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Journal of Chromatography B, 819 (2005) 229–235
JOURNAL OF CHROMATOGRAPHY B
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Simultaneous determination of 16 purine derivatives in urinary calculi by gradient reversed-phase high-performance liquid chromatography with UV detection

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Received 3 September 2004; accepted 5 November 2004
Available online 16 March 2005

Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method with ultraviolet detection has been developed for the analysis of purines in urinary calculi. The method using gradient of methanol concentration and pH was able to separate 16 compounds: uric acid, 2,8-dihydroxyadenine, xanthine, hypoxanthine, allopurinol and oxypurinol as well as 10 methyl derivatives of uric acid or xanthine (1-, 3-, 7- and 9-methyluric acid, 1,3-, 1,7- and 3,7-dimethyluric acid, 1-, 3- and 7-methylxanthine). Limits of detection for individual compounds ranged from 0.006 to 0.035 mg purine/g of the stone weight and precision (CV%) was 0.5–2.4%. The method enabled us to detect in human uric acid stones admixtures of nine other purine derivatives: natural metabolites (hypoxanthine, xanthine, 2,8-dihydroxyadenine) and methylated purines (1-, 3- and 7-methyluric acid, 1,3-dimethyluric acid, 3- and 7-methylxanthine) originating from the metabolism of methylxanthines (caffeine, theophylline and theobromine). The method allows simultaneous quantitation of all known purine constituents of urinary stones, including methylated purines, and may be used as a reference one for diagnosing disorders of purine metabolism and research on the pathogenesis of urolithiasis.

Keywords: Purines; Uric acid; Allopurinol; Methylxanthines; Urolithiasis; Urinary calculi; Inborn errors of purine metabolism; Xanthinuria; Dihydroxyadeninuria; High-performance liquid chromatography

1. Introduction

Low solubility in aqueous solutions is a characteristic feature of numerous purines (e.g. uric acid, xanthine, 2,8-dihydroxyadenine) [1,2]. These compounds may precipitate from urine and form urinary calculi if their concentrations exceed limits of saturation.

According to the previous reports, uric acid (UA) is the only purine component of urinary calculi occurring at normal activities of purine metabolising enzymes. Calculi built of xanthine (Xan) may appear in a hereditary defect of xanthine oxidoreductase called xanthinuria [3] as well as during excessive inhibition of xanthine oxidoreductase with hypoxanthine isomer, allopurinol (Alp) [4–6]. Xanthine isomer oxypurinol (Oxp), the metabolite of Alp, was also found as constituent of stones formed during Alp treatment [7,8]. 2,8-Dihydroxyadenine (2,8-DHA) is excreted in urine and can precipitate in renal tissue and urinary tract in patients with dihydroxyadeninuria—an inherited deficiency of adenine phosphoribosyltransferase [9].

Xan, 2,8-DHA and methyluric acids are indistinguishable from UA by simple methods routinely used in clinical laboratories, i.e. murexide or phosphomolybdate tests. The tech-

Abbreviations: 1-MUA, 1-methyluric acid; 3-MUA, 3-methyluric acid; 7-MUA, 7-methyluric acid; 9-MUA, 9-methyluric acid; 1,3-DMU, 1,3-dimethyluric acid; 1,7-DMU, 1,7-dimethyluric acid; 3,7-DMU, 3,7-dimethyluric acid; 1-MX, 1-methylxanthine; 3-MX, 3-methylxanthine; 7-MX, 7-methylxanthine; 2,8-DHA, 2,8-dihydroxyadenine; Alp, allopurinol; HPLC, high-performance liquid chromatography; Hyp, hypoxanthine; Oxp, oxypurinol; UA, uric acid; UV, ultraviolet; Xan, xanthine

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doi:10.1016/j.jchromb.2004.11.013

nique of choice used for purine research is high-performance liquid chromatography (HPLC), but until now only a few earlier reports have dealt with urinary calculi [10–12]. We have recently described a simple isocratic HPLC method for analysis of these six purines (UA, Hyp, Xan, 2,8-DHA, Oxp, Alp) in urinary calculi [13], which enabled us to find that all uric acid calculi also contained 1-methyluric (1-MUA) and 7-methyluric (7-MUA) acids. These methylated purines are known as metabolites of methylxanthines present in plants. There are however many more methylated purines excreted in urine, which the isocratic method could not separate. It is noticeable that their average daily output (depending on the ingestion of methylxanthines) in urine is about 500 mg/day [14], which equals the output of UA. Methyluric acids have similar properties like UA, including low solubility in water solutions. Therefore, they may play a role in the processes of urinary stone formation.

