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A High-Performance Liquid Chromatographic Method for the Determination of Hypoxanthine, Xanthine, Uric Acid and Allantoin in Serum

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Summary: A method was developed for the simultaneous determination of hypoxanthine, xanthine, uric acid and allantoin based on isocratic reversed-phase chromatography. This HPLC-method additionally allows the direct determination with UV-detection of inosine-5′-phosphate, uridine, thymine, orotic acid, allopurinol and oxipurinol, besides hypoxanthine, xanthine and uric acid in the same chromatographic run. Allantoin elutes in this system near the void volume and a fraction is collected covering the retention time range for this substance. After hydrolysis allantoin is converted to glyoxylate-2,4-dinitrophenylhydrazone, rechromatographed and detected at 360 nm.

The coefficient of variation for this method does not exceed 5.0% for a serum concentration of 0.3 μmol/l hypoxanthine and is not greater than 5.3% for a xanthine concentration of 0.3 μmol/l serum. Recoveries were 90–110% for both hypoxanthine and xanthine. The determination of uric acid had an imprecision and inaccuracy not exceeding 1.45% in the concentration range of 103–568 μmol/l. Due to the more complex procedure required for the determination of allantoin, the coefficient of variation between days was 13.6% for a sample containing 0.8 μmol/l allantoin and the recoveries for this analyte were in the range of 86–93%.

Reference ranges (mean ± SD) determined on 171 serum samples from healthy adults were 12.7 ± 6.6 μmol/l for hypoxanthine, 3.3 ± 1.4 μmol/l for xanthine, and 15.7 ± 7.9 μmol/l for allantoin. No significant age or sex dependence was observed. Uric acid concentrations were 320 ± 55 μmol/l serum for men and 206 ± 55 μmol/l for women.

Introduction

The myocytes as well as the vascular endothelial cells of the heart are both targets for damage by free radicals generated during myocardial ischemia and reperfusion. A possibly important process for the generation of oxygen radicals is the conversion of hypoxanthine to xanthine and finally uric acid by the enzyme xanthine oxido-reductase (EC 1.2.3.2) localized in the endothelial cell. Normally this enzyme reduces NAD^+ and does not form free oxygen radicals, but it has been shown by *McCord* et al. (1), that this enzyme is converted during ischemia to the oxidase form, which uses molecular oxygen as an oxidizing agent instead of NAD^+.

Several studies have been performed to assess the relevance of purine metabolism in ischemic tissue (2–4), but no attempt has been made to determine the metabolites arising from the action of xanthine oxido-reductase on allantoin, the product resulting from the oxidation of uric acid by free oxygen radicals. The function of uric acid as an antioxidant in vitro has been shown for several targets of free radical action (5–7); furthermore, it has been shown that the concentration of allantoin is elevated in synovia and serum of rheumatoid patients (8).

Here we report the optimization of an HPLC method and its analytical properties for the determination of

terminal metabolites of purine metabolism including "non-physiological" allantoin. Reference ranges were assessed for hypoxanthine, xanthine, uric acid and allantoin.

Materials and Methods

Chemicals

Allantoin, allopurinol, hyoxanthine, inosine-5′-phosphate, orotic acid, oxipurinol, thymine, uridine and xanthine were obtained from SIGMA, Munich, Germany in the highest available grade of purity and used for preparation of the standard solutions. Uric acid was Standard Reference Material with a purity of 99.7% (SRM 913, National Bureau of Standards, Washington D. C. 20234). All other chemicals were purchased from Merck (Darmstadt, Germany) and were of analytical grade; methanol was HPLC-grade. Demineralized water was prepared with a Milli-Q apparatus from Millipore GmbH (Eschborn, Germany). The control material for the uric acid determination was Standard Reference Material (Human Serum SRM 909, National Bureau of Standards, Washington D. C. 20234).

Samples

The serum samples for the reference range assessment were from healthy blood donors. The Sarstedt system with separation gel was used for withdrawn blood, and the time between blood withdrawal and centrifugation never exceeded 30 min.

