Journal of Chromatography, 274 (1983) 75—85
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1591

SIMULTANEOUS DETERMINATION OF PLASMA AND URINARY URIC ACID, XANTHINE, HYPOXANTHINE, ALLOPURINOL, OXIPURINOL, OROTIC ACID, OROTIDINE AND CREATININE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received August 27th, 1982; revised manuscript received November 24th, 1982)

SUMMARY

A new high-performance liquid chromatographic procedure is described for the simultaneous determination of plasma and urinary uric acid, xanthine, hypoxanthine, allopurinol, oxipurinol, orotic acid and orotidine whose quantities are varied by allopurinol treatment in man. Creatinine was also measurable. The method was established by high-performance liquid chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

Allopurinol, 4-hydroxypyrazolo[3,4-d]pyrimidine, a xanthine oxidase (E.C. 1.2.3.2) inhibitor [1], is a most widely used purine analogue in clinical practice, and is converted mainly to a metabolite, oxipurinol, 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine [2]. Treatment of gouty and hyperuricemic patients with the drug results in decreased uric acid production and a concomitant increase of hypoxanthine and xanthine [3], which are substrates of xanthine oxidase. The importance of monitoring the purine analogue and purines in blood and urine is obvious in handling these diseases.

There are many methods (see, for example, refs. 4—8) for estimating allopurinol, oxipurinol and oxipurines, but their combined use to estimate all these compounds in plasma and urine is time-consuming. It is therefore convenient if plasma and urinary allopurinol, its metabolite and the variable purines described above can be simply measured. In establishing such an assay by high-performance liquid chromatography (HPLC) we found a few unidentified

0378-4347/83/$03.00

materials in the HPLC profiles of urine samples of normal and gouty subjects. These unidentified materials were obtained in the HPLC eluates and subjected to identification by ultraviolet (UV) spectrometry and gas chromatography—mass spectrometry (GC—MS), and together with confirmation by the retention times of authentic compounds their estimation became possible.

This paper describes those identification studies and the simultaneous HPLC assay procedure for plasma and urinary allopurinol, oxipurinol, uric acid, xanthine, hypoxanthine, creatinine, orotic acid and orotidine. The latter two pyrimidines are known [9] to be increased in urine following allopurinol ingestion. Therefore, the amount of compounds varying following allopurinol therapy can be estimated by the present systems, as well as creatinine, an essentially invariable indicator.

EXPERIMENTAL

Chemicals

Uric acid, hypoxanthine, xanthine, orotic acid, orotidine and creatinine were obtained commercially (Wako, Kohjin, and Calbiochem-Behring). Allopurinol was our own product. Oxipurinol was synthesized as described previously [2]. All other chemicals were of analytical reagent grade.

HPLC apparatus

The HPLC apparatus used was a Waters Model ALC/GPC-204 liquid chromatograph consisting of a Model 6000A high-performance pump and a Model 440 absorbance detector at a wavelength of 254 nm, equipped with either a U6K injector and a National pen recorder VP-6511W or a Waters WISP 710A sample processor and Waters data module.

GC—MS apparatus

The GC—MS apparatus used was a JMS-D300 mass spectrometer equipped with a JGC-20K gas chromatograph and JMA-2000 data anlysis system (JEOL).

Procedure for HPLC

A 30 cm × 3.9 mm μBondapak C_{18} (particle size 8—10 μm) column (Waters Assoc.), connected to a pre-column (Waters guard column) packed with μBondapak C_{18}, was used for HPLC. The mobile phase was 4 m*M* sodium phosphate buffer (pH 5.0—8.0). For routine work, the pH of the buffer was 6.0. The mobile phase was pumped at a flow-rate of 1.0 ml/min. Column pressure ranged between 70 and 84 bars. Separation was done at ambient temperature. Sample solutions of 1—100 μl were introduced through the injector or the processor. Peak heights were measured either manually or by an on-line computer in the data module equipment.