This paper presents a novel gradient HPLC method for analysis of 16 purine derivatives, including 10 methylated purines, in urinary calculi.

2. Experimental

2.1. Urinary calculi

We examined stones or "sand", removed from urinary tracts of 48 adult patients (31 men, 17 women) aged 49 ± 15 years during their operation at urological ward. If they were heterogeneous—their respective parts or layers differed in colour or structure from each other—separate samples were taken from each part, e.g. nucleus and outer layer. Altogether, 65 samples were analysed.

2.2. Chemicals

We used NaOH, KH_2PO_4, K_2HPO_4 from Fluka (puriss. p.a.), water from Milli-Q system (Millipore), methanol (gradient grade) from Merck. Phosphate buffers were filtered before use (Schleicher & Schuell RC 55, pore diameter 0.45 μm). Standards of analysed compounds came from Sigma (Xan, 2,8-DHA, Oxp, 1-MX, 1-MUA, 3-MUA, 7-MUA, 1,3-DMU, 1,7-DMU, 3,7-DMU), Fluka (Hyp, Alp, 3-MX, 7-MX, 9-MUA) and Merck (UA).

2.3. Preparation of samples and standards

Procedure of sample preparation was described in detail previously [13]. Urinary calculi were dried for 24 h at room temperature and crushed in a porcelain mortar. Five milligrams of powder was inundated with 10 mL of 0.1 M NaOH and placed on a magnetic stirrer at room temperature for 10 min. The extract from above the sediment was diluted with an adequate volume of 50 mM KH_2PO_4, to obtain 100 mg/L solution (five-fold dilution). It was filtered through ACRO LC13 (0.45 μm, Gelman Sciences) before injection.

Solutions of the 16 calibration standards at final concentrations 5, 25 and 100 mg/L as well as blank samples were prepared similarly to calculi samples. 2,8-DHA precipitated from the buffered solution at concentrations above 5 mg/L, therefore higher concentrations of this compound could not be used. Standard mixture containing 5 mg/L of each standard was prepared in the same way and used for the determination of sensitivity and precision of the method, as well as for daily quality control.

Filtered solutions of samples and standards were stored at −75 °C, thawed at room temperature and mixed for a few seconds before injection to avoid partial crystallisation of the dissolved compounds in the course of freezing and thawing.

2.4. HPLC equipment

A Hewlett-Packard Series 1050 chromatographic system consisted of a quaternary gradient pump with low-pressure mixer (dwell volume approximately 1 mL), UV variable wavelength detector (VWD), Rheodyne 7125 manual injection valve with 20 μL loop, helium solvent degassing system and column thermostating compartment HP G1316A. Chromatographic data were processed by HP Chemstation software (Hewlett-Packard, now Agilent).

2.5. Column

We used 125 mm × 4 mm column pre-packed with LiChrospher 100 RP-18 (5 μm particles, 0.08 nm pores) (Merck). A cartridge 4 mm × 4 mm, packed with the same material, served as precolumn.

2.6. Chromatographic conditions

A new gradient method was worked out, with two parameters of mobile phase changing during analysis: methanol content and pH. The change of mobile phase composition was achieved by mixing of three solvents in controlled proportions. The gradient programme is shown in Table 1 and Fig. 1.

