Journal of Chromatography, 221 (1980) 9—17
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 650

MASS FRAGMENTOGRAPHIC DETERMINATION OF XANTHINE AND HYPOXANTHINE IN BIOLOGICAL FLUIDS

J.L. CHABARD, C. LARTIGUE-MATTEI, F. VEDRINE, J. PETIT and J.A. BERGER*

Laboratoire de Chimie analytique de la Faculté de Pharmacie et Unité No 71 de l'I.N.S.E.R.M., Boite Postale No 38, 63001 Clermont-Ferrand Cedex (France)

(First received March 31st, 1980; revised manuscript received June 10th, 1980)

SUMMARY

The method presented for the simultaneous determination of xanthine and hypoxanthine, uses mass-fragmentography in the electron impact (EI) mode, after the gas chromatographic separation of butylated derivatives. Butylation, rather than methylation, is used in order to avoid interference coming from exogenous caffeine, which is frequently encountered. [7,9-^{15}N]Xanthine is used as the internal standard, and for each sample, a blank is obtained by xanthine oxidase reaction. In the biological fluids studied the sensitivity was about 50 ng/ml.

INTRODUCTION

Analytical methods for oxypurines determination have been restricted for a long time to an overall estimation of xanthine and hypoxanthine by UV absorption spectrophotometric methods [1, 2]. More recent reports, including enzymatic spectrophotometry [3], thin-layer chromatography combined with spectrophotometry [4], high-performance liquid chromatography (HPLC) [5, 6], or ion-exchange column chromatography [7] have allowed the simultaneous determination of xanthine and hypoxanthine. These methods, often requiring a large amount of sample [3], give no confirmation as to the identity of the substances quantitated, and also yield non-consistent data as Pfadenhauer [5] pointed out. Confirmation of identity was obtained by Snedden and Parker [8] who used high-resolution mass spectrometry with direct introduction into the mass spectrometer of lyophilized samples of blood and muscle. This method does not require previous separation of the different substances since it allows a selection of accurate mass peaks, but it involves tedious equipment calibration. Not having a high-resolution mass spectrometric

0378-4347/80/0000—0000/$02.25

system at our disposal, we used a classical quadrupolar gas chromatographic—mass spectrometric (GC—MS) system, which allowed previous separation of the oxypurines.

MATERIALS AND METHODS

Chemicals

Standards. Xanthine and hypoxanthine (Merck, Darmstadt, G.F.R.), and [7,9-^{15}N]xanthine (C.E.A., Saclay, France) prepared in weak ammoniacal solutions were found to be stable for several months at 4°C.

0.5 M formiate buffer. Prepared by dissolving 23 g formic acid (Riedel de Haën, Hoescht, G.F.R.) in 1 l of distilled water, and adjusting to pH 3.65 with 5 *N* sodium hydroxide.

Solvents. n-Butanol obtained from Prolabo (Paris, France), and methanol GR obtained from Merck.

Reagents. N,N-Dimethylacetamide (Fluka, Buchs, Switzerland) was stored in the dark over anhydrous sodium sulfate. The tetrabutylammonium hydroxide, 0.1 *N* in methanol and butyl iodide were purchased from Merck. The Silyl-8 was from Pierce (Rockford, IL, U.S.A.).

Enzymatic preparations. Xanthine oxidase, pronase and subtilisin were purchased from Boehringer (Mannheim, G.F.R.).

GC—MS system

Experiments were performed by mass fragmentography, on a quadrupolar GC—MS Hewlett-Packard 5985 system, equipped with an electron impact—chemical ionization (EI—CI) source. Scanning, and selected ion monitoring were carried out under the software control of a computer (Hewlett-Packard 1000 integrated data system). Mass spectrometry operating conditions were set up as follows: electron impact, 70 eV and emission current, 300 μA. Helium was used as the carrier gas at a flow-rate of 20 ml/min, and gave a pressure of $2 \cdot 10^{-6}$ torr in the source. Methane was used as the reactant gas for chemical ionization.