High performance liquid chromatography

The HPLC was performed on a Bruker Model LC-31 Chromatograph equipped with Rheodyne injection valve (100 µl injection-loop), 20 x 4 mm RP-18 guard-column, 250 × 4 mm RP-18 analytical colum, both packed with LiChrosorb RP-18, 5 µm material from Merck (Darmstadt, Germany). The guard column had to be replaced every 30 injections to avoid significant reduction of the separation properties.

UV-detection was performed with a photo-diode-array-detector (Millipore-Waters PDA-990+) using the following settings: wavelength range 200–400 nm, resolution 2 nm, 1 scan per second, 27 ms sampling time; 27 sampling times were averaged per scan; sensitivity was set to high (7 points).

The eluents used were:

Solvent A:	demineralized water
Solvent B:	$K_2HPO_4/KH_2PO_4/H_3PO_4$-buffer with 100 mmol/l phosphate, pH ranging from 2.50 to 7.50
Solvent C:	KH_2PO_4/H_3PO_4-buffer with 50 mmol/l phosphate, pH 4.60
Solvent D:	tri-sodium-citrate 30 mmol/l, sodium-acetate 27.7 mmol/l, pH 4.75, methanol, volume fraction 0.25

The flow-rate was 1 ml/min, corresponding to a back-pressure of 130–145 bar.

Procedures and measurements

Preparation of the stock-standards

The final standard concentration for all substances was 500 µmol/l. Allantoin, creatinine, inosine-5′-phosphate, orotic acid and uridine were dissolved in demineralized water. Allopurinol, hypoxanthine, oxipurinol, thymine and xanthine were dissolved in 0.1 mol/l NaOH, and uric acid was dissolved in 0.013 mol/l NH_3.

Preparation of the standard mixture for the solvent system optimization

For every pH the standard mixture was prepared from the stock-standards separately. A solution consisting of 1 ml of each stock-standard (total volume 11 ml) was mixed with 9 ml solvent B to give a final volume of 20 ml. The concentrations of each constituent in the standard mixture was 25 µmol/l. An aliquot (50 µl) of this standard mixture, containing 1.25 nmol of each component, was injected for the determination of the retention times.

Preparation of the standards for the determination of hypoxanthine, xanthine, uric acid and allantoin in serum

Stock-standards of hypoxanthine, xanthine and allantoin (1 ml of each) were mixed with 20 ml of the stock-standard of uric acid and diluted with solvent C to 50 ml in a volumetric flask. This standard, which contained 10 µmol/l hypoxanthine, xanthine and allantoin and 200 µmol/l uric acid, was diluted to produce a calibration curve covering the range from 25 pmol to 500 pmol injected hypoxanthine, xanthine and allantoin. Uric acid was injected to produce a range between 0.5 nmol and 100 nmol. Hypoxanthine was detected at a wavelength of 248 nm, xanthine at 264 nm, and uric acid at 285 nm. The calibration was based on the areas calculated with the Waters PDA-990+ photo-diode-array integration software.

Preparation of the serum samples

A serum sample (100 µl) was mixed with 400 µl of solvent C, then filtered through a membrane filter with a pore diameter of 0.22 µm (Millipore GmbH, Eschborn, Germany). An aliquot (50 µl) of the filtrate was directly injected into the rheodyne injector of the HPLC device. The quantification of hypoxanthine, xanthine and uric acid is based on the peak areas calculated for the wavelength described in the previous section.

Determination of allantoin

Allantoin was determined with a method according to *Grootveld* (8) with minor modifications. Serum samples and standards were treated in the same way. The fraction between elution time t_0 of the void volume and t_0 + 2 min was collected and evaporated under a nitrogen stream at 60 °C. The residue was reconstituted in 250 µl 0.12 mol/l NaOH and incubated for 20 min at 100 °C. In this step allantoin is hydrolysed to allantoic acid. After adding 250 µl 1 mol/l HCl the mixture was incubated for 5 min at 100 °C. This step hydrolyses allantoic to glyoxylic acid. Finally 25 µl 3 mmol/l 2,4-dinitrophenylhydraziniumhydrochloride in 1 mol/l HCl was added and the mixture incubated for 5 min at 100 °C. To enhance the precision of the determination, the final incubation mixture was evaporated to dryness under a nitrogen stream at 60 °C. The residue was dissolved in 250 µl solvent D and 50 µl were injected into the HPLC-apparatus for isocratic rechromatography with solvent D, using the same column as before. The detection was performed at a wavelength of 360 nm.