The flow rate was 1 mL/min. The 100 μL samples (five-fold loop volume) were injected every 17 min. The column temperature was 27 ± 0.1 °C. Samples of stone extract containing purines were injected twice. Detection wavelength for the first run was 254 nm, which allowed to detect all analytes but was not optimal for quantitation of Xan and UA derivatives. When some peaks of purines were detected the analysis was repeated with wavelength changing during separation according to the programme (Table 2). Data from two separations were used to calculate characteristic ratios of peak areas for confirmation of compound identification.

The obtained chromatograms were corrected on the basis of blank samples using the software option "subtract blank run" to compensate for baseline deviations dependent on gradient. Pressure of the mobile phase oscillated from 60 to 85 bar during run. The quantitation was based on peak ar-

Table 1
Timetable of mobile phase gradient programme

Solvent	Content of solvents (%, v/v)										
	0.00 (min)[a]	2.40 (min)	2.41 (min)	3.00 (min)	6.30 (min)	6.80 (min)	8.70 (min)	8.71 (min)	13.00 (min)	13.01 (min)	17.00 (min)
A	85.5	85.5	100	100	98	0	0	91.2	82.6	85.5	85.5
B	14.5	14.5	0	0	0	95	91.2	0	0	14.5	14.5
C	0	0	0	0	2	5	8.8	8.8	17.4	0	0

Content of the three solvents (A–C) in the mixture is expressed as percent of mobile phase volume at the indicated moments of analysis time. It is changing linearly between these values. Solvent A: 50 mM KH_2PO_4 (pH 4.6), prepared by dissolving KH_2PO_4 in water; solvent B: 50 mM solution KH_2PO_4/K_2HPO_4 (pH 6.4), prepared by mixing 50 mM solutions of KH_2PO_4 and K_2HPO_4 to obtain target pH; solvent C: methanol.
[a] Analysis time since injection (min).

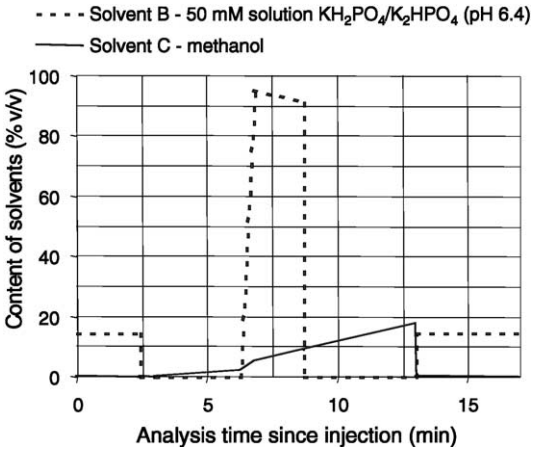

Fig. 1. Graphical presentation of HPLC mobile phase gradient programme. Content of solvent A (50 mM KH_2PO_4, pH 4.6) is equal to 100% − (solvent B + solvent C). Content of methanol gradually increases to elute less polar compounds while content of solvent B (less acidic than A) determines mobile phase pH to adjust retention times for uric acid derivatives.

Table 2
Programme of UV detector wavelength used during the second analysis of the stone extract samples

Range of analysis time (min)	Compounds analysed	Detection wavelength (nm)
0–3.25	UA	290
3.25–3.9	2,8-DHA	320
3.9–5.1	9-MUA, Hyp	290
5.1–6.7	Xan	270
6.7–10.45	Oxp, 3-MUA, Alp, 7-MUA, 1-MUA, 3,7-DMU	290
10.45–12.45	7-MX, 3-MX, 1-MX	270
12.45–14.5	1,3-DMU, 1,7-DMU	290

eas with external standard calibration. The column was filled with 100% methanol between working days.

3. Results

Fig. 2 presents chromatogram of the 16-standard mixture (5 mg/L each) at $\lambda = 254$ nm. Retention times of analysed compounds were changing within narrow range ±0.05 min during 1 day and ±0.1 min on consecutive days. So far, over 300 analyses have been performed on the LiChrospher column without significant deterioration of separation quality.

