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DETERMINATION OF SELECTED PYRIMIDINES, PURINES AND THEIR METABOLITES IN SERUM AND URINE BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY*

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SUMMARY

The qualitative and quantitative determination of selected pyrimidines, purines and azapurines and their metabolites by reversed-phase ion-pair high-performance liquid chromatography, using tetrabutylammonium hydroxide as pairing ion and isocratic chromatographic conditions, is described. The method provides a selective and sensitive assay for these classes of compounds examined in complex biological fluids.

INTRODUCTION

The analysis of pyrimidine and purine derivatives and their biotransformation products is important from a clinical point of view. This applies to disorders of purine and pyrimidine metabolism (gout) and subsequent treatment with azapurine[1,2] and inter-individual studies of the bioavailability and biotransformation of theophylline[3,4].

Several workers described the determination of selected purines and pyrimidines and their derivatives and metabolites in body fluids by high-performance liquid chromatography (HPLC)[5–8]. However, the separation conditions used in these methods are only suitable for a limited number of compounds. Ion-exchange HPLC is used for the determination of uric acid[10] in biological fluids and also caffeine, theopylline and hypoxanthine are separated by the same principle[11,12]. For the separation of the azapurines allopurinol and oxipurinol, three different HPLC methods are described: reversed-phase HPLC[7], ion-exchange HPLC[5] and ion-exchange HPLC in combination with purification on Chelex-100 resin[6]. Recently Brown *et al.*[8] described a reversed-phase paired-ion chromatographic system for the simultaneous determination of hypoxanthine, xanthine, allopurinol and uric acid. Sodium acetate (pH 4.00)–1-heptane sulfone acid was used as the mobile phase. However, under these

* Dedicated to Professor Dr. Dr. G. Weitzel on the occasion of his 65th birthday.

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conditions it was not possible to separate hypoxanthine, xanthine, uric acid, allopurinol and oxipurinol.

For the studies of reactions with the flavoprotein xanthine oxidase the simultaneous determination of all these compounds was essential. Therefore a more efficient reversed-phase paired-ion liquid chromatographic system was developed in our laboratories and will be discussed here.

The separation method is also suitable to determine substituted xanthine and their metabolites simultaneously in body fluids and appears to be an excellent tool for the study of the biotransformation of theophylline.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (HP) Model 1084B liquid chromatograph, equipped with an HP 798775A UV detector, was used. A ready-to-use column (250 × 4.6 mm I.D.; Knauer, Berlin, G.F.R.), filled with RP-18 (10 μm), was employed.

Analytical procedures

Separation of purines, pyrimidines and their metabolites was achieved by means of ion-pair HPLC. Tetrabutylammonium hydroxide was added to the mobile phase as counter-ion.

All separations were performed in the isocratic mode after addition of different amounts of acetonitrile to the aqueous mobile phase. Equilibration of the column was usually obtained with 50 ml of mobile phase.

Chemicals and compounds investigated

The purines, pyrimidines and their derivatives were obtained from commercial sources (Serva, Heidelberg, G.F.R.; Ferak, Berlin, G.F.R.). Acetonitrile (LiChrosolv®) was purchased from Merck (Darmstadt, G.F.R.) and tetrabutylammonium hydroxide from Fluka (Buchs, Switzerland).

The structures of the compounds investigated are given in Table I.

Sample preparation

Urine. An aliquot of a urine sample was adjusted to pH 7.5 and diluted with the eluent in the ratio of 1:2. A 10–15-μl volume of this solution was injected directly into the separation system.

Serum. A 0.5–1.0-ml volume of a serum sample was centrifuged for 20 min at *ca.* 150 *g* using core-shaped filter inserts (Centriflo type 2100 CF 50) and a 40–100-μl aliquot of the ultrafiltrate obtained was injected directly[13–15].

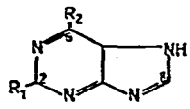
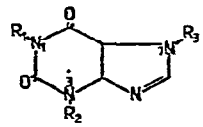
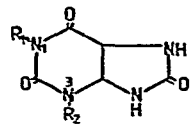
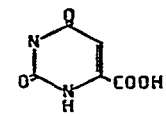
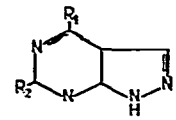
RESULTS AND DISCUSSION

Chromatography

In order to obtain optimal selectivity and resolution for the analysis of purines and pyrimidines, the dependence of the capacity ratio (k') on the column temperature, molarity of counter-ion, pH and concentration of acetonitrile in the mobile phase were investigated.

