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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYPOXANTHINE AND XANTHINE IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid and selective reversed-phase high-performance liquid chromatographic method for the simultaneous determination of hypoxanthine and xanthine in biological fluids was developed. The identification of hypoxanthine and xanthine was confirmed by xanthine oxidase reaction. This method was applied to the investigation of purine metabolism in subjects with xanthine oxidase deficiency or gout. Hypoxanthine concentrations three to ten times higher than those determined in plasma were found in erythrocyte samples from normal subjects and from patients with xanthine oxidase deficiency or hyperuricemia under allopurinol therapy.

INTRODUCTION

Purine metabolism is complex. It is composed of five steps, one of which corresponds to purine nucleotide degradation and leads to hypoxanthine and xanthine formation. Hypoxanthine and xanthine are oxidized to uric acid by xanthine oxidase (EC 1.2.3.2.). Uric acid is the final product of purine metabolism in humans. A block of purine nucleotide degradation due to a deficiency of xanthine oxidase occurs in xanthinuria. This enzyme defect causes increased serum and urinary concentrations of hypoxanthine and

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xanthine and decreased serum and urinary uric acid concentrations. Hypoxanthine and xanthine are, therefore, the final products of purine degradation. This block also occurs during allopurinol therapy. Allopurinol, used in gouty subjects, is a potent inhibitor of xanthine oxidase.

Several methods for the determination of oxypurines (hypoxanthine and xanthine) in biological fluids have been published. Enzymatic spectrophotometric [1] and column chromatographic [2] methods have been used; but these methods presented a major disadvantage, namely they did not separate hypoxanthine and xanthine. Thin-layer chromatography [3] and gas—liquid chromatography coupled to mass spectrometry, requiring derivatization of compounds [4, 5] have also been used. Different methods have been reported for the separation of nucleosides or bases using high-performance liquid chromatography [6—15]. Ion-exchange chromatography using the possibility of ionisation of bases and nucleosides has been developed [9, 10]. Nowadays, reversed-phase systems have become the most popular method of liquid chromatography. The retention of charged molecules due to the presence of appropriate modifiers is a useful alternative to the ion-exchange method. Of the methods which have heretofore been considered, a great number did not resolve hypoxanthine from guanine [6, 7, 11—13]. Other works dis not consider the presence of guanine in the chromatographic systems [8, 14, 15]. It is important to separate hypoxanthine and guanine since, in purine metabolism, xanthine has two origins — from hypoxanthine after the action of xanthine oxidase and from guanine after the action of guanase. In xanthine oxidase deficiency, purine nucleotide degradation is disrupted. The possible presence of guanine at significant levels in biological fluids must not be ignored. Studies were principally carried out on serum or plasma [8, 12–14] but the specific analysis of hypoxanthine and xanthine in various biological fluids (especially urine and erythrocytes) has not been examined.

We therefore developed a reversed-phase method allowing a rapid and selective analysis of oxypurines in different biological fluids, without interference by purines and pyrimidines, especially guanine. Confirmation of peak identity was made by an enzymatic technique. This method was applied to the analysis of plasma, urine and erythrocytes in normal subjects, and in patients with xanthinuria and in hyperuricemic subjects undergoing allopurinol therapy.

EXPERIMENTAL

Reagents

Xanthine and hypoxanthine were purchased from Sigma (St. Louis, MO, U.S.A.), 9-methylxanthine was from Fluka (Buchs, Switzerland), and potassium dihydrogen phosphate, phosphoric acid and methanol (Uvasol) were from Merck (Darmstadt, G.F.R.).

Xanthine oxidase in suspension (activity 10 U/ml) was from PL Biochemicals (Milwaukee, WI, U.S.A.). Water throughout these experiments was deionized distilled water (Laboratoire Aguettant, Lyon, France).

Blank serum was a lyophilized control serum (serum Lyotrol N; Biomerieux, Charbonnières les Bains, France).