Calibration curves were linear up to concentration of 100 mg/L for all the standards except 2,8-DHA and Xan. Due to their limited solubility in the buffer, the upper limits of linearity were 5 mg/L for 2,8-DHA and 25 mg/L for Xan. Results of analyses were not reproducible for higher concentrations, which was probably caused by partial crystallisation in solutions.

Table 3 presents inter-day precision and limits of detection (S/N = 3) for each compound of 16-standard mixture. Limit of detection is calculated for the wavelength giving higher absorption coefficient for particular compound. It was 254 nm for Hyp, Alp and Oxp. For the other compounds, it was consistent with the detector wavelength timetable (Table 2). Precision is calculated as coefficient of variation (CV%) of six analyses of the 16-standard mixture components on consecutive days.

3.1. The chromatographic analysis of stone samples

Uric acid was the main component (>50 wt.%) of 18 stone samples from 11 patients. No xanthine or 2,8-dihydroxyadenine stones were found. Figs. 3a and b and 4a and b show chromatograms of 100 mg/L extract of a representative uric acid stone (85% UA content), obtained with constant (Fig. 3) and variable (Fig. 4) detection wavelength.

The peaks of purines accompanying UA are 2,8-DHA (trace amounts detected only at 320 nm), Hyp, Xan, 3-MUA, 7-MUA, 1-MUA, 7-MX, 3-MX, 1,3-DMU. Analysis of all chromatograms revealed that the same 10 compounds were present in all 18 uric acid calculi samples, though in different proportions. 9-MUA, 1-MX, 1,7-DMU, 3,7-DMU, Alp and Oxp were not detected in any sample. Table 3 presents maximal content of purines detected in the examined stones. These compounds were never found in stones containing no UA.

4. Discussion

4.1. Methodological aspects of extraction and chromatographic separation

The procedure of sample separation was the same as described and validated previously [13] for the analysis of UA,

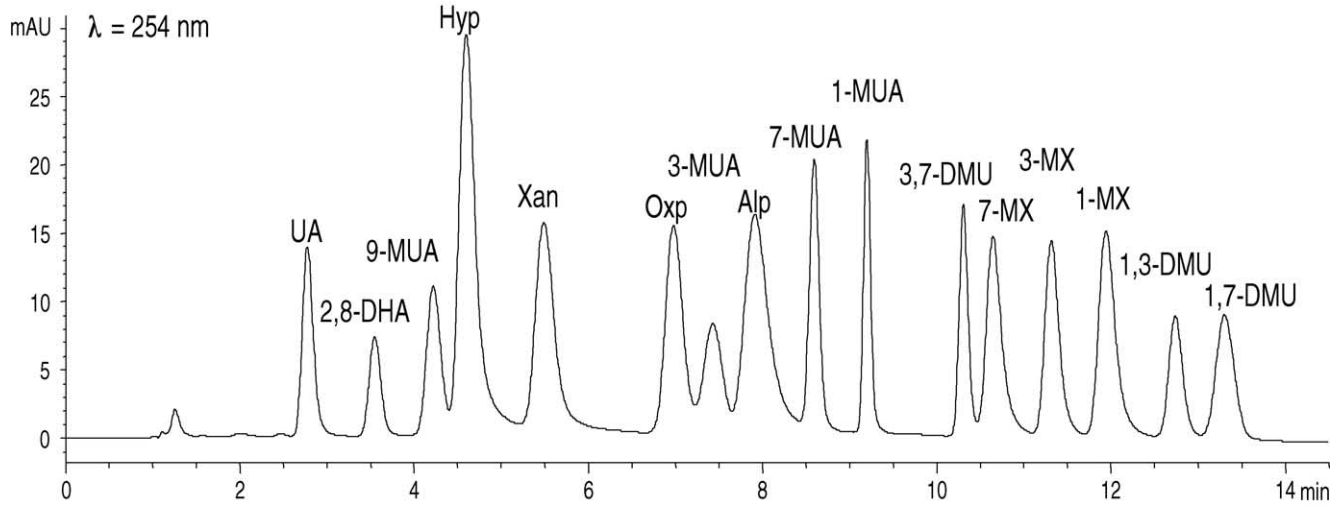

Fig. 2. Chromatogram of the 16-standard mixture at λ = 254 nm. Concentration of each standard is 5 mg/L. Chromatographic conditions are described in Section 2. Gradient programme is shown in Table 1 and Fig. 1.