TABLE I

STRUCTURES OF INVESTIGATED PURINES AND PYRIMIDINES

Type of compound	*Formula*	R_1	R_2	R_3	*Name*
Purines		HO	HO	HO	Uric acid
		HO	HO	H	Xanthine
		H	HO	H	Hypoxanthine
Substituted xanthines		CH_3	CH_3	H	Theophylline
		H	CH_3	CH_3	Theobromine
		CH_3	CH_3	CH_3	Caffeine
		CH_3	H	H	1-Methylxanthine
		H	CH_3	H	3-Methylxanthine
Substituted uric acids		CH_3	H		1-Methyluric acid
		CH_2	CH_2		1,3-Dimethyluric acid
Purine metabolite					Orotic acid
Azapurines		HO	H		Allopurinol
		HO	HO		Oxipurinol

Influence of counter-ion concentration on the capacity ratio. The influence of the molarity of the counter-ion and the column temperature at pH 7.5 and an acetonitrile concentration of 2.5% on k' for compounds 1–8 is demonstrated in Tables II–IV.

The values in Tables II–IV clearly demonstrate a general trend for the capacity factors (with the exception of theophylline, 7): the highest k' values are found at a

TABLE II

k' VALUES FOR 5 m*M* COUNTER- ION CONCENTRATION

Column: 125 × 4.6 mm I.D. Stationary phase: RP-18 (5 μm). Flow-rate: 1 ml/min. Compounds investigated: theobromine (1), uric acid (2), 3-methylxanthine (3), 1-methylxanthine (4), 1-methyluric acid (5), caffeine (6), theophylline (7) and 1,3-dimethyluric acid (8).

Compound	*Temperature (°C)*			
	35	*50*	*60*	*70*
1	3.47	2.71	2.02	1.75
2	3.47	3.34	2.79	2.52
3	3.47	3.34	3.05	2.90
4	4.58	4.07	3.56	3.27
5	7.43	6.16	5.07	4.37
6	10.63	8.23	7.05	6.17
7	15.12	9.86	12.83	10.28

TABLE III

k' VALUES FOR 10 m*M* COUNTER-ION CONCENTRATION

Conditions as in Table II.

Compound	*Temperature (°C)*				
	30	*40*	*50*	*60*	*70*
1	3.44	2.84	2.37	2.07	1.74
2	4.01	3.66	3.34	3.06	2.67
3	4.40	4.08	3.88	3.75	3.45
4	5.71	5.16	4.72	4.35	3.82
5	8.67	7.49	6.41	5.62	4.69
6	11.11	9.86	8.58	7.79	7.06
7	12.73	9.86	7.80	6.24	4.96
8	23.77	19.71	16.21	13.31	10.59

TABLE IV

k' VALUES FOR 20 m*M* COUNTER-ION CONCENTRATION

Conditions as in Table II.

Compound	*Temperature (°C)*			
	35	*50*	*60*	*70*
1	2.10	1.66	1.44	1.30
3	4.40	3.99	3.71	3.11
4	3.27	3.21	3.15	3.49
6	9.07	7.71	7.03	6.64
7	11.00	7.29	5.70	4.65
8	—	14.76	11.89	10.19

concentration of 10 m*M*; at the higher and lower concentrations lower values of *k'* are observed.

Influence of pH on the capacity ratio. The influence of pH and column temperature on the capacity ratios for the eight compounds is obvious from Figs. 1–3.

As expected, the *k'* values decrease with increasing column temperature. At pH 7 (Fig. 1), theobromine (1), uric acid (2) and 3-methylxanthine (3) (at column temperatures of 35–50°C), and 1-methyluric acid (5) and caffeine (6), have identical *k'* values. Much better resolution can be achieved at pH 8.0; however, the *k'* values of 3-methylyanthine (3) and 1-methylxanthine (4) coincide at higher temperatures. As indicated in Fig. 2, the compounds are best separated at pH 7.5. However, the elution order may be dependent on the column temperature for purine derivatives, as is seen for caffeine (6) and theophylline (7) (Fig. 2).