Procedure for GC—MS

A glass column (1 m × 2 mm I.D.) containing 3% OV-17 on Chromosorb W AW DMCS (80—100 mesh) was used under the following conditions: oven temperature 150°C to 260°C programmed at 8°C/min, injection port tempera-

ture 300°C, helium as carrier gas at 0.9 kg/cm^2, ionization voltage of 70 V, trap current of 300 μA, and ionization chamber temperature of 150°C.

Direct mass spectra were also measured under conditions similar to above using the direct inlet system.

Sample preparation for HPLC

Urine. Human urine (0.5 ml) was mixed with 5 ml of 0.02 *M* sodium phosphate buffer (pH 8.0). Ten to fifty microlitres of the mixture were injected for HPLC.

Plasma. Plasma (0.5 ml) was mixed with 0.4 ml of water and then with 0.1 ml of 20% perchloric acid in an ice-bath for deproteinization. The mixture was centrifuged at 1300 *g* at 4°C for 10 min. An aliquot (0.5 ml) of the supernatant solution was combined with 0.5 ml of 0.2 *M* disodium phosphate and 50 μl of the mixture were injected for HPLC.

Sample storage. Urine and plasma were stored at −20°C until analyzed. Urine yielded precipitates which contained no significant amounts of interfering compounds except in the case of the Lesch-Nyhan [10] patient. The precipitates were soluble in 0.1 *N* sodium hydroxide and the solution was processed in the same way as the urine samples.

Sample processing for mass spectrometry

The aqueous samples separated and recovered from HPLC, which contained the material to be identified and an appreciable amount of sodium phosphate, were freeze-dried. The residue was divided into two portions, one of which was introduced directly into the mass spectrometer via the direct inlet system; the other portion was dissolved in 50 μl of 0.05 *M* trimethylphenylammonium hydroxide (TMPAH) in methanol for analysis by GC—MS. TMPAH was successfully used for methylation of the samples in these studies; the usual trimethylsilylation procedure [11] failed to derivatize the samples, possibly due to the presence of phosphate.

Urine and plasma samples of human subjects

Urine and plasma of normal human subjects were supplied to our laboratory during a bioavailability test of our allopurinol tablets. Normal healthy males ingested a single oral dose of 200 mg of allopurinol tablets with informed consent. Samples of a gouty subject,who is a colleague, were kindly supplied by him in our laboratory. Urine of a Lesch-Nyhan syndrome patient [10,12] was kindly supplied by the Department of Urology, Tokushima University Hospital, School of Medicine, Tokushima University, Tokushima, Japan. The patients had been under therapy with a daily dose of 200 mg of allopurinol.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Authenthic compounds. After examination of columns and solvents it was decided to use the HPLC systems described in the Experimental section based on the retention time of uric acid, hypoxanthine, xanthine, oxipurinol and allopurinol which were around 4.5 min, 11.2 min, 12.8 min, 16.0 min and 18.8

min, respectively. Besides these compounds, orotic acid, orotidine and creatinine were identified in the identification studies described in the latter part of this paper. Their retention times were around 2.9 min (orotidine), 3.4 min (orotic acid) and 6.1 min (creatinine). Fig. 1 is an HPLC profile of a mixture of authentic samples of these compounds, 5—20 ng of each. The resolution was satisfactory under the present conditions.

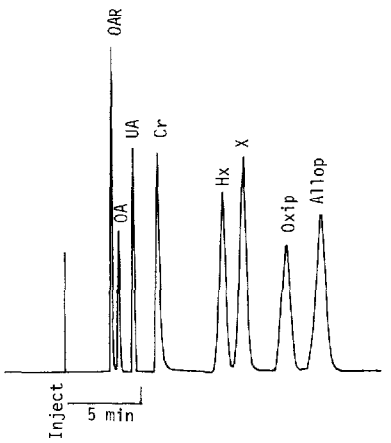

Fig. 1. HPLC profile of a standard mixture. Abbreviations: OAR = orotidine; OA = orotic acid; UA = uric acid; Cr = creatinine; Hx = hypoxanthine; X = xanthine; Oxip = oxipurinol; Allop = allopurinol.