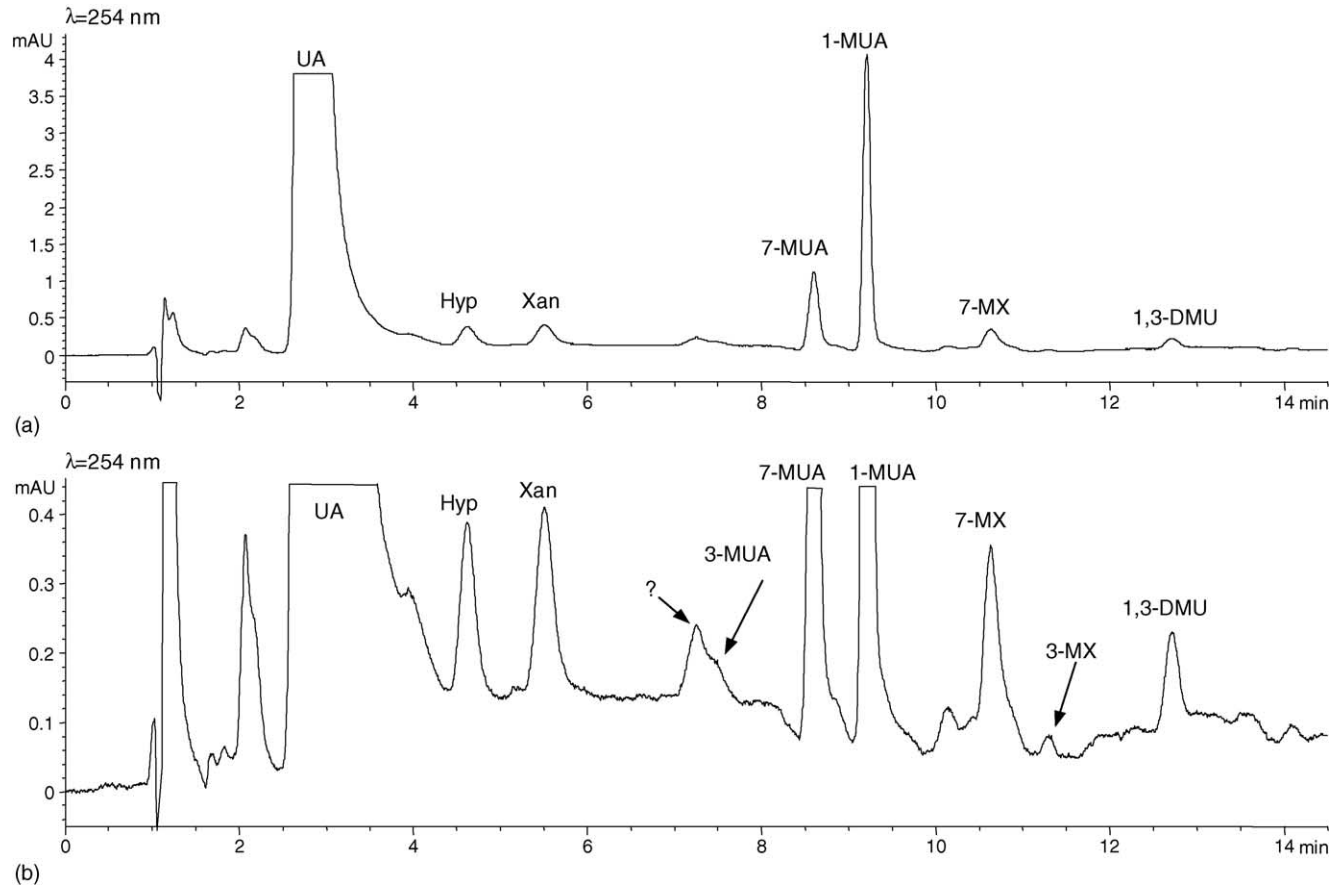

Fig. 3. (a and b) Chromatogram of a representative uric acid stone extract obtained with constant detection wavelength at λ = 254 nm. The stone contained 85% UA. Extract concentration is 100 mg/L. The chromatogram is shown at two absorbance scales because of range of peak heights. Some peaks were truncated due to spreading of the absorbance scale.