The GC column was a 150 cm × 2 mm I.D. glass column filled with 3% OV 17 on Gas Chrom Q 100—120 mesh. The column was conditioned and then silylated at 150°C, by injecting 10 μl of Silyl-8 five times. The chromatograph was operated with temperature programming from 220°C to 260°C at 10°/min and the injection port was maintained at 250°C.

Glassware problem

During preliminary studies, the calibration curves obtained with extracts from standard solutions, did not go through zero, which indicated a possible adsorption on glass. This phenomenon was also observed by Driessen et al. [9] with 5-fluorouracil. The use of polyethylene vials for the different steps of the oxypurines evaluation eliminated this problem.

Ultrafiltration

Serum was deproteinized by ultrafiltration in Amicon CF 25 centriflo cones (Lexington, MA, U.S.A.) according to the methods of Lakings et al. [10]

or Yamamoto et al. [11]. Serum was introduced into a cone and centrifuged at 600 *g* for 15 min. The residue was then washed with distilled water and centrifuged again.

Procedure

Each serum and urine sample was analyzed according to the scheme described in Fig. 1.

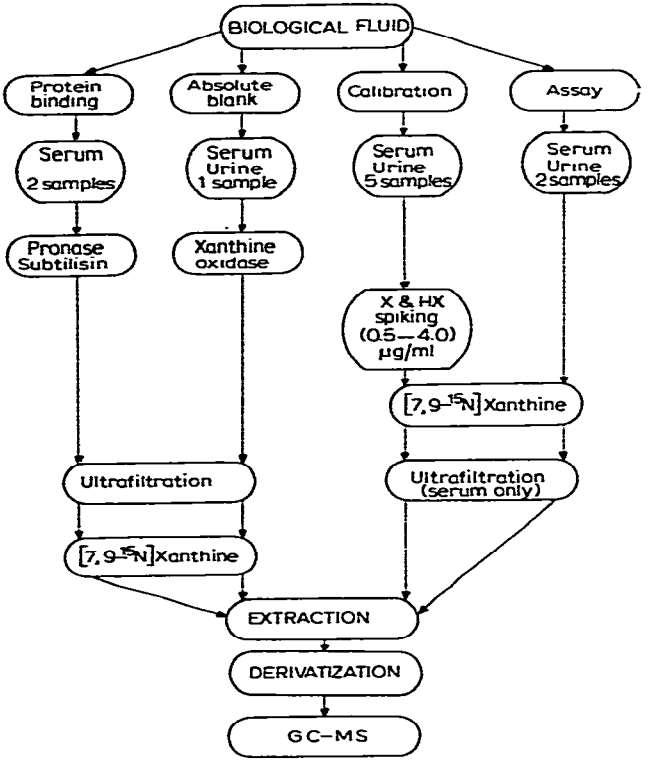

Fig. 1. Scheme for the analysis of xanthine and hypoxanthine in biological fluids.

Serum analysis

Each sample (0.5 ml) was spiked with [7,9-^{15}N]xanthine and then ultrafiltered. The ultrafiltrate was buffered to pH 4.20 with the formiate buffer solution (200 µl) and extracted by shaking for 1 min, on a vortex mixer, with *n*-butanol (2 ml) in 15-ml polyethylene vials.

Different solvents commonly used to extract other xanthines, such as theophylline [12, 13] or allopurinol [14], from biological fluids were also checked. However, it appeared that only *n*-butanol, used by Pantarotto et al. [15] to quantitate nucleoside analogues (5-fluorouracil, 6-mercaptopurine, 5-fluoro-2′-deoxyuridine) gave an acceptable recovery.

After centrifugation, the organic layer was evaporated to dryness, at 50°C under a stream of nitrogen. The residue was then dissolved in 120 µl of N,N-dimethylacetamide, and 50 µl of tetrabutylammonium hydroxide. After vortex

mixing for 10 sec, a 20-μl aliquot of butyl iodide was then added to the solution. The container was capped, then vortexed for 1 min and allowed to stand for 15 min at room temperature. After evaporation at 50°C under a stream of nitrogen, the residue was redissolved in 20 μl of methanol, and 2-μl aliquots were injected into the GC—MS system.