Solvent system optimization

The eluent optimization was performed for pH values between 2.50 and 7.50 and for phosphate ion concentrations between 20 mmol/l and 100 mmol/l. At every pH the retention times of

the components allantoin, allopurinol, creatinine, hypoxanthine, inosine-5'-phosphate, orotic acid, oxipurinol, thymine, uridine, xanthine, uric acid were measured five times.

For calculation of the optimized eluent composition the treatment described by *Glajch* (1980) was used. The chromatographic-response-function (CRF) has the general form:

$$CRF = \sum_{i=1}^{k} \ln (P_i/P_0) + \alpha (t_m - t_L) \qquad \text{(Eq. 1)}$$

where

P_i = f/g is a measure of resolution of a pair of adjacent peaks;

f is the vertical distance of the valley-point between the two peaks and the line connecting the two peak maxima;

g is the vertical distance of the baseline to the line connecting the two peaks. If there is baseline-separation, f is equal to g and this results in $P_i = 1$ or $\ln pI_i = 0$;

P_0 is the desired peak separation and it is taken as 1 in this solvent system optimization.

The second expression contains

t_M, the maximum acceptable analysis time for one chromatographic run;

t_L, the actual analysis time and

α, an arbitrary weighting factor.

For simplification of the calculation presented here, the second expression is ignored. Only the separation of uric acid at a wavelength of 285 nm, hypoxanthine at 248 nm and xanthine at 264 nm from their adjacent peaks is of interest and needs to be considered. The function CRF defined in this way has an absolute extremum of CRF = 0 only when all substances of interest are baseline-separated from their neighbouring peaks. It is necessary to find one local extremum describing the solvent composition for the best separation of hypoxanthine, xanthine and uric acid from their neighbouring peaks.

The function CRF is then plotted as a function of pH and phosphate concentration *c*. The two variables of the elution system and the hyperplane should be described by a polynomial dependence of CRF (called z) on pH (called x) and *c* (called x):

$$z = f(x,y) = \sum_{n=0}^{5} \sum_{k=0}^{n} c_{nk}\, x^{n-k}\, y^{k} \qquad \text{(Eq. 2)}$$

The results found for the chromatographic response function have to be fitted according to equation 2 with a method for a constrained multidimensional nonlinear-least-square fit (10) using the constraint $z \leq 0$. Finally the result for the pH and the phosphate concentration yielding the best separation is found from the total differential of CRF, as the highest local maximum of this function in the range $2.5 \leq pH \leq 7.5$ and 20 mmol/l $\leq c \leq 100$ mmol/l.

Results

Solvent optimization for the determination of uric acid, hypoxanthine, xanthine and allantoin

The results for the determination of the chromatographic response function for the separation of uric acid, hypoxanthine and xanthine from the neighbouring peaks are shown in figure 1. The optimization converged and the local maximum of the function