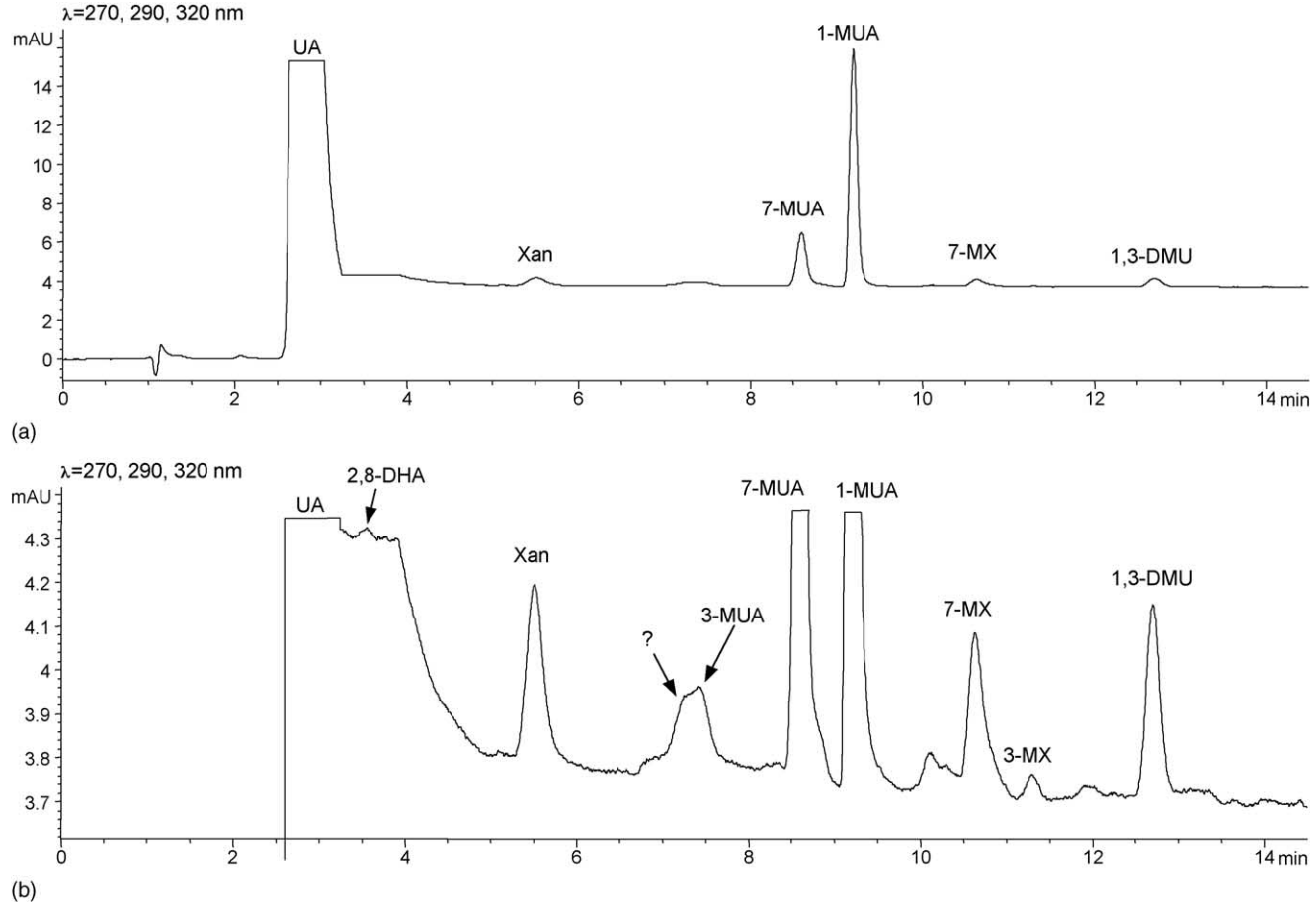

Fig. 4. (a and b) Chromatogram of a representative uric acid stone extract obtained with variable detection wavelength. The chromatogram presents the same stone extract as in Fig. 3 analysed at variable detection wavelength according to the programme (Table 2). Two absorbance scales are used because of range of peak heights. Some peaks were truncated due to spreading of the absorbance scale.

Table 3
Precision, limits of detection of the gradient HPLC method and maximal content in the examined stones for 16 analysed compounds

Compound	Precision[a] (CV%, $n = 6$, $c = 5$ mg/L)	Limits of detection at the concentration of the extract 100 mg/L[b] (mg/g of the stone)	Maximal content in the 65 analysed stone samples[c] (mg/g of the stone)
UA	1.1	0.010	907
2,8-DHA	1.5	0.025	0.12
9-MUA	1.2	0.010	Not detected
Hyp	1.3	0.011	0.40
Xan	2.1	0.017	1.20
Oxp	1.2	0.035	Not detected
3-MUA	1.4	0.015	0.50
Alp	1.7	0.026	Not detected
7-MUA	0.8	0.009	3.10
1-MUA	1.8	0.006	12.00
3,7-DMU	1.6	0.009	Not detected
7-MX	2.4	0.021	1.60
3-MX	2.1	0.018	0.40
1-MX	1.8	0.030	Not detected
1,3-DMU	0.5	0.019	Not detected
1,7-DMU	1.3	0.024	Not detected

[a] Inter-day standard deviation (CV%) of six analyses of the 16-standard mixture components.
[b] Limit of detection, S/N = 3 (S/N = peak height/standard deviation of baseline noise).
[c] Purines other than uric acid could be detected only in stones containing at least 4% UA.

2,8-DHA, Hyp, Xan, Oxp and Alp. The methyl derivatives of UA and Xan have similar physicochemical properties (e.g. pK_a [15,16]) to their parent compounds so the presented method proved to be efficient for their extraction from stones.