Apparatus and chromatographic conditions

The chromatograph was a Chromatem 800 (Touzart et Matignon, Vitry, France) equipped with a variable-wavelength absorbance detector LC—UV Pye Unicam (Philips, Bobigny, France).

The column (15 cm × 4.6 mm I.D.) was packed with Hypersil ODS 3 μm (Shandon, Cheshire, Great Britain) by the slurry packing technique as described by Coq et al. [16]. A precolumn (5 cm × 4.6 mm I.D.) used as a guard column was filled with Hypersil ODS 5 μm by the same technique. Separations were carried out using an isocratic method. The mobile phase consisted of a solution of 0.02 *M* KH_2PO_4, the pH of which was adjusted to 3.65 with phosphoric acid. The flow-rate was 1.5 ml min^{-1}; detection was performed at 254 nm.

Hypoxanthine and xanthine peaks were initially identified on the basis of their retention times. Peak identification was confirmed by the enzymatic peak-shift technique. Indeed, xanthine oxidase catalyses the following transformations

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{xanthine} + H_2O_2$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{uric acid} + H_2O_2$$

Plasma, urine and erythrocyte samples were incubated with xanthine oxidase at 25°C (pH 7.8) for 10 min (10 μl of xanthine oxidase + 50 μl of sample). Chromatographic profile observation before and after addition of xanthine oxidase makes it possible to confirm (or not) the presence of oxypurines in the samples.

Sample preparation

Standard solutions were prepared by dissolving hypoxanthine and 9-methylxanthine (internal standard) in water (1 mmol l^{-1}). Due to its weak solubility, xanthine was used at the concentration of 0.125 mmol l^{-1} in water. Standard solutions used to spike samples were first evaporated to dryness under nitrogen (to prevent sample dilution).

Blood (5 ml) was collected in heparinized tubes and immediately centrifuged. The plasma samples were then stored at −20°C. Erythrocytes were subjected to mild sonication and immediately analysed or stored at −20°C. A 1-ml aliquot of plasma spiked with the internal standard (25 μmol l^{-1} of 9-methylxanthine) was deproteinized by heating (2 min in a boiling water-bath). Deproteinized samples were centrifuged for 10 min at 23,500 *g* in an ultracentrifuge MSE Superspeed 65. Erythrocytes were treated in the same way.

Urine samples (24-h) were collected and stored at −20°C; 2 ml of spiked urine (25 μmol l^{-1} of internal standard) were analysed without further treatment.

RESULTS AND DISCUSSION

Chromatographic method

A preliminary study involving the separation of purine and pyrimidine bases and nucleosides led us to consider effect of the pH of the mobile phase on hypoxanthine/xanthine separation and possible interference with other com-

pounds, especially guanine. Fig. 1 shows that oxypurine separation is obtained without interference from other bases and nucleosides at pH 3.65. Separation of a standard mixture containing hypoxanthine, xanthine and 9-methylxanthine with the addition of allopurinol and guanine is shown in Fig. 2.

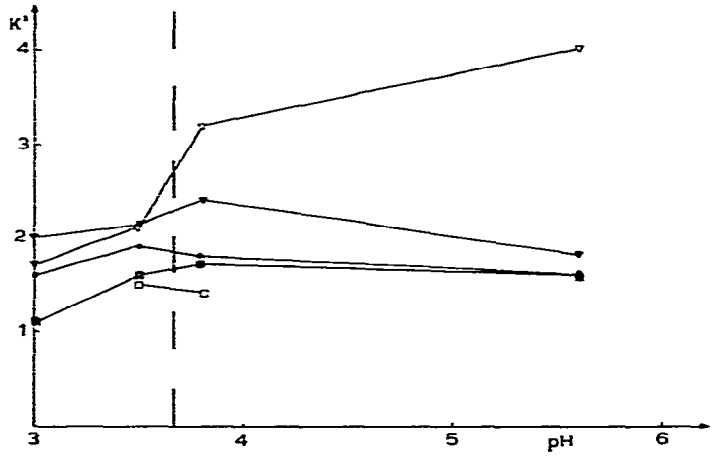

Fig. 1. Effect of eluent pH on retention of bases: (▽) adenine, (▼) xanthine, (●) hypoxanthine, (■) guanine, (□) uric acid.