Urine. In chromatograms of urine of normal subjects, a significant peak of uric acid was seen as well as smaller peaks of hypoxanthine and xanthine (a typical chromatogram is shown in Fig. 2a). Addition of authentic hypoxanthine and xanthine to the urine resulted in the corresponding increase and appearance of each compound, confirming that for urine they are recovered from the HPLC column with good resolution. When the man ingested 200 mg of allopurinol, peaks of the unchanged allopurinol and metabolite oxipurinol were seen in urine pooled 0—8 h after ingestion (Fig. 2b). The identity and resolution of these compounds were also confirmed by the addition of authentic compounds to the urine, and GC—MS as described below.

Peak I seen in the urine of the normal subject was identified as creatinine by mass spectrometry as described below, UV spectra and then co-elution with the authentic compound. Peak II may be adenine on the basis of retention time and that of authentic adenine determined separately, but no further confirmation was made.

As above, it is possible to estimate uric acid, xanthine, hypoxanthine, allopurinol, oxipurinol and creatinine in the present systems.

Fig. 2c is the HPLC profile of urine of a gouty subject where xanthine and hypoxanthine were more significant than those in normal subject's urine. Their identity was confirmed by co-elution with authentic purines and GC—MS as described later. As well as peaks of purines and the drug and its metabolite, unidentified peaks III and IV were also seen in his urine. They were identified as orotidine and orotic acid, respectively, as described later.

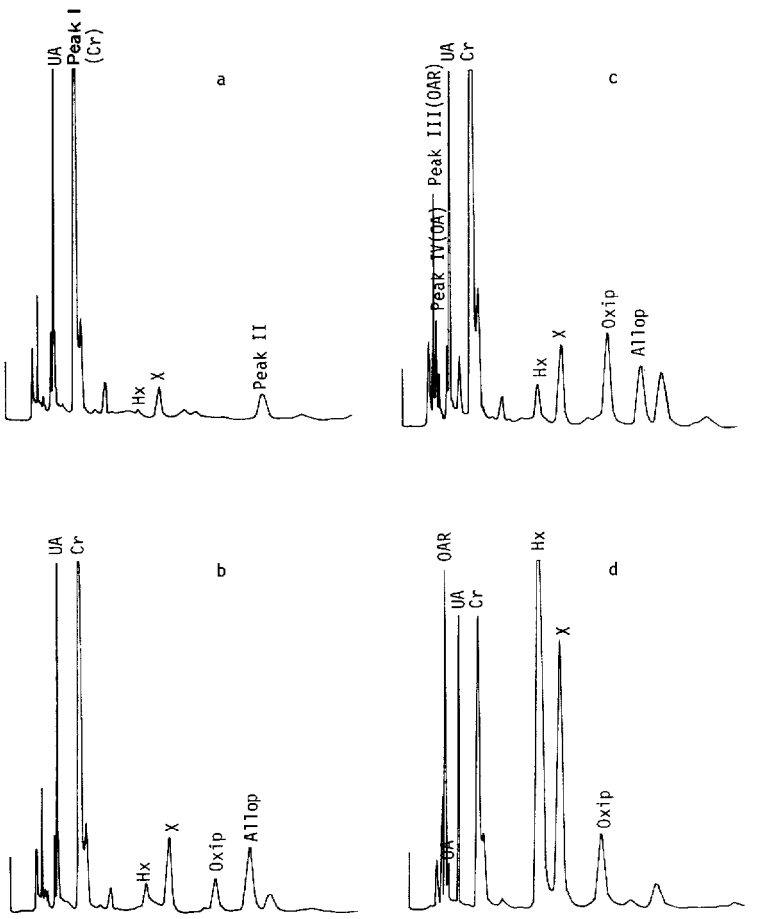

Fig. 2. HPLC profiles of (a) a normal subject's urine, (b) 0—8-h pooled urine after he had ingested 200 mg of allopurinol, (c) 0—8-h pooled urine after a gouty subject under allopurinol therapy (200 mg/day) had ingested the drug, and (d) 24-h pooled urine of a Lesch-Nyhan syndrome patient under allopurinol therapy (200 mg/day). Abbreviations as in Fig. 1.