Urine analysis

Urine samples were diluted ten times and were then assayed as the serum samples, but without undergoing the ultrafiltration step.

RESULTS AND DISCUSSION

Preliminary assays to procedure

Protein binding. The ultrafiltration of the serum is reliable, because oxypurines are not bound to serum proteins. This was verified by reacting pronase an subtilisin with the serum, according to the method of Osselton et al. [16], before the ultrafiltration and extraction steps. The enzymatic hydrolysis performed at pH 8.0, for 60 min at 55°C (enzymatic preparation: 2 μg/ml of biological fluid) never generated a significant increase in the oxypurines recovery.

Absolute blanks. A blank was prepared by reacting each sample with xanthine oxidase at 37°C for 1 h, prior to the ultrafiltration, spiking of [7,9-^{15}N] xanthine, and extraction steps. The reaction was carried out by using 50 μl of enzymatic suspension for the serum and 10 μl for the urine, according to the mechanism given in Fig. 2.

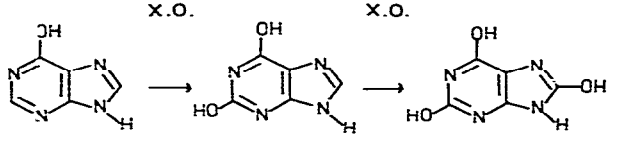

Fig. 2. Hypoxanthine oxidative transformation in uric acid by xanthine oxidase (X.O.).

Derivatization. As oxypurines are polar molecules, they must be derivatized before GC analysis. First derivatization attempts, by flash methylation with 0.2 *N* trimethylanilinium hydroxide in methanol, currently used with barbiturates [17] or purines [18], were not successful for two reasons:

(1) no chromatographic conditions could be found to separate the trimethylated derivative of xanthine (or caffeine) from endogenous stearic acid methyl ester and

(2) the preparation of blanks, by the reaction of xanthine oxidase with the biological fluids, revealed the presence of exogenous caffeine, leading therefore to an important error in the xanthine evaluation; caffeine and stearic acid were effectively identified by their mass spectra.

Derivatization by butylation, according to Greeley's method [19], modified by Pranskevich et al. [20], eliminated these interferences.

Mass spectra

Mass spectra obtained in the EI mode, showed that tributylated derivatives of xanthine and [7,9-^{15}N]xanthine gave molecular ions with *m/z* of 320 and 322 respectively. Because of their relative intensity of 60% and because of the absence of any interfering peak in this part of the spectrum, they were found to be suitable for the mass fragmentographic quantitation (Fig. 3).

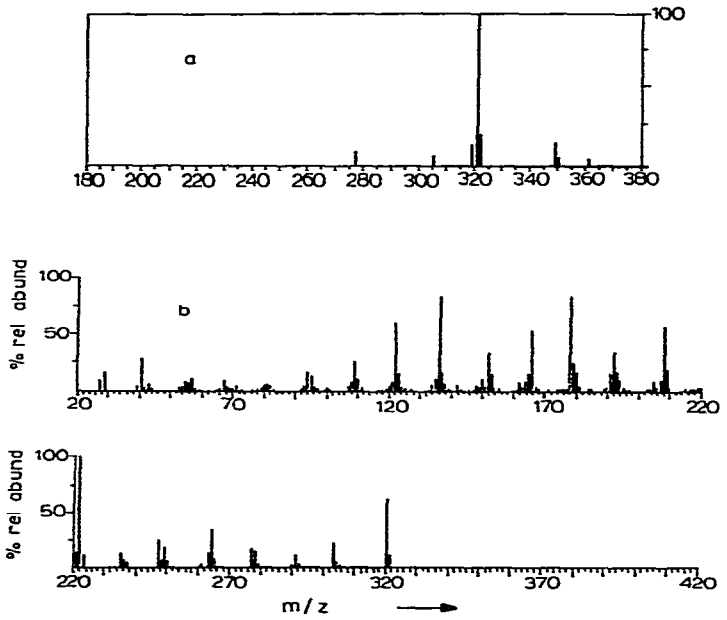

Fig. 3. Mass spectra of tributylated derivative of xanthine: (a) in CI mode and (b) in EI mode.