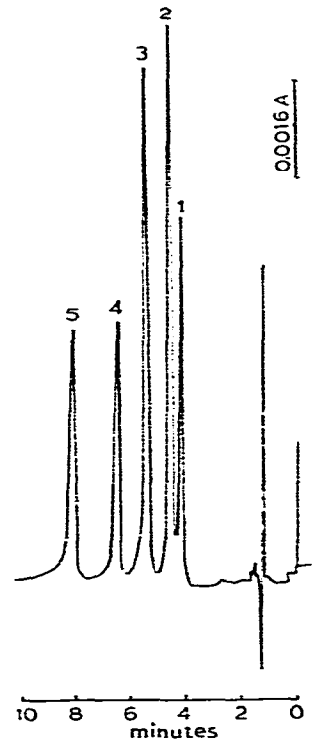

Fig. 2. Separation of guanine (1), hypoxanthine (2), xanthine (3), 9-methylxanthine (4), and allopurinol (5). Injection volume: 10 μl. Column: Hypersil ODS 3 μm. Precolumn: Hypersil ODS 5 μm. Mobile phase: 0.02 *M* KH_2PO_4 pH 3.65. Flow-rate: 1.5 ml min^{-1}. Detection: 254 nm.

Linearity (peak height versus concentration) was tested on standards in aqueous solution and in control serum spiked at various concentrations. The linearity is excellent up to 50 μmol l^{-1} for hypoxanthine, xanthine and 9-methylxanthine. Furthermore, slopes corresponding to aqueous solutions and serum are identical. This confirms that no compound of interest was lost during deproteinization.

Reproducibility and accuracy studies were performed at three different concentrations of hypoxanthine and xanthine in spiked control serum. The coefficient of variation was about 1.5%. The results are given in Table I.

The detection limit was 0.5 μmol l^{-1} for hypoxanthine and 1 μmol l^{-1} for xanthine (injection volume = 10 μl).

TABLE I

REPRODUCIBILITY AND ACCURACY OF HYPOXANTHINE AND XANTHINE ANALYSIS IN SPIKED CONTROL SERUM

	Amount added (μmol l^{-1})	Amount found (μmol l^{-1}; mean ± S.D., n = 10)	C.V. (%)
Hypoxanthine	2.5	2.51 ± 0.04	1.7
	5.0	5.01 ± 0.07	1.4
	15.0	14.95 ± 0.30	2.0
Xanthine	6.25	6.25 ± 0.07	1.2
	12.50	12.47 ± 0.05	0.4
	25.0	25.01 ± 0.25	1.0

The column used in this study has demonstrated a high degree of efficiency and a long lifetime (several hundred samples were injected on to the column for seven months). The above procedure was also applicable to the determination of hypoxanthine and xanthine in liver extracts. The use of an enzymatic technique to identify compounds provides a very specific technique. We observed in our study that hypoxanthine and guanine can be separated only at pH values below 4. This is why authors working at pH values ranging from 4.8 to 6.0 could not separate hypoxanthine from guanine.

Biological samples

This method was applied to biological samples from three normal subjects, two patients with xanthinuria and one with hyperuricemia undergoing allopurinol therapy. The results are listed in Table II. A chromatogram of control serum spiked with a standard mixture is shown in Fig. 3. Sample chromatograms of plasma, erythrocytes and urine from a normal subject are presented in Fig. 4. Sample chromatograms from a patient with xanthinuria are shown in Fig. 5.