It is now possible to estimate two pyrimidines, three purines and allopurinol including its metabolite, all of whose amounts are varied by allopurinol ingestion. Besides these, creatinine is also measurable, whose amount is essentially unrelated to allopurinol ingestion. The amount of creatinine excreted in the urine within a given time interval is virtually constant in man. Therefore, the ratio of peak height in one HPLC chromatogram of the purines and pyrimidines to that of creatinine is variable, and dependent on allopurinol ingestion alone. The creatinine peak in an HPLC chromatogram can thus be a measure for comparing the relative quantity of those variable compounds; i.e. the HPLC profile can be used as a follow-up of allopurinol therapy to provide the biochemical status of patients for purines and pyrimidines.

For instance, the HPLC profile of the Lesch-Nyhan patient's urine (Fig. 2d) shows unusually large peaks of hypoxanthine and xanthine relative to creatinine due to his lack [10] of hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8) and partly due to allopurinol therapy, the latter possibly

causing the elevated orotidine and orotic acid excretion seen in the figure. The identity of the compounds appearing in the HPLC chromatogram was confirmed by the following GC—MS studies. Precipitates occurring in his urine during storage at −20°C were found to contain uric acid, xanthine, hypoxanthine and oxipurinol when processed and analysed as described in the Experimental section.

Plasma. HPLC profiles of normal human plasma gave distinct peaks of creatinine and uric acid (e.g., Fig. 3a) and smaller ones of two oxipurines. Allopurinol and oxipurinol were seen as well in this subject's plasma 2 h after he had ingested 200 mg of allopurinol (Fig. 3b).

Two hours after the gouty subject ingested 200 mg of allopurinol, peaks of creatinine, uric acid, hypoxanthine, xanthine, allopurinol and oxipurinol were significant (Fig. 3c). Oxipurinol and two oxipurine peaks were distinct relative to those in the normal subject's urine, possibly because he had been repeatedly taking the drug. The elimination rate of oxipurinol in plasma is known [2] to be much longer than that of unchanged allopurinol.

The identity of the compounds in plasma was confirmed first by their retention times and by the addition of authentic compounds to plasma and the appearance of the corresponding peak in the HPLC chromatogram.

Calibration and recovery. Aqueous mixtures containing known amounts of uric acid, xanthine, hypoxanthine, creatinine, orotic acid and orotidine were prepared and injected into the HPLC apparatus. Plots of their peak heights on chromatograms versus their quantity gave a straight calibration line (usually, correlation coefficient > 0.99) for each compound. Known amounts of the compounds were added to a known volume of urine or plasma and HPLC was carried out according to the standard procedure. The increment of peak height