Hypoxanthine gave a dibutylated derivative whose molecular ion *m/z* 248 (Fig. 4) has a relative intensity of about 40%. Because this mass is subject to interference in some samples, we selected the ion *m/z* 231, whose relative intensity is about 60%, for the quantitation of hypoxanthine.

A correction was made to the [7,9-^{15}N]xanthine peak evaluation, to take into account the contribution of the xanthine peak at mass M + 2. (The M + 2 xanthine peak whose relative intensity 2.5% adds to the [7,9-^{15}N]xanthine peak).

Mass spectra obtained in the EI mode were confirmed in the CI mode (Figs. 3 and 4). Fig. 5 shows a selected ion monitoring (SIM) recording of xanthine, [7,9-^{15}N]xanthine, and hypoxanthine from a serum extract.

Recovery

The extraction recovery was determined from samples of serum spiked with [7,9-^{15}N]xanthine (1.25 μg/ml). In spite of an extraction recovery that appears rather low (55 ± 4%) the analytical method is still worthwhile for two reasons: [7,9-^{15}N]xanthine is practically the ideal internal standard, because its physico-chemical behaviour, in the extraction and derivatization steps,

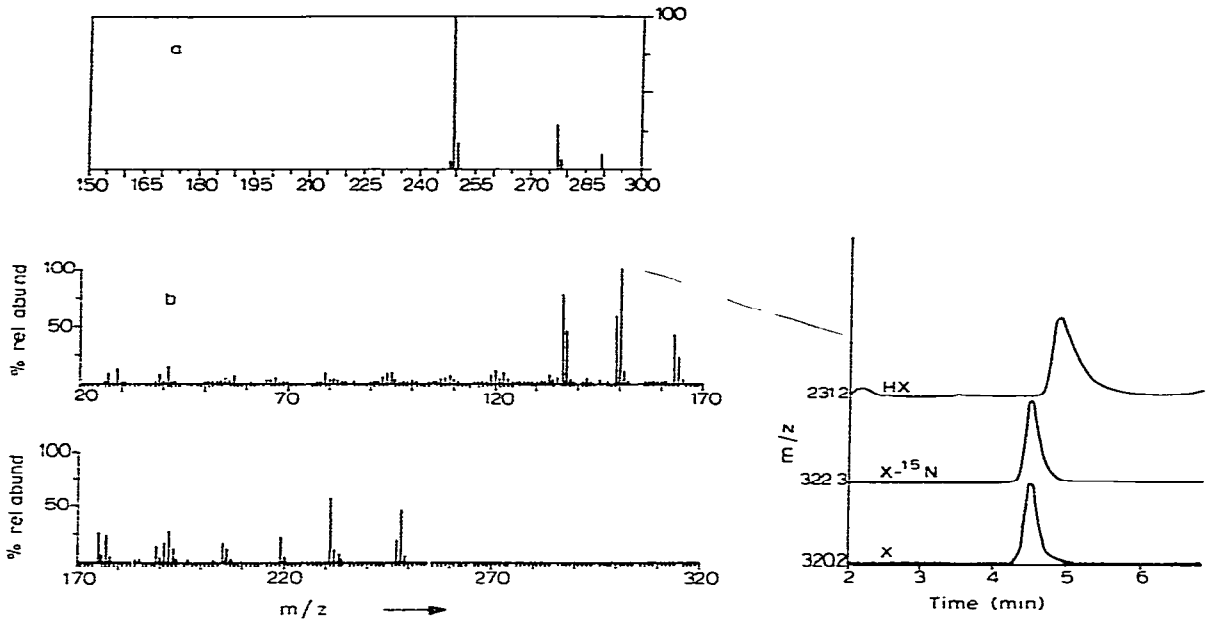

Fig. 4. Mass spectra of dibutylated hypoxanthine: (a) in CI mode and (b) in EI mode.