Influence of acetonitrile concentration on the capacity ratio. From the above results, purine derivatives are best separated at pH 7.5, a column temperature of 35°C and a counter-ion concentration of 10 m*M*. Using these parameters, the dependence of the capacity ratio on the acetonitrile concentration was studied (Fig. 4).

Similar investigations were undertaken for the separation of xanthine, hypoxanthine, uric acid, the azapurine allopurinol, its metabolite oxipurinol and orotic

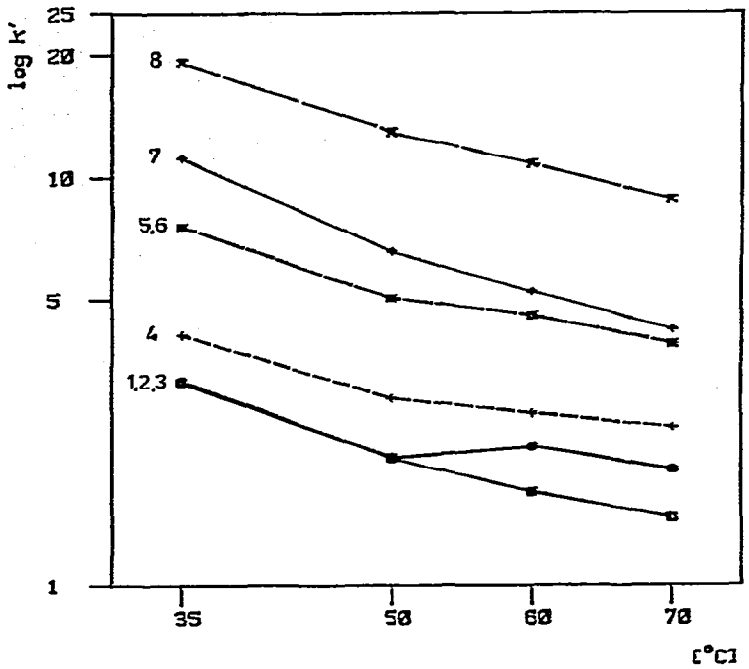


Fig. 1. Influence of column temperature on the capacity ratio (k') at pH 7.0. Column: 125 × 4.6 mm I.D. Stationary phase: RP-18 (5 μm). Mobile phase: 10 mM tetrabutylammonium hydroxide in water–acetonitrile (97.5:2.5). Flow-rate: 1 ml/min. Compounds investigated: theobromine (1), uric acid (2), 3-methylxanthine (3), 1-methylxanthine (4), 1-methyluric acid (5), caffeine (6), theophylline (7) and 1,3-dimethyluric acid (8).

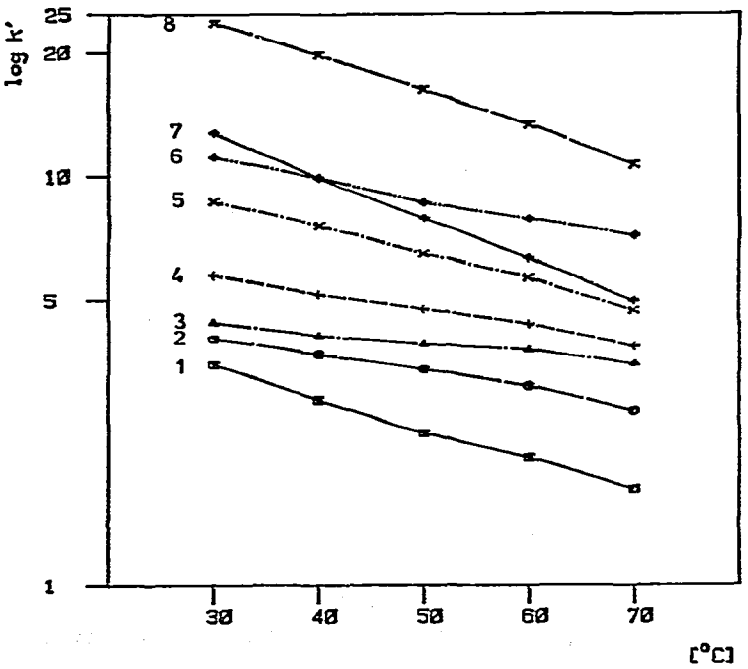


Fig. 2. Influence of column temperature on the capacity ratio (k') at pH 7.5. Separation conditions and compounds as in Fig. 1.