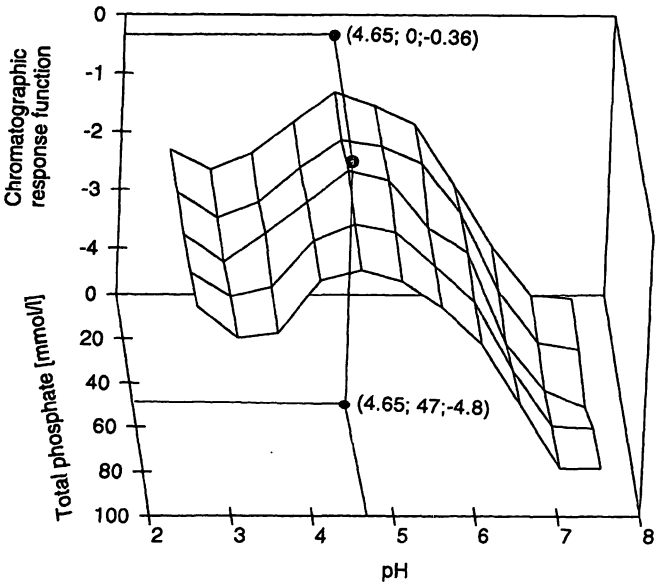

Fig. 1. Results for the calculation of the chromatographic response function (CRF) in dependence on the pH and the total phosphate ion concentration of the eluent. The filled circle with the falling line on the plane of the independent variable indicates the solvent composition determined with the procedure described in methods. The optimized solvent composition was pH 4.65 and 47 mmol/l total phosphate ion concentration. The obtained chromatographic response function was CRF = −0.36.

CRF was found at a solvent pH of 4.65 and a phosphate ion concentration of 47 mmol/l. The calculated value at this eluent composition was CRF = −0.36. All following data were obtained with this eluent system and the CRF calculated from a chromatogram of a standard mixture (fig. 2) was CRF = −0.39 ± 0.02 ($\bar{x} \pm s$). Due to the tailing of the peaks a full baseline separation could not be obtained with this chromatographic system, but due to the relative concentrations of uric acid, hypoxanthine, uridine, and xanthine in serum this was satisfactory for all serum samples analysed. A chromatogram of a serum sample drawn from a healthy male adult is shown in figure 3.

Imprecision data for the determination of hypoxanthine and xanthine

Table 1 shows the data for the within-run imprecisions and the day-to-day imprecisions for serum samples supplemented with hypoxanthine and xanthine standard to cover the measuring range of interest. For these measurements, 50 µl aliquots of fivefold dilutions of the standard-substituted pooled serum samples were injected. The lowest concentration of 0.3 µmol/l hypoxanthine and xanthine corresponds to the injection of 3 pmol analyte. Within-run imprecision for the concentration range considered was 1.9% – 4.5% for hypoxanthine and 1.5% – 4.7% for xan-

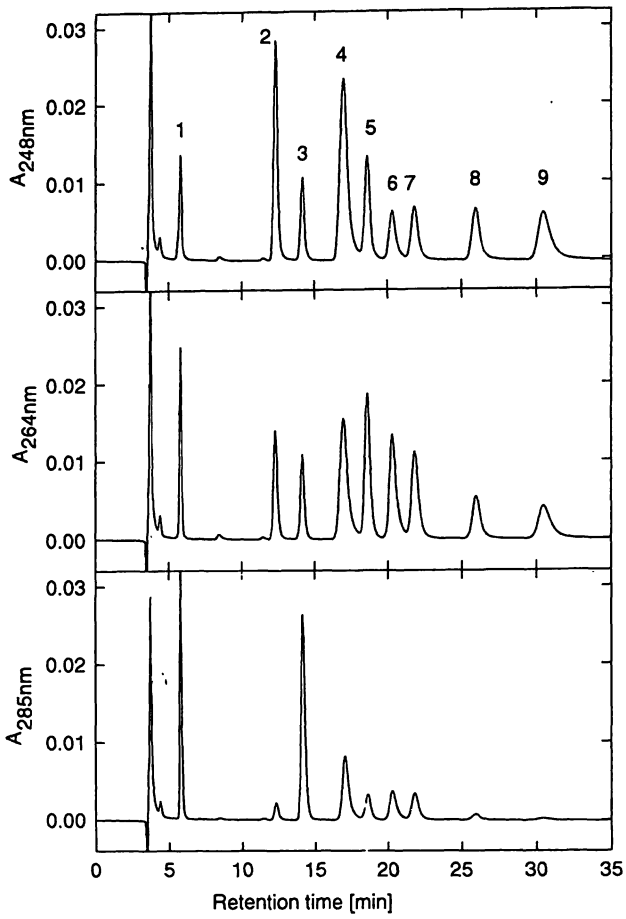

Fig. 2. Multiwavelength chromatogram of a standard mixture performed at 248 nm, 264 nm and 285 nm. The standard contained 45 µmol/l of each substance. The eluent was the optimized phosphate buffer with 47 mmol/l phosphate and pH 4.65.
Peak identification:
1. orotic acid
2. inosine-5'-phosphate
3. uric acid
4. hypoxanthine
5. uridine
6. xanthine
7. thymine
8. oxipurinol
9. allopurinol