Fig. 5. SIM recording of xanthine (X), [7,9-^{15}N]xanthine (X-^{15}N) and hypoxanthine (HX) from serum extract.

is similar to that of the molecule quantitated. The lack of any significant protein binding for oxypurines, as was demonstrated by the action of proteolytic enzymes, such as pronase and subtilisin. The relatively low recovery of the extraction seems to be due only to an unfavourable partition coefficient of these substances between the fairly polar solvent used and water.

Sensitivity, reproducibility and calibration

The detection limit for oxypurines quantitation in biological fluids is 50 ng/ml, for 0.5 ml of serum and 0.1 ml of urine. The reproducibility determined on ten replicate analyses of serum and spiked serum (two concentrations) is given in Table I and the intra-assay coefficient of variation is about 5%.

TABLE I

REPRODUCIBILITY OF THE ASSAY OF XANTHINE (X) AND HYPOXANTHINE (HX) IN SERUM

	Concentration (μg/ml) (n= 10)	Coefficient of variation (%)
X	0.5	5.0
X	1.0	5.2
HX	1.5	5.7
HX	2.0	6.8

Calibration curves, obtained for serum samples spiked with xanthine and hypoxanthine, were found to be linear for concentrations ranging from 0.5 to 4.0 μg/ml. Correlation coefficients are greater than 0.995 for the xanthine calibration, and greater than 0.970 for the hypoxanthine calibration. An example is given in Fig. 6.

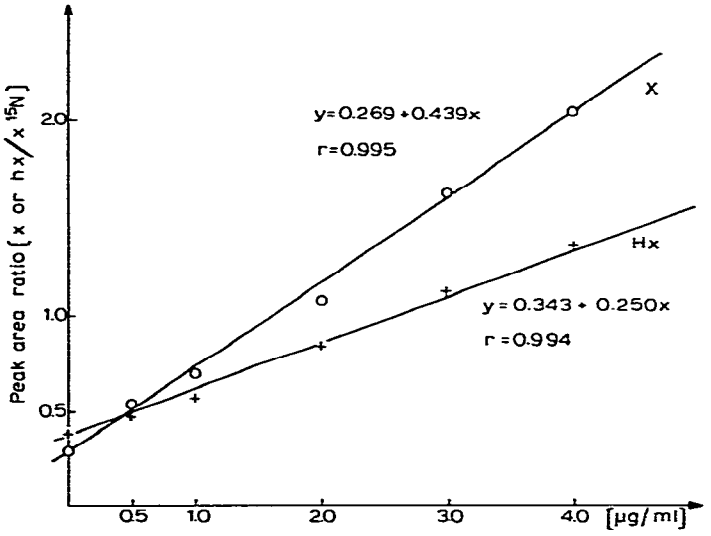

Fig. 6. Calibration curves of xanthine (X) and hypoxanthine (HX), an example given for subject No. 12.

TABLE II

SERUM CONCENTRATION AND URINARY ELIMINATION RATE OF XANTHINE (X) AND HYPOXANTHINE (HX) IN NORMAL SUBJECTS

Subject No.	Serum (μg/ml)		Urine (mg/24 h)	
	X	HX	X	HX
1	0.61	1.50	6.5	6.3
2	0.69	2.50	6.5	5.5
3	0.57	1.20	4.5	5.4
4	0.95	3.25	12.8	11.4
5	0.69	3.80	14.5	13.8
6	0.53	1.45	8.6	6.4
7	0.62	1.10	11.6	11.8
8	0.52	0.90	8.3	13.5
9	0.65	1.55	7.9	8.6
10	0.58	1.52	8.8	6.8
11	0.60	1.31	9.5	11.6
12	0.62	1.56	10.6	6.3
13	0.60	1.36	9.0	7.6
14	0.60	1.55	6.8	5.3
15	0.63	1.65	9.4	6.4
Mean	0.63	1.75	9.0	8.5
σ	0.10	0.80	2.6	3.1