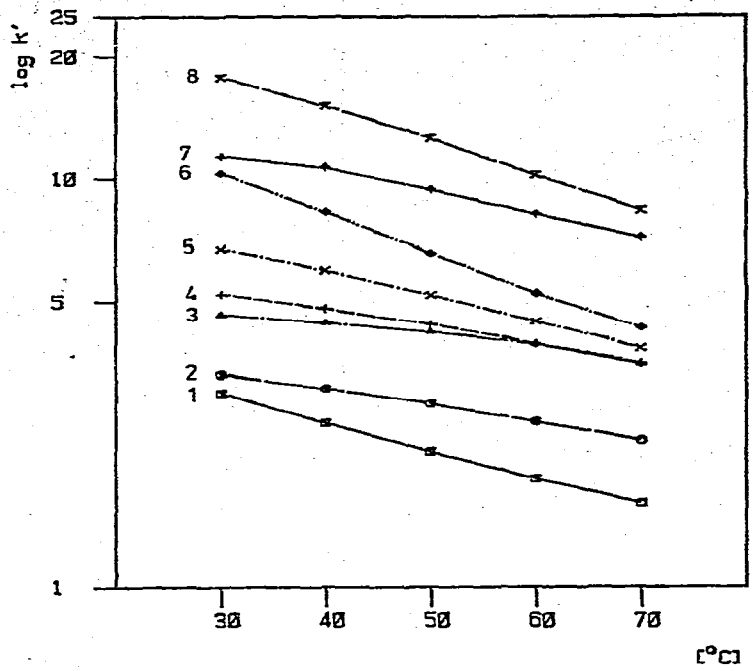


Fig. 3. Influence of column temperature on the capacity ratio (k') at pH 8.0. Separation conditions and compounds as in Fig. 1.

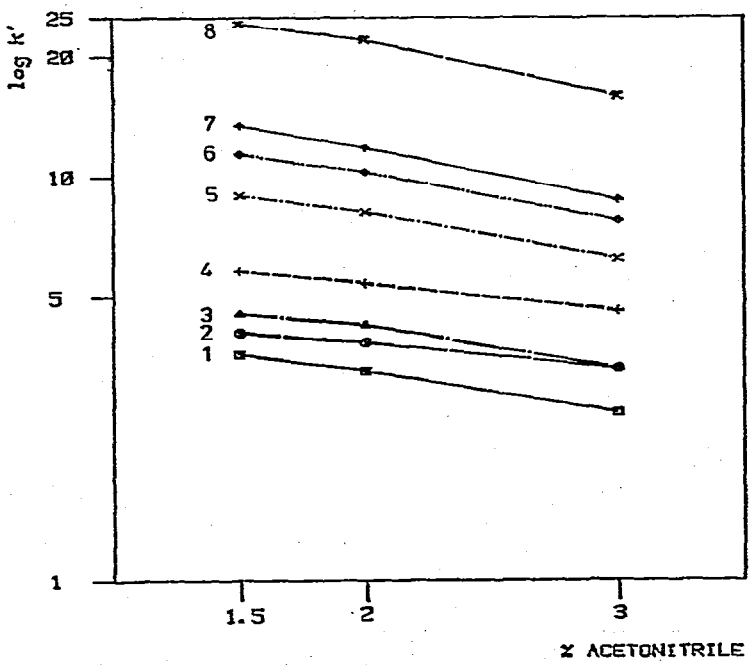


Fig. 4. Influence of acetonitrile concentration on the capacity ratio (k') at a column temperature of 35°C. Separation conditions and compounds as in Fig. 1.

acid. The optimal separation conditions were found to be as follows: tetrabutylammonium ion concentration, 10 m*M*; pH, 8.0; acetonitrile concentration, 0.2%; and column temperature, 35°C.

Bioanalyses of purines and azapurines

Serum. Fig. 5 shows the analysis of a serum sample after addition of hypoxanthine, xanthine, orotic acid, allopurinol and oxipurinol. The uric acid identified corresponds to endogenic serum uric acid, the only peak found in the analysis of a blank sample using the above-mentioned detector setting and separating conditions.