In plasma and urine, as a consequence of the addition of xanthine oxidase, we observed a complete disappearance of hypoxanthine and xanthine peaks

TABLE II

HYPOXANTHINE AND XANTHINE LEVELS IN PLASMA, ERYTHROCYTE AND URINE SAMPLES FROM NORMAL SUBJECTS AND PATIENTS

	Normal subjects (mean conc. found, $n = 3$)		Patients with xanthine oxidase deficiency		Patient with hyperuricemia (allopurinol treatment)	
	Hypoxanthine	Xanthine	Hypoxanthine	Xanthine	Hypoxanthine	Xanthine
Plasma (μmol l^{-1})	3.2	2.0	24.7*	32.5*		
			43.5**	20*	6.6	26.5
Erythrocyte (μmol l^{-1})	31.8	<1	397*	<1*		
			106.2**	<1**	58	<1
Urine [μmol (24 h)$^{-1}$]	50.2	77	333*	2640*	—***	—
			399**	2620**		

*Patient S.A.
**Patient J.S.
***—, no sample.

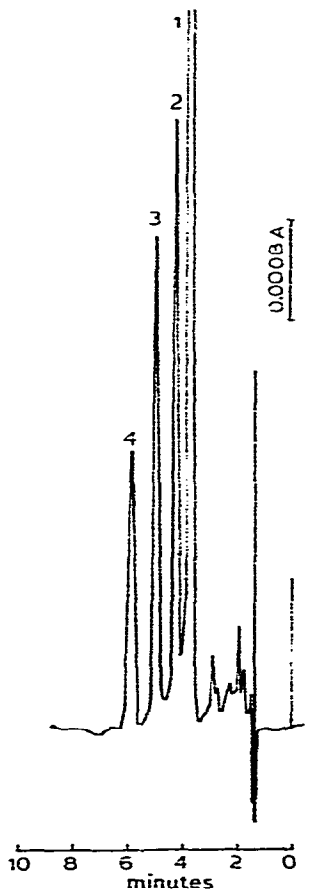

Fig. 3. Chromatogram of a control serum spiked with hypoxanthine (12.5 μmol l^{-1}), xanthine (30 μmol l^{-1}) and 9-methylxanthine (12.5 μmol l^{-1}). Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