Applications in humans

The study was conducted with 15 healthy volunteers, who were fasted overnight. Blood was collected by venipuncture (20 ml in dry tubes) and urine was collected over the following 24 h. Serum and urine samples were stored frozen (−20°C) until analysis. The results summarized in Table II are in good agreement with those obtained by HPLC [5]. In Table II, the serum concentrations are expressed in μg/ml, and urinary elimination is expressed in mg/24 h. It can be noted that the ratio of xanthine to hypoxanthine serum concentrations ranges from 0.25 to 0.50, and that the ratio of xanthine to hypoxanthine urinary elimination rates is about 1.0.

CONCLUSION

In comparison with the spectroenzymatic or the HPLC methods, the GC—MS method presented here offers the advantage of combining accuracy, sensitivity and specificity.

The different techniques used during the development of the procedure have eliminated any interference, and have also established the reliability of the method.

The preparation of absolute blanks was carried out by treating each urine and serum sample with xanthine oxidase. The action of pronase and subtilisin on the serum samples confirmed the absence of protein binding for these oxypurines and allowed the precise determination of the extraction recoveries. Finally, the use of [7,9-^{15}N]xanthine as the internal standard allowed us to obtain an optimal accuracy for the calibration curves. When first applied to normal subjects, this method allowed us to obtain accurate and relatively homogenous results, which were used to define a mean serum concentration and a mean daily urinary elimination rate for both xanthine and hypoxanthine. This method proved to be also extremely effective in following the development of oxypurines serum concentrations in hyperuricemic subjects, during their treatment with allopurinol, a xanthine oxidase inhibitor.

REFERENCES

1 H.M. Kalchar, J. Biol. Chem., 429 (1947) 429.
2 B. Petersen, J. Jorni and S. Jorgensen, Scand. J. Clin. Lab. Invest., 17 (1965) 454.
3 R.A. Chalmers and R.W.E. Watts, Analyst (London), 94 (1969) 226.
4 P.J. Orsulak, W. Haab and M.D. Appleton, Anal. Biochem., 23 (1968) 156.
5 E.H. Pfadenhauer, J. Chromatogr., 81 (1973) 85.
6 P.R. Brown, S. Bobick and F.L. Hanley, J. Chromatogr., 99 (1974) 587.
7 H.A. Simmonds and J.D. Wilson, Clin. Chim. Acta, 16 (1967) 155.
8 W. Snedden and R.B. Parker, Anal. Chem., 43 (1971) 1651.
9 O. Driessen, D. de Vos and P.J.A. Timmermans, J. Pharm. Sci., 67 (1978) 1494.
10 D.B. Lakings, R.H. Adamson and R.B. Diasio, J. Chromatogr., 146 (1978) 512.
11 Y. Yamamoto, T. Manji, A. Saito, K. Maeda and K. Ohta, J. Chromatogr., 162 (1979) 327.
12 D.G. Bailey, H.L. Davis and G.E. Johnson, J. Chromatogr., 121 (1976) 263.
13 R.F. Adams, F.L. Vandemark and G.J. Schmidt, Clin. Chem., 22 (1977) 1903.
14 R. Endele and G. Lettenbauer, J. Chromatogr., 115 (1975) 228.

15 C. Pantarotto, A. Martini, G. Belvedere, A. Bossi, M.G. Donelli and A. Frigerio, J. Chromatogr., 99 (1974) 519.
16 M.D. Osselton, J.C. Shaw and H.M. Stevens, Analyst (London), 103 (1978) 1160.
17 R.J. Perchalski, K.N. Scott, B.J. Wilder and R.H. Hammer, J. Pharm. Sci., 62 (1973) 1735.
18 J. Mac Gee, Anal. Biochem., 14 (1966) 305.
19 R.H. Greeley, J. Chromatogr., 88 (1974) 229.
20 C.A. Pranskevich, J.L. Swihart and J.J. Thoma, J. Anal. Toxicol., 2 (1978) 3.