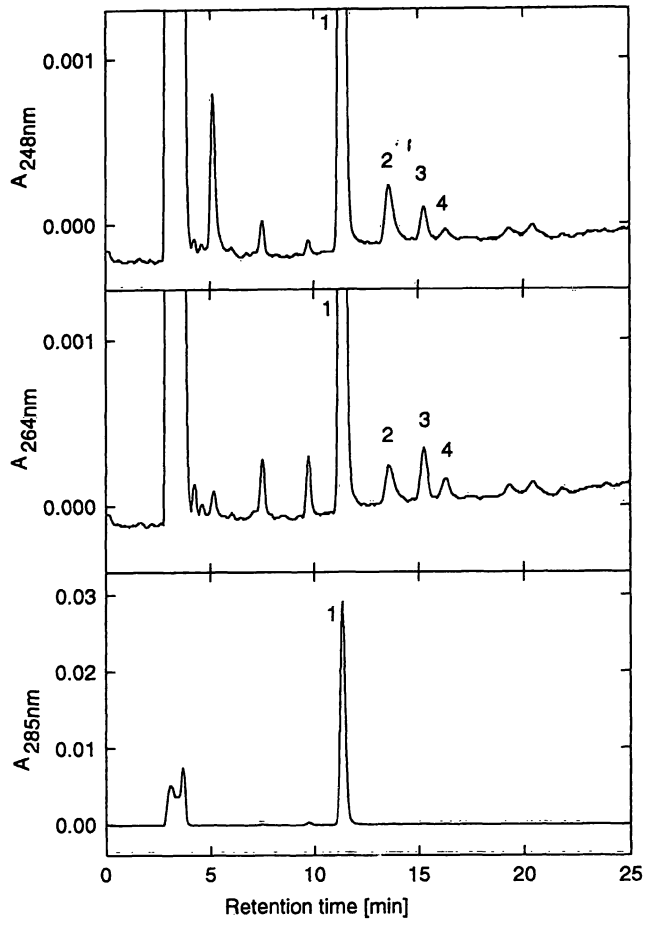

Fig. 3. Multiwavelength chromatogram of a normal serum sample with 208 µmol/l uric acid (peak 1), 3.1 µmol/l hypoxanthine (peak 2), uridine (peak 3) and 0.56 µmol/l xanthine (peak 4), performed at 248 nm, 264 nm and 285 nm. The eluent was the optimized phosphate buffer with 47 mmol/l phosphate and pH 4.65.

thine. Due to the error introduced from the calibration, the day-to-day imprecision was 2.2–5.0% for hypoxanthine and 2.7%–5.3% for xanthine.

Tab. 1. Imprecision data for the determination of hypoxanthine, xanthine and uric acid

Analyte	Imprecision within-run (n = 20)		Imprecision day-to-day (n = 20)	
	Mean concentration [µmol/l]	Coefficient of variation [%]	Mean concentration [µmol/l]	CV [%]
Hypoxanthine	27.6	1.9	25.3	2.2
	5.5	2.3	4.8	3.1
	1.1	3.1	1.0	3.9
	0.3	4.5	0.3	5.0
Xanthine	26.9	1.5	27.2	2.7
	6.1	2.0	1.2	2.9
	1.2	2.9	1.4	4.6
	0.3	4.7	0.3	5.3
Uric acid	510	1.11	568	1.37
	366	0.95	331	1.31
	189	1.34	219	1.23
	123	1.18	103	1.45

Tab. 2. Recoveries of hypoxanthine and xanthine (n = 20)

Analyte	Added concentration [μmol/l]	Mean recovery [%]	Standard deviation of recovery [%]
Hypoxanthine	20.3	101.2	2.8
	4.1	94.3	3.2
	1.0	95.8	4.6
	0.2	90.2	5.4
Xanthine	20.5	97.9	2.4
	4.1	90.8	2.9
	1.0	108.4	3.8
	0.2	107.3	4.9

Recovery data for hypoxanthine and xanthine

Table 2 shows the standard recoveries for hypoxanthine and xanthine. For these experiments 200 μl pooled serum, 200 μl standard of the concentration shown in table 2 and 600 μl eluent were mixed, and 50 μl of this dilution were injected. In the case of hypoxanthine recoveries ranged from 90.2% to 101.2% with standard deviations not different from the imprecisions found. For xanthine the situation is similar to that of hypoxanthine, with recoveries ranging from 97.9% to 107.3%.