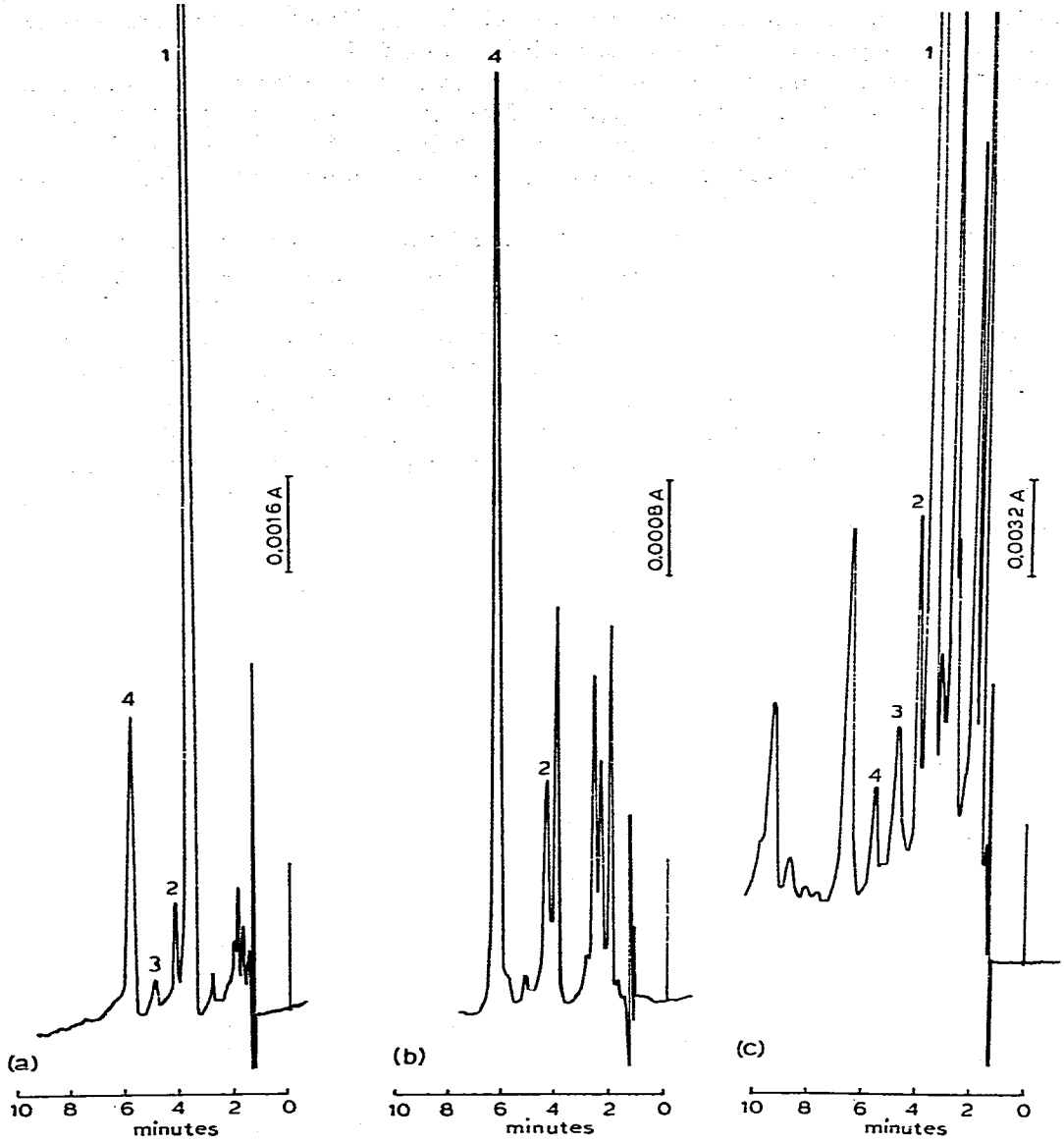

Fig. 4. Chromatograms of plasma (a), erythrocytes diluted five times (b), and urine (c) from a normal subject. Injection volume: 10 μl. Chromatographic conditions as in Fig. 2. Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

and, simultaneously, an increase of the uric acid peak. For low xanthine oxidase activities, the oxidation of hypoxanthine into xanthine, and then of xanthine into uric acid, was observed successively and in that order. This result confirmed the presence of hypoxanthine and xanthine in the samples. In erythrocyte samples from patients, in spite of existence of two peaks which seemed to correspond to hypoxanthine and xanthine, enzymatic identification

showed that only hypoxanthine was present in erythrocytes, the other peak being due to the presence of an unidentified compound.

Results obtained from normal human samples agreed well with others in the literature; in serum samples, hypoxanthine concentrations ranged from 1.5 to 12.8 μmol l^{-1} and xanthine concentrations from 0.5 to 4.7 μmol l^{-1} [8]. The daily oxypurine excretion has been found by Desbois et al. [17] to range from 70 to 140 μmol $(24\ h)^{-1}$.

In patients with xanthinuria, only total oxypurine levels (hypoxanthine + xanthine) have usually been determined. They were found to range from 20 to 65 μmol l^{-1} in serum and from 700 to 4300 μmol $(24\ h)^{-1}$ in urine [17]. For the two patients studied, the sum of the values (hypoxanthine + xanthine) were in good agreement. Furthermore, our analysis being specific with regard to hypoxanthine and xanthine, makes it possible to specify that xanthine is predominant in urine whereas in plasma xanthine or hypoxanthine are found to be predominant depending on the samples. In the case of a high concentration of xanthine, therapy must be rapidly established to prevent xanthine stone formation due to the low solubility of xanthine.