Final concentration of stone extracts was 100 mg/L to obtain high sensitivity of measurements. However, there is a danger of 2,8-DHA and Xan crystallisation in such concentrated solution if their content exceeds 5 and 25% of stone mass, respectively. Other purines are better soluble and should not crystallise, though 100 mg/L UA solution is close to saturation at the buffer pH value. Linearity of their calibration curves up to 100 mg/L confirms lack of crystallisation. To exclude errors caused by possible precipitation of examined compounds from 100 mg/L extract, laboratory procedure should include additional analysis of 5 mg/L extract (prepared by 100-fold dilution of the initial NaOH extract) if concentrations measured with 100 mg/L extract exceeded 5 mg/L for 2,8-DHA and 25 mg/L for Xan. There were no samples fulfilling the criteria in the examined material.

Freezing of extracts at −75 °C ensured stability of analysed compounds. Their concentrations did not change after 2-year storage.

According to our knowledge, this is the first HPLC method suitable for the analysis of 16 mentioned purines in urinary calculi. Although numerous reversed-phase chromatographic methods are used for analysis of oxypurines [11,17–21], methylated purines [18,22–24], 2,8-dihydroxyadenine [12,19,20], allopurinol and oxypurinol [11,19–21,23,25], they are not designed to separate simultaneously all the purines specific for urinary calculi. Original double gradient of pH and methanol content in mobile phase was used, because we could not achieve satisfactory separation of all analytes with constant buffer pH. Higher pH significantly shortens retention time of uric and methyluric acids. Its gradient was worked out empirically so that peaks of UA derivatives were placed between other peaks.

Thermostating of chromatographic column was necessary since even little changes in ambient temperature (1–2 °C) caused changes of retention times and deterioration of separation quality.