Imprecision and inaccuracy of the uric acid determination

The imprecision of the uric acid determination was determined separately from 4 pooled serum samples (data shown in tab. 1); the within-run imprecision was 0.95% − 1.34%, between-run 1.23% − 1.45%. The inaccuracy, determined with the reference material, human serum SRM 909 (certified uric acid concentration 480 μmol/l), was 485 ± 6 μmol/l (n = 20).

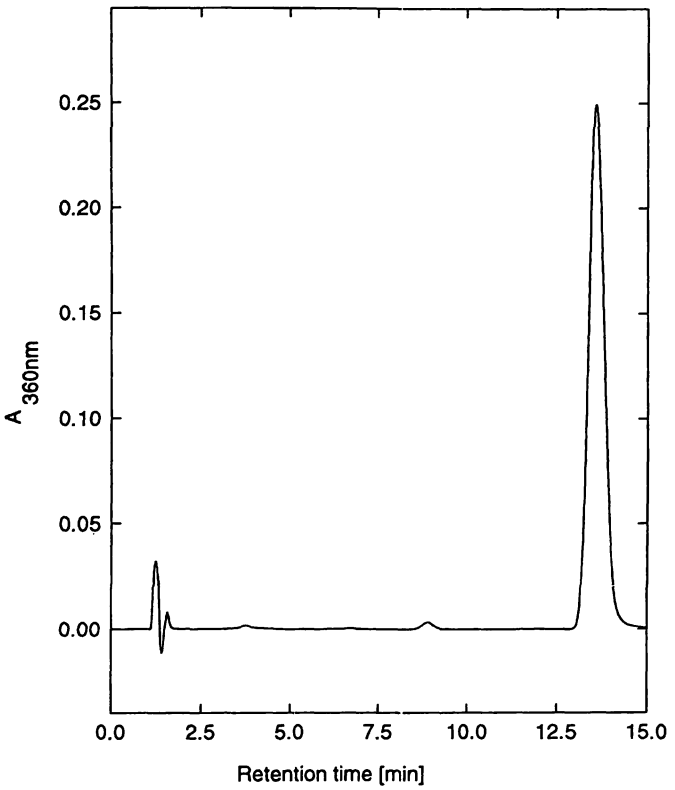

Fig. 4. Chromatogram (with detection at 360 nm) of the glyoxylate-2,4-dinitrophenylhydrazone, derived from the serum sample shown in figure 3, which contained 20 μmol/l allantoin.

Tab. 3. Imprecision within-run, imprecision day-to-day and recoveries for the determination of allantoin

Imprecision within-run (n = 20)		Imprecision day-to-day (n = 20)	
Mean concentration [μmol/l]	Coefficient of variation [%]	Mean concentration [μmol/l]	Coefficient of variation [%]
32.3	5.8	42.4	6.3
16.4	6.9	11.3	8.1
3.1	9.2	5.2	11.6
0.5	11.7	0.8	13.6

Standard recoveries (n = 20)

Added concentration [μmol/l]	Mean recovery [%]	Standard deviation of recovery [%]
36.4	86.3	7.0
8.5	82.7	8.9
3.1	90.1	10.2
0.6	93.2	14.4

Imprecision and recovery for the determination of allantoin

Table 3 shows the data for the imprecision and recovery experiments for allantoin. Figure 4 shows the chromatogram of the glyoxylate-2,4-dinitrophenylhydrazone from a serum sample containing 20 µmol/l allantoin. Pooled serum was substituted with an allantoin standard solution and diluted with eluent C to give a five-fold serum dilution; 50 µl of this dilution were subsequently analysed. The imprecision was 5.8% – 11.7% within-run and 6.3% – 13.6% day-to-day. The recoveries, determined in samples treated as described for hypoxanthine and xanthine, were 86.3% to 93.2%.