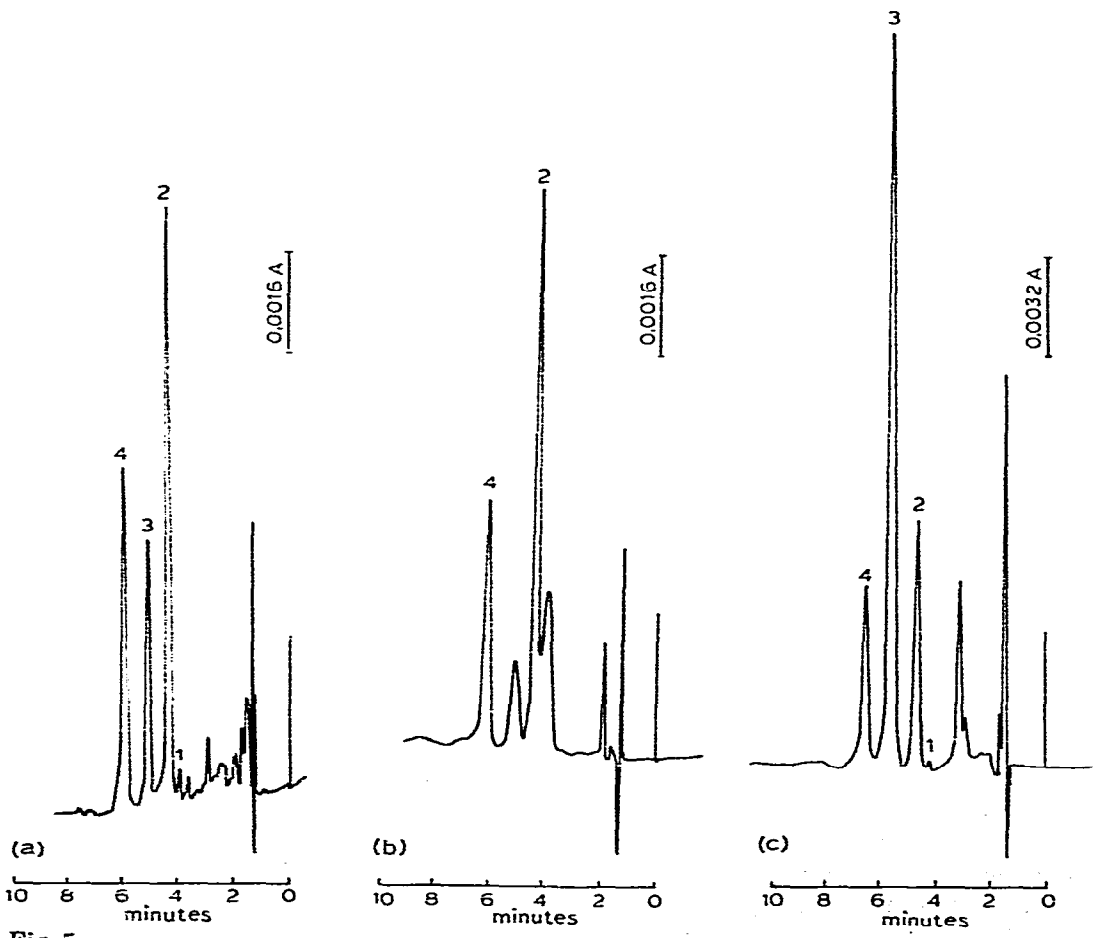

Fig. 5.

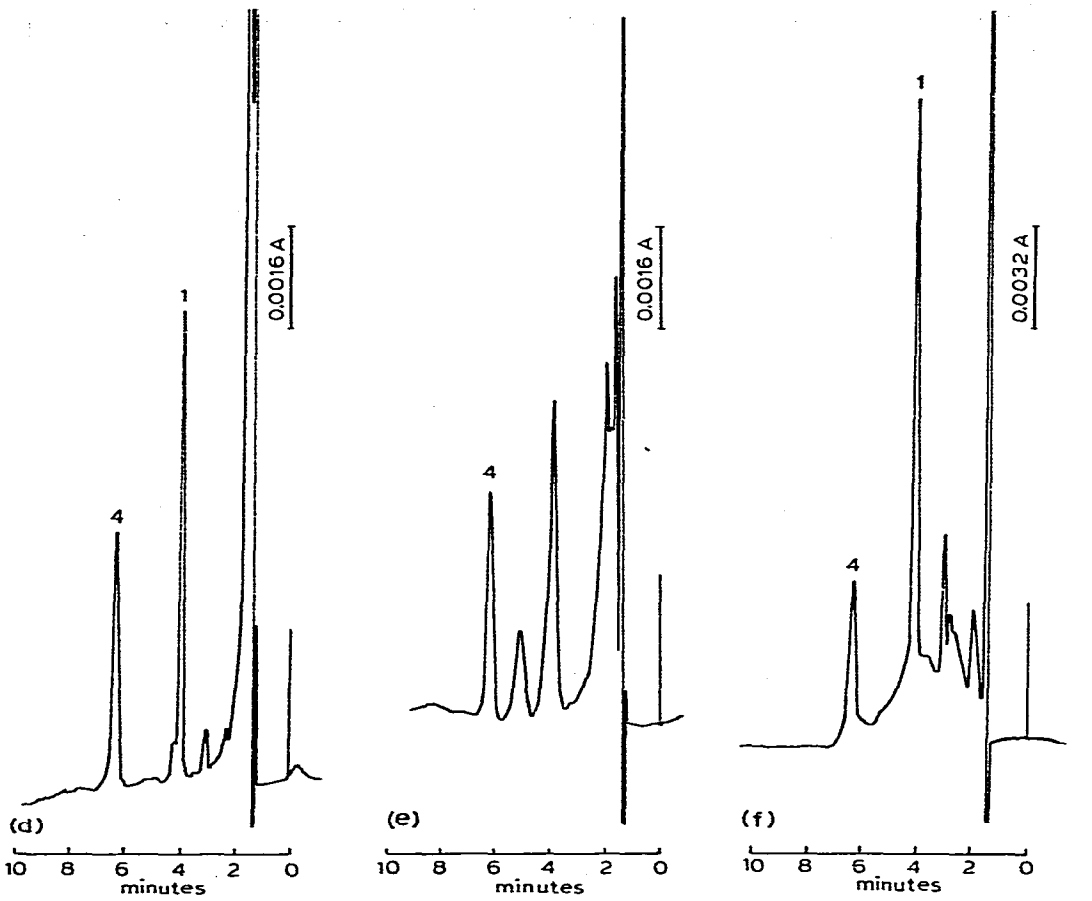

Fig. 5. (a—c) Chromatograms of samples from patient S.A. with xanthine oxidase deficiency. Plasma (a), erythrocyte diluted twenty times (b), urine diluted ten times (c). (d—f) Chromatograms of the same samples after incubation with xanthine oxidase: plasma (d); erythrocyte (e) and urine (f). Injection volume: 10 µl. Chromatographic conditions as in Fig. 2. Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

For all the subjects studied, erythrocyte hypoxanthine concentrations were found to be three to ten times higher than those determined in plasma. This would explain the large differences in hypoxanthine concentrations noticed by Wung and Howell [13] in plasma, when the formed elements of blood were not immediately separated. Marz et al. [18] have found a rapid transport of hypoxanthine in cultured cells; they have shown that, at high or low concentrations of extracellular hypoxanthine, the steady-state concentrations of hypoxanthine in the cells approached those in the extracellular medium. Our results show that erythrocytes behave differently: they seem able to accumulate very large amounts of hypoxanthine. Furthermore, we have shown a specificity in the accumulation phenomenon of hypoxanthine: in spite of its non-negligible levels

in plasma there is no xanthine in erythrocytes. At the present time, this mechanism of accumulation is not known; it is under investigation in our laboratory.

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