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Determination of purines including 2,8-dihydroxyadenine in urine using capillary electrophoresis

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Abstract

For the purpose of rapid drug monitoring, methods have been developed for the determination of 2,8-dihydroxyadenine, allopurinol, oxypurinol, adenine, hypoxanthine, hippuric acid and xanthine in urine with and without sodium dodecyl sulfate as additive in sodium tetraborate running buffer. No sample preparation is necessary. 6-methylmercaptopurine and etofylline have been used as the internal standards. The limit of detection is 5 μ*M* and the range of quantification stretches from 20 to 2000 μ*M*. The capillary electrophoresis methods are simple, fast and robust.

Keywords: Purines; Dihydroxyadenine

1. Introduction

The rare deficiency of the purine salvage enzyme adenine phosphoribosyltransferase (APRT: EC 2.4.2.7), which converts adenine into adenosine monophosphate in the presence of 5-phosphoribosyl-1-pyrophosphate, is inherited as an autosomal recessive trait [1,2]. The main clinical manifestation directly related to the metabolic defect is 2,8-dihydroxyadenine (2,8-DHA) lithiasis [1]. In patients with APRT deficiency, the accumulated adenine is converted by xanthine oxidase first into 8-hydroxyadenine and then into 2,8-DHA (Fig. 1). Another problem is the high renal clearance: 2,8-DHA is protein bound and is actively excreted into the urine [1]. Thus, a patient on our ward excretes large amounts of 2,8-DHA, which crystallises out very easily in the nephro-tubular system (solubility 15–20 μ*M* in urine [3]) causing the loss of one kidney so far. The only therapy, besides continuous and high fluid intake (up to 3 L/d) [1,4], is the lifelong use of allopurinol to block the responsible enzyme xanthine oxidase (XO) and thus to reduce the amount of 2,8-DHA excreted (Fig. 1). Because of the extended use of allopurinol the excretion of hypoxanthine and mainly xanthine increases and can lead to the formation of xanthine stones [1,2]. Therefore, the aim was to adjust the optimal dosage of allopurinol at the lowest possible excretion level of 2,8-DHA. The influences of a purine-restricted diet and non-compliance are further reasons to establish a non-invasive and long-term therapeutic drug monitoring (TDM) of 2,8-DHA, other purine end products and allopurinol in urine. For routine clinical examination of urine levels we developed a simple, robust and easy-to-use method using capillary electrophoresis (CE). The method allows direct and fast analysis

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