Reference ranges

Table 4a shows the results for the reference ranges determined for hypoxanthine, xanthine, allantoin, table 4b the results for uric acid, and figure 5a – 5d the corresponding reference-ranges for all women in

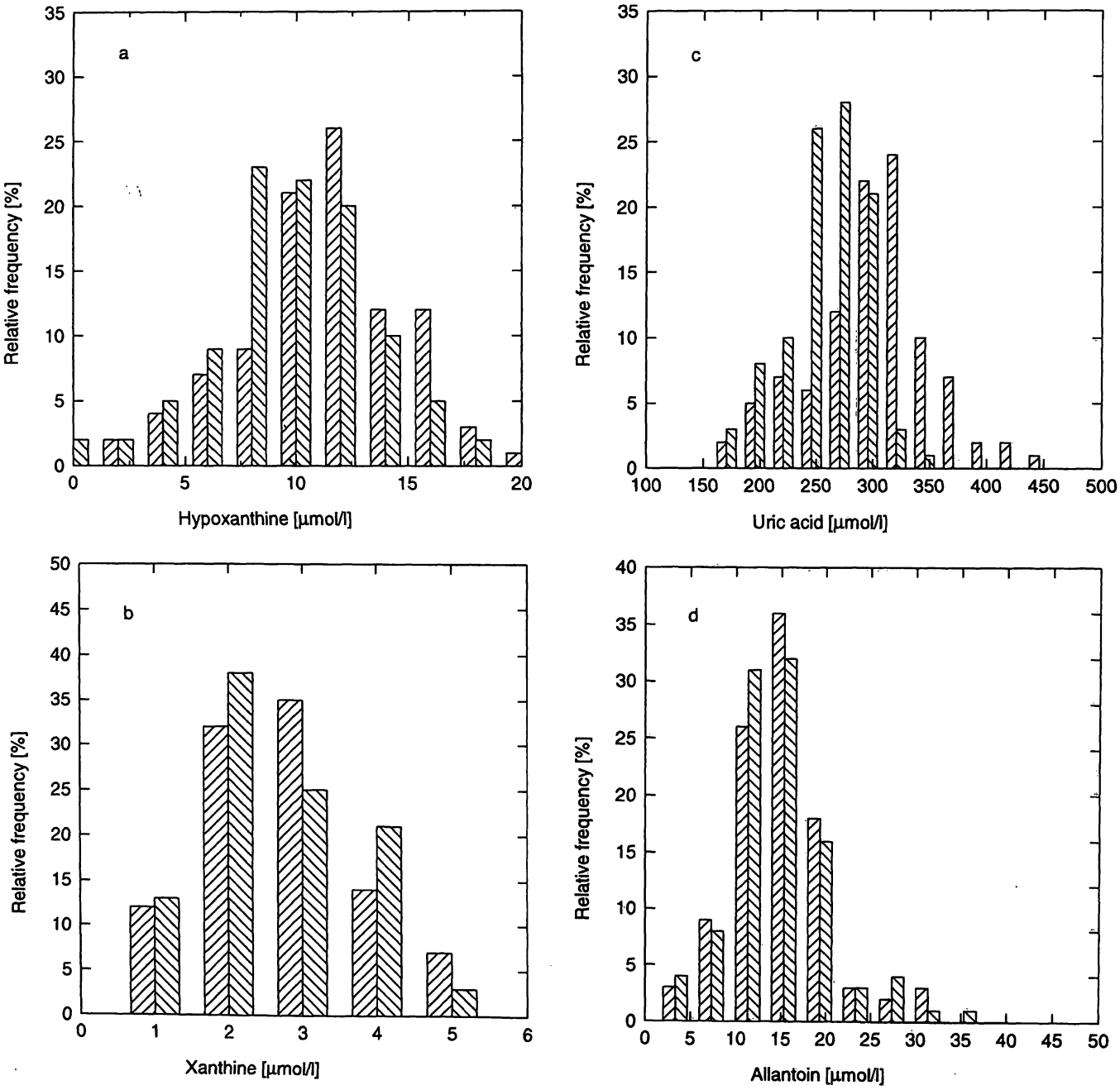

Fig. 5a – d. The reference range of serum of
a) hypoxanthine
b) xanthine,
c) uric acid and
d) allantoin
for 77 adult males and 94 adult females. For age-and sex-dependence of the reference range for uric acid see table 4b.
▨ Reference range adult females
▨ Reference range adult males