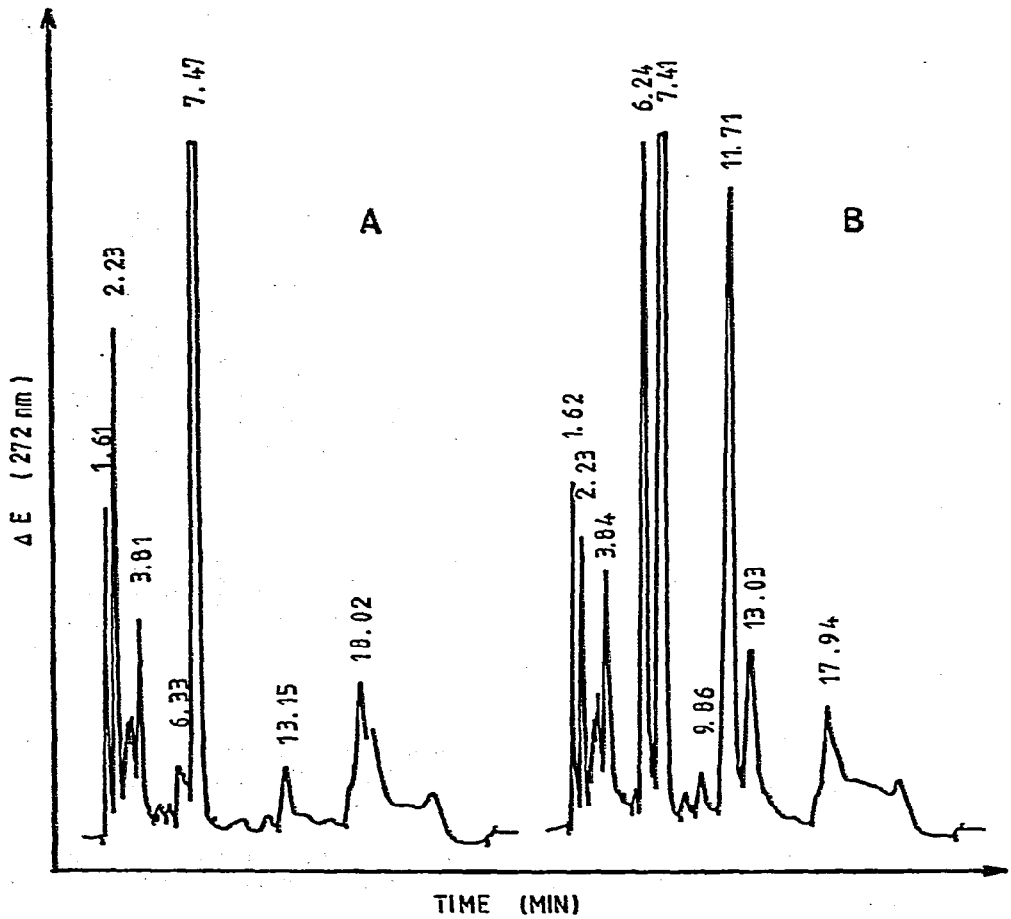


Fig. 5. Chromatogram of the serum filtrate from a patient (blank serum) (A) and of the serum filtrate (identical patient) under treatment with allopurinol (B). Each peak in chromatogram B corresponds to the following quantity: hypoxanthine (retention time, t_R = 3.8 min), 1.2 nmole; xanthine (t_R = 6.2 min), 0.2 nmole; uric acid (t_R = 7.4 min), 24.3 nmole; orotic acid (t_R = 9.8 min), 0.08 nmole; and oxipurinol (t_R = 11.7 min), 0.8 nmole. Mobile phase, 10 m*M* tetrabutylammonium hydroxide in water (pH 8.0)–acetonitrile (99.8:0.2). Flow-rate, 2 ml/min. Injection volume, 40 μl.

Urine. In Fig. 6 the chromatographic analysis of a urine sample (pooled 24-h urine) from a patient after administration of 300 mg of allopurinol is shown. The experimental conditions (isocratic mode) were optimized to permit the determination of larger amounts of xanthine than hypoxanthine. If desired, the separation can be improved in the first portion of the chromatogram by a slight reduction in the acetonitrile content of the mobile phase.