Sample extracts were injected twice with different detection wavelength to confirm correct identification of detected compounds and increase sensitivity of measurements because our UV detector could analyse only one wavelength at a time. The wavelength of 254 nm was optimal for detection of Hyp, Oxp and Alp, but made possible detection of all 16 purines (Fig. 1). The programme of wavelength changes in the second analysis adjusted the current wavelength to the value close to absorbance maximum of the compounds eluted from the column. For detection of Xan derivatives, $\lambda = 270$ nm was used, for UA it was $\lambda = 290$ nm. 2,8-DHA was detected at $\lambda = 320$ nm, which gave the most advantageous ratio of specific absorbance coefficients of 2,8-DHA to UA. Then, characteristic ratios of peak areas at both wavelengths were calculated and compared to standards to confirm compound identity.

It was difficult to quantify exactly trace amounts of 2,8-DHA found in stone samples. An unknown compound with spectral characteristics different from UA derivatives (denoted by question mark in Figs. 3 and 4) sometimes interfered with 3-MUA and hampered its quantitation. No other interfering peaks were observed. Application of multiple wavelength detector would make one chromatographic run sufficient to detect and confirm identity of all 16 compounds.

4.2. Methyl derivatives of uric acid and xanthine

According to our knowledge the presented method is the first one to show presence of six methylated purines in urinary stones. All of the examined uric acid calculi contained *N*-methyl derivatives of UA (1-MUA, 7-MUA, 3-MUA, 1,3-DMU) and Xan (3-MX, 7-MX). No traces of 9-MUA, 1-MX, 1,7-DMU, 3,7-DMU were detected. Methylated purines were not just contamination of stone surface coming from urine, since they were often present in larger amounts in stone nuclei than in the outer layers. The methylated purines detected in stones are excreted in urine as the products of metabolism of methylxanthines: 1,3,7-trimethylxanthine (caffeine) [14], 1,3-dimethylxanthine (theophylline) [26] and 3,7-dimethylxanthine (theobromine) [27]. These compounds are present in popular diet components: coffee and some carbonated beverages (caffeine), tea (caffeine, theophylline), cocoa and chocolate (theobromine). Absence of 9-MUA confirms that methyluric acids in stones are metabolites of methylxanthines originating from plants, since they never contain methyl group at N_9 of purine ring.

The maximal total content of methylated purines in the examined calculi was 1.7% of stone mass. This is relatively small amount, but considerably greater than the sum of endogenous purines (Hyp, Xan, 2,8-DHA), which amounted to 0.16 wt.%. Possible role of methylated purines in urinary stone formation needs further investigation.

4.3. Comparison of HPLC and other methods of urinary stone analysis

The conducted research has pointed out that HPLC is a valuable method for the analysis of various purine compounds in urinary calculi. X-ray diffraction and infrared spectroscopy allow determination of all components of the calculi. However, HPLC is much more sensitive and specific in relation to purines. X-ray diffraction is capable of detection of compounds constituting at least a few percent of stone mass, and did not allow detection of purines accompanying UA in stones [28].

The presented gradient method is superior to the isocratic HPLC method described previously [13] allowing to separate 16 (instead of 6) compounds with comparable sensitivity. It solves the earlier problem of overlapping Oxp and 7-MUA peaks. On the other hand, the presented method requires a pump with at least ternary gradient mixer and column thermostat to obtain reproducible separations; the run

time (17 min) is much longer than for the isocratic method (8 min).

The earlier fast isocratic method is simpler in terms of technique and equipment, and therefore more suitable for routine clinical analysis of urinary stones in hospital laboratories. The presented gradient separation requiring more sophisticated instrument can be used as a reference method for research purposes when the content of all known purine constituents of urinary stones, including recently reported methylated purines, must be known. An estimation of possible role of these derivatives in the pathogenesis of urolithiasis can be the objective for further studies. HPLC methods should be treated as complementary to X-ray diffraction and infrared spectroscopy for the analysis of urinary stone composition.

Acknowledgement

This work was supported by a grant (4 P05B 028 15) from the Polish Committee for Scientific Research in the years 1998–1999.

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