comparison with all men. No significant age or sex dependence was found for hypoxanthine, xanthine and allantoin based on a distribution comparison by means of a non-parametric two-sided *Komolgoroff-Smirnoff*-test with a 5%-significance level. Only uric acid shows differences between males and females and an age dependence in males.

Discussion

Optimization of the chromatographic procedure showed that the best separation between the serum constituents inosine-5′-phosphate, uric acid, hypoxanthine, uridine and xanthine could be obtained with a potassium dihydrogenphosphate buffer with a pH of 4.60 and a phosphate concentration of 50 mmol/l. For this solvent system one chromatographic run takes 20 min and a sufficient separation is established for all compounds in the serum samples tested. Due to the dynamic measuring range of the photo-diode-array-detector, uric acid could be quantified from the same chromatographic run as hypoxanthine and xanthine, whose concentrations are about 30 and 150 times lower, respectively. Neither normal sera nor pathological sera from patients caused any observable chromatographic interference. The use of a guard column in the method presented eliminates the requirement for sample deproteinisation techniques like ultrafiltration or protein precipitation with for example trichloroacetic acid. In contrast to the procedure originally presented by *Grootveld* (8), the determination of allantoin presented here is not sensitive to unintentional evaporation of the incubation mixture, because the glyoxylic acid-2,4-dinitrophenylhydrazone is finally dried and reconstituted in a well defined volume of solvent.

For hypoxanthine a coefficient of variation between days (CV_{dd}) of 5.0% was found for an injection of 3 pmol substance. For 3 pmol injected xanthine a slightly higher CV_{dd} of 5.3% was found; this is due to a broader elution band shape. The recoveries in the standard recovery experiments were 90–110%, determined by means of standard additions covering the reference range. The much more complicated de-

Tab. 4a. Reference — ranges for hypoxanthine, xanthine and allantoin in serum

	Mean	Standard diviation	Range
Hypoxanthine [µmol/l]	12.7	6.6	1.2–17.9
Xanthine [µmol/l]	3.3	1.4	0.2– 5.8
Allantoin [µmol/l]	15.7	7.9	3.1–36.4

Tab. 4b. Reference range for uric acid in serum

a) Men

Age [a]	n	Concentration of uric acid [µmol/l]		
		mean	standard deviation	range
10–20	32	288	25	207–369
21–30	19	321	36	231–398
31–40	14	305	31	256–407
41–50	12	346	47	216–442
51–60	12	378	35	265–403
>60	5	361	27	281–417
all	94	320	55	207–442

b) Women

Age [a]	n	Concentration of uric acid [µmol/l]		
		mean	standard deviation	range
10–20	21	207	25	162–314
21–30	14	178	34	151–279
31–40	12	213	31	167–296
41–50	10	231	26	181–343
51–60	12	180	40	224–297
>60	8	250	62	211–367
all	77	206	61	151–367

termination procedure for allantoin resulted in a CV_{dd} of 13.6% when analysing a 8 pmol sample, and the recoveries found for allantoin were 86–93%. The results for uric acid were comparable to those previously published for a HPLC procedure optimized for the determination of uric acid.

The reference ranges of hypoxanthine, xanthine and allantoin did not show a significant dependence on age or sex. Only uric acid showed the well established higher reference range for men and an increase of this reference range for men with age.

With the method presented, a sensitive simultaneous determination of the metabolites of terminal purine metabolism, hypoxanthine, xanthine, uric acid and allantoin is possible. The method may also be used for other body fluids like urine and cerebrospinal fluid.

A paper is in preparation, in which we describe the changes of hypoxanthine, xanthine, uric acid and allantoin in patients with clincally well defined acute myocardial infarction in comparison with other diseases with muscle destruction.

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