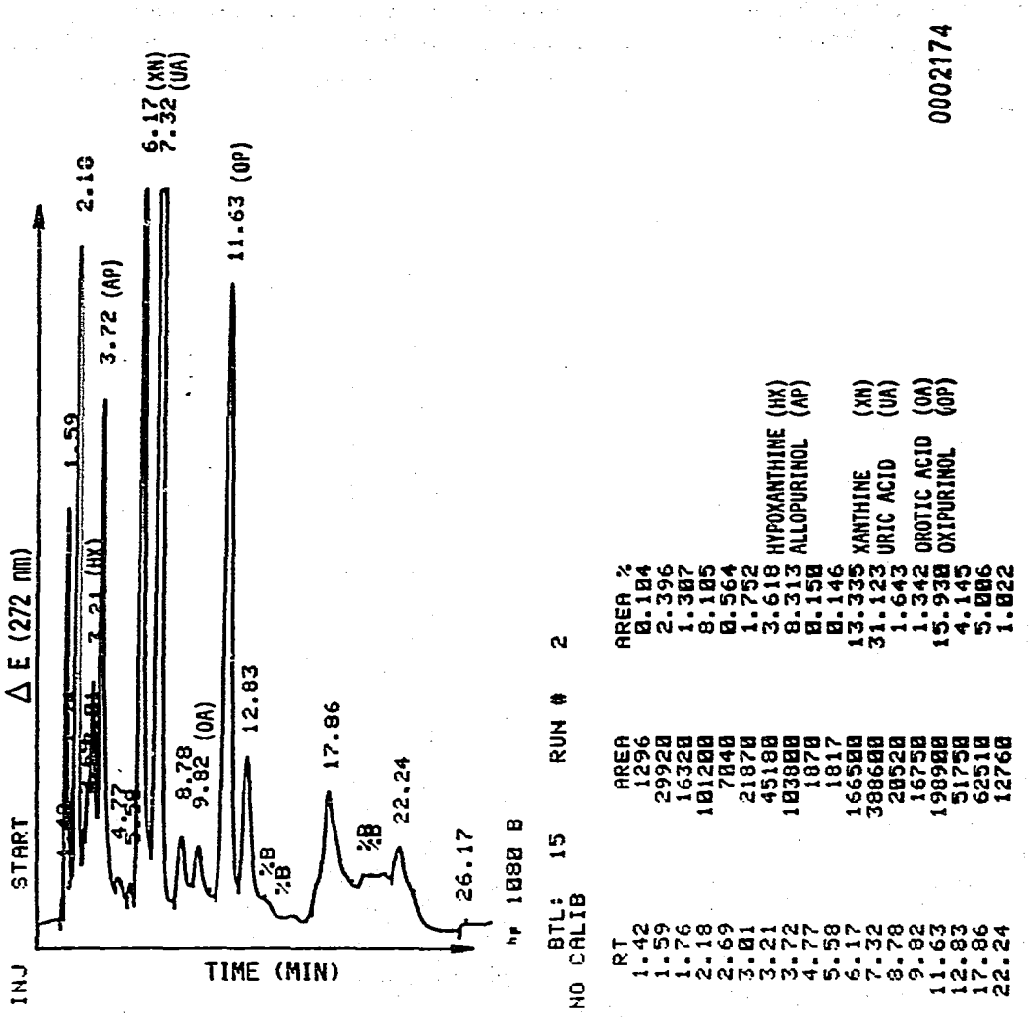


Fig. 6. Analysis of a urine sample (pooled 24-h urine) from a patient after treatment with 300 mg of allopurinol. Separation conditions as in Fig. 5. Abbreviations on the peaks are indicated on the right-hand side of the figure.

Comparison of chromatographic and enzymatic uric acid determinations. To examine the accuracy of the chromatographic uric acid determination, the uric acid concentration from an identical sample was determined separately by two enzymatic methods of analysis (uricase method[9]) with and without deproteinization of the serum

TABLE V

SERUM URIC ACID CONCENTRATIONS DETERMINED BY DIFFERENT METHODS (REPEATED TESTS)

Uric acid (μmol/l)		
Uricase method		*HPLC*
Without deproteinization	*With deproteinization*	
354.5	321.4	354.7
377.7	348.8	365.3
397.4	367.0	392.4
403.9	368.0	410.2