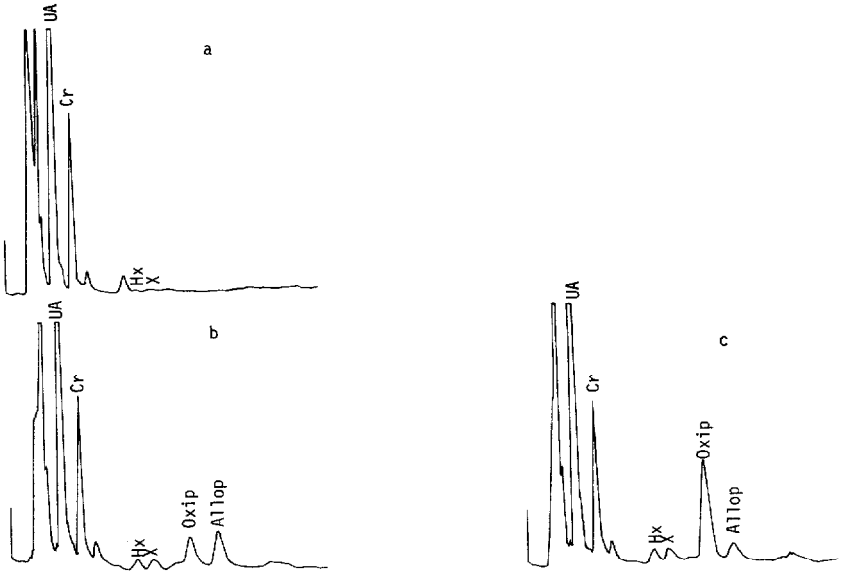

Fig. 3. HPLC profiles of (a) a normal subject's plasma (b) plasma 2 h after he had ingested 200 mg of allopurinol, and (c) a gouty subject's plasma 2 h after he had ingested his daily 200 mg of allopurinol for therapy. Abbreviations as in Fig. 1.

gave their recoveries on chromatography (Table I). The precision was satisfactory when judged from the reproducibility, indicated by standard error means in Table I. The reliable limits of estimation of compounds were different due to their different molar extinction coefficients and were virtually in the range of 3—9 ng (approximately 0.2—0.7 μg/ml plasma and 0.7—2 μg/ml urine). When values were expressed as the mean ± S.E.M. of five normal men, the urinary excretion of uric acid and creatinine determined by the present HPLC procedure was 440 ± 53 and 1350 ± 84 mg/day, respectively. Their plasma levels were 5.86 ± 0.50 and 0.83 ± 0.06 mg per 100 ml, respectively, in the morning. The values are in the well-known normal range in clinical chemistry.

Peak heights of xenobiotic allopurinol and oxipurinol added in a known volume of normal urine or plasma were linear (correlation coefficients > 0.99) when plotted against the concentration in the specimen. Detection of these compounds was possible at > 0.1 μg/ml of plasma and at > 0.3 μg/ml of urine.

Identification of peak components by mass spectrometry

Peak I. The component of peak I gave a molecular ion at *m/z* 113, and fragments at *m/z* 112, 84 and 69 in the direct electron-impact (EI) mass spectrum (Fig. 4), suggesting that it was either creatine or creatinine. Since creatine is easily dehydrated at low temperature to give creatinine, it is inadequate to distinguish the two compounds by EI mass spectrometry where the sample is vaporized by heat. However, peak I was finally identified as creatinine by comparison with the retention time and UV spectrum of authentic creatinine.

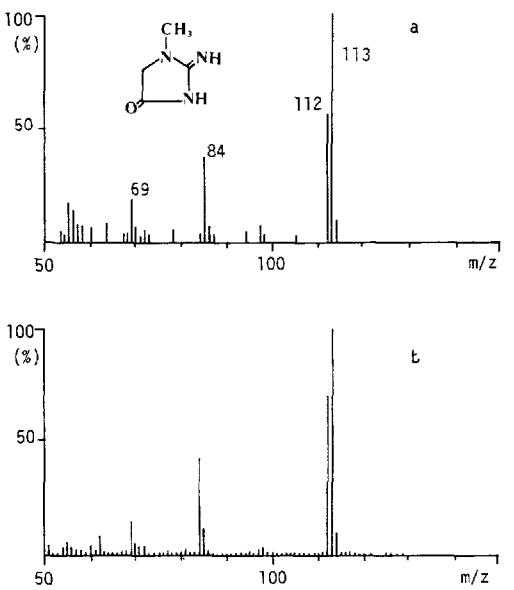

Fig. 4. Mass spectra of (a) creatinine and (b) peak I (EI, 70 V, direct inlet system).

