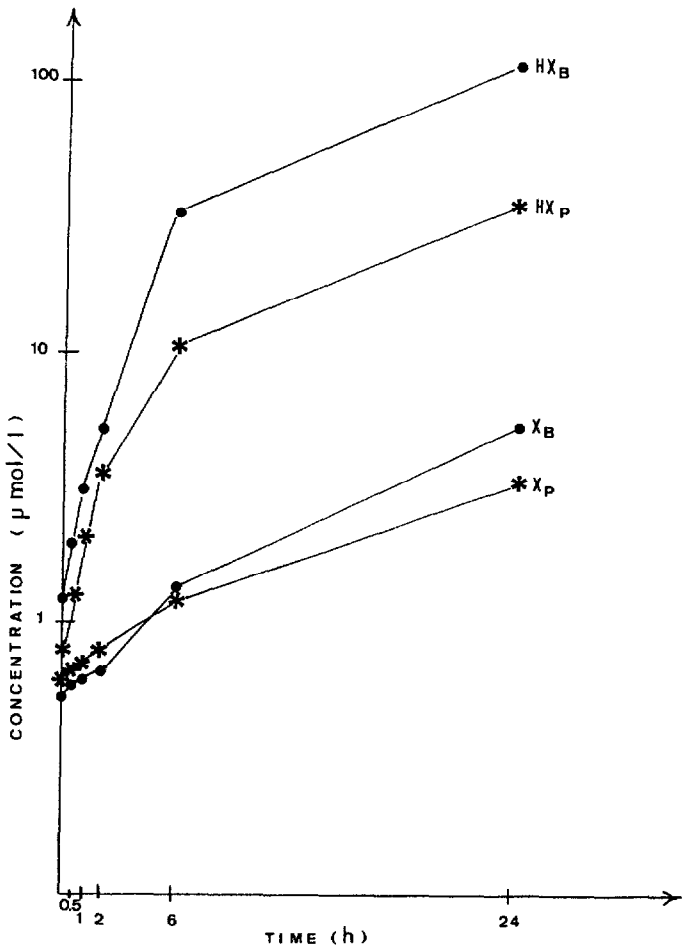

Fig. 3. Plot of plasma (P) and blood (B) levels versus time of contact between the plasma and the other blood components. HX = hypoxanthine; X = xanthine.

intra-erythrocytal purine metabolism. The initial xan levels were comparable with those obtained previously in subjects aged 40 and 50, whereas the initial hyp levels were lower than the mean value for these subjects. This can be attributed to the small size of the sample, the higher inter-individual variation for hyp than for xan and the age dependency, particularly marked in the females.

DISCUSSION

The GC–MS method described here for xan and hyp in plasma and blood associated with a precisely codified sample preparation procedure, revealed in

224 subjects with no purine metabolism disorders a marked age dependency for both xan and hyp and a sex dependency in subjects aged 40 ($n=57$). The groups of age below 40 studied here were statistically too small to permit any correlation. The inter-individual variability was marked, more so for hyp than for xan. Comparison of concentrations obtained in plasma and in blood showed a significant difference of less than 10%, but a kinetic study showed that the rate of in vitro evolution of oxypurine levels is such that if plasma separation is delayed for half an hour after sampling, hyp is overestimated by more than 10%. Hence it would appear preferable to use blood for assay whenever samples cannot be promptly spun, provided they are deproteinated immediately after collection. Harkness [1], in a recent bibliographical review concerning the assay of hyp and xan in biological fluids as an indication of ATP depletion, stressed its importance and the essential role it may play in the future. Though high-performance liquid chromatography (HPLC) might theoretically be expected to be ideal for routine analysis, its application in clinical practice remains to be achieved. Also, though the importance of sampling conditions is widely recognized, given the rapidity of release of hyp from erythrocytes, they have never been standardized, in particular as regards choice of anticoagulant. In previous work [3] we confirmed its crucial role by comparing oxypurine titers obtained for various anticoagulants, in which EDTA proved to be the best.

As a reference method, GC–MS enabled us to compare the values obtained from a large set of subjects with reported values obtained by HPLC from various sources. The most marked discrepancies, in the form of overestimation of oxypurine levels, particularly of hyp, were mostly due to sampling conditions or to the use of heparin [6,7] or because assay was performed on serum [8,9]. When sampling conditions are identical, the values obtained by HPLC are either comparable with those obtained by GC–MS [5,10] or overestimated for xan [11,12], suggesting some spurious effect in the HPLC procedure.

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