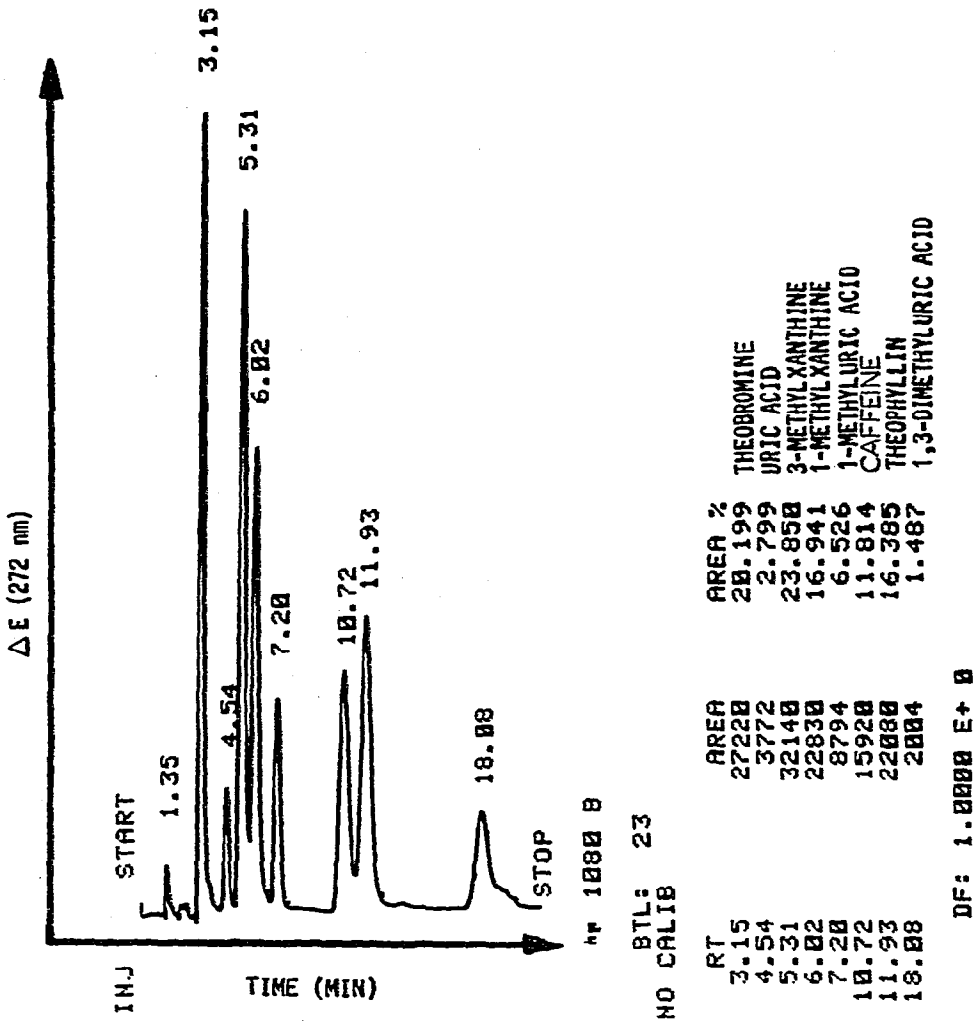


Fig. 7. Analysis of a spiked control serum sample. Each peak corresponds to theobromine 50 ng, 3-methylxanthine 20 ng, 1-methylxanthine 50 ng, 1-methyluric acid 100 ng, caffeine 50 ng, theophylline 50 ng and 1,3-dimethyluric acid 200 ng. Separation conditions as in Fig. 5, except water–acetonitrile (97.5:2.5) was used.

sample, and the results were compared with those obtained by the chromatographic method (Table V).

The values obtained for uric acid in serum by the uricase method without deproteinization are almost identical with those obtained by HPLC. If the uricase method with deproteinization is used, the values were about 8–10% lower, owing to a constant portion of uric acid being lost during protein precipitation.

Bioanalyses of substituted xanthines and uric acids (theophylline and metabolites)

To demonstrate the high resolution of the proposed separation technique, a serum sample spiked with theobromine, 3-methylxanthine, 1-methylxanthine, 1-methyluric acid, caffeine, theophylline and 1,3-dimethyluric acid was investigated. The results are shown in Fig. 7.

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