TABLE I

RECOVERIES OF STANDARDS FROM URINE AND PLASMA SPECIMENS

Sample	Urine				Plasma			
	Amount added* (ng)	Amount recovered (ng)	Recovery (%)	*n*	Amount added* (ng)	Amount recovered (ng)	Recovery (%)	*n*
Orotidine	20.0—79.0	18.6—87.3	103 ± 4	8	62.5—500	59.1—372	83 ± 5	6
Orotic acid	100 —400	91.3—381	94 ± 1	8	250 —500	212 —302	73 ± 7	4
Uric acid	217 —1740	189 —1590	92 ± 4	6	125 —1000	104 —802	82 ± 1	6
Creatinine	217 —1740	217 —1840	102 ± 2	6	15.6—62.5	16.4—59.9	95 ± 6	6
Hypoxanthine	63.0—189	58.4—189	90 ± 2	8	3.1—21.0	2.9—21.3	90 ± 3	6
Xanthine	91.0—364	106 —385	112 ± 2	8	3.1—100	3.1—99.5	92 ± 3	9

*Absolute amount injected for HPLC. Therefore, 100 ng for urine and plasma is equivalent to 23 μg/ml of urine and 8 μg/ml of plasma when 50 μl of sample solution prepared as described in the text were injected.

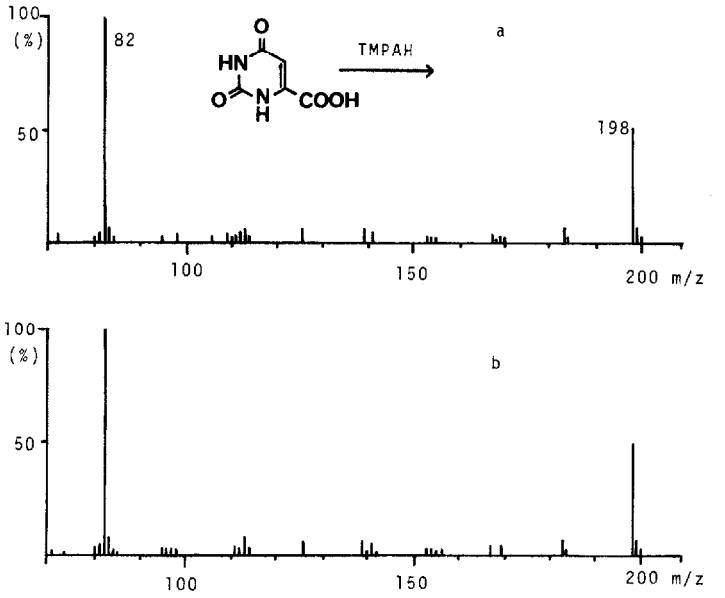

Fig. 5. Mass spectra of (a) orotic acid and (b) peak III after co-injection with TMPAH (EI, 70 V, GC—MS).

Peaks III and IV. The direct mass spectra of peaks III and IV were essentially the same as each other, giving rise to prominent ions at *m/z* 112 and 69 attributable to uracil. However, their behaviour in HPLC was quite different from that of uracil. This suggested that uracil was their thermal decomposition product in the sample probe of the mass spectrometer.

For further clarification, GC—MS was carried out after derivatization. Under the conditions described in the Experimental section, peak III gave a response at 3 min in the total ion monitor chromatogram and the mass spectrum showing ions at *m/z* 198 and 82 attributable to trimethylated orotic acid. Peak III was confirmed as orotic acid by agreement of the mass spectrum with that of authentic orotic acid as shown in Fig. 5. The retention time of peak III in HPLC also agreed with that of the authentic compound.

Peak IV also gave a response at 3 min, the mass spectrum being identical to that of peak III, trimethylorotic acid. The orotic acid detected was thought to be a product by heat yielded from a non-volatile derivative of the acid, i.e., orotidine, since methylation by TMPAH is a flash-heater reaction involving pyrolysis of the quarternary ammonium salt which often causes decomposition of samples [13]. Finally, peak IV was confirmed as orotidine by coelution with the authentic compound in HPLC.

Other peaks. The peak components, the behaviour of which in HPLC was identical to uric acid, xanthine, hypoxanthine, allopurinol and oxipurinol, were also confirmed by selected ion monitoring (SIM). Each ion selected was the molecular ion of the respective derivative: *m/z* 224 corresponds to that of tetramethylated uric acid, *m/z* 194 to trimethylated xanthine and oxipurinol, and *m/z* 164 to dimethylated allopurinol and hypoxanthine. Typical SIM chromatograms are shown in Fig. 6, indicating fair agreement of urinary

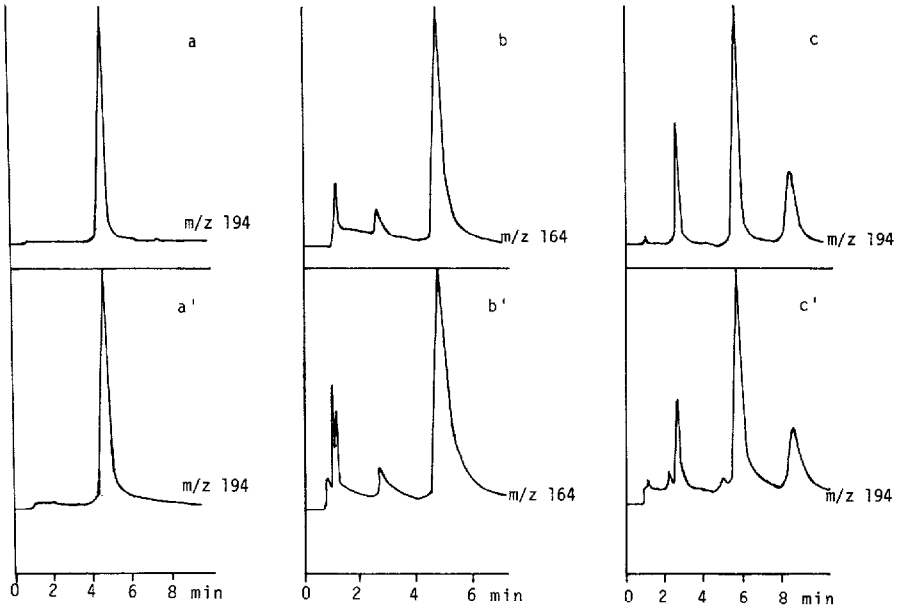

Fig. 6. Identification of xanthine, hypoxanthine and oxipurinol: SIM of authentic (a) xanthine, (b) hypoxanthine and (c) oxipurinol and HPLC eluate corresponding to each compound (a', b' and c', respectively) after co-injection with TMPAH at 300°C and isothermal analysis at 190°C. Each ion selected was the molecular ion of the corresponding derivative.

xanthine, hypoxanthine and oxipurinol with the authentic compounds. Multiple peaks in the chromatograms indicate that, under the conditions employed, methylation did not result in a single product of each purine and analogue due to the formation of either the O-methyl or the N-methyl isomer.

CONCLUDING REMARKS

In an attempt to establish a system to determine simultaneously uric acid, xanthine, hypoxanthine, allopurinol and its metabolite by HPLC, two pyrimidines and creatinine were identified with the aid of GC—MS, and became measurable. As pointed out, since the quantity of creatinine excreted following allopurinol treatment does not change, and can be determined as well as the variable purines and pyrimidines, the HPLC profile, particularly that of urine, could help us to know the biochemical status of patients receiving the drug. Combination of HPLC with GC—MS as described in this study is conceivably of great importance for developing a new assay system for multiples of compounds.

ACKNOWLEDGEMENTS

We wish to thank Dr. K. Hiraishi, Department of Urology, Tokushima University Hospital, for his kind supply of urine of a Lesch-Nyhan syndrome patient, and Drs. M. Shimizu and H. Nishimura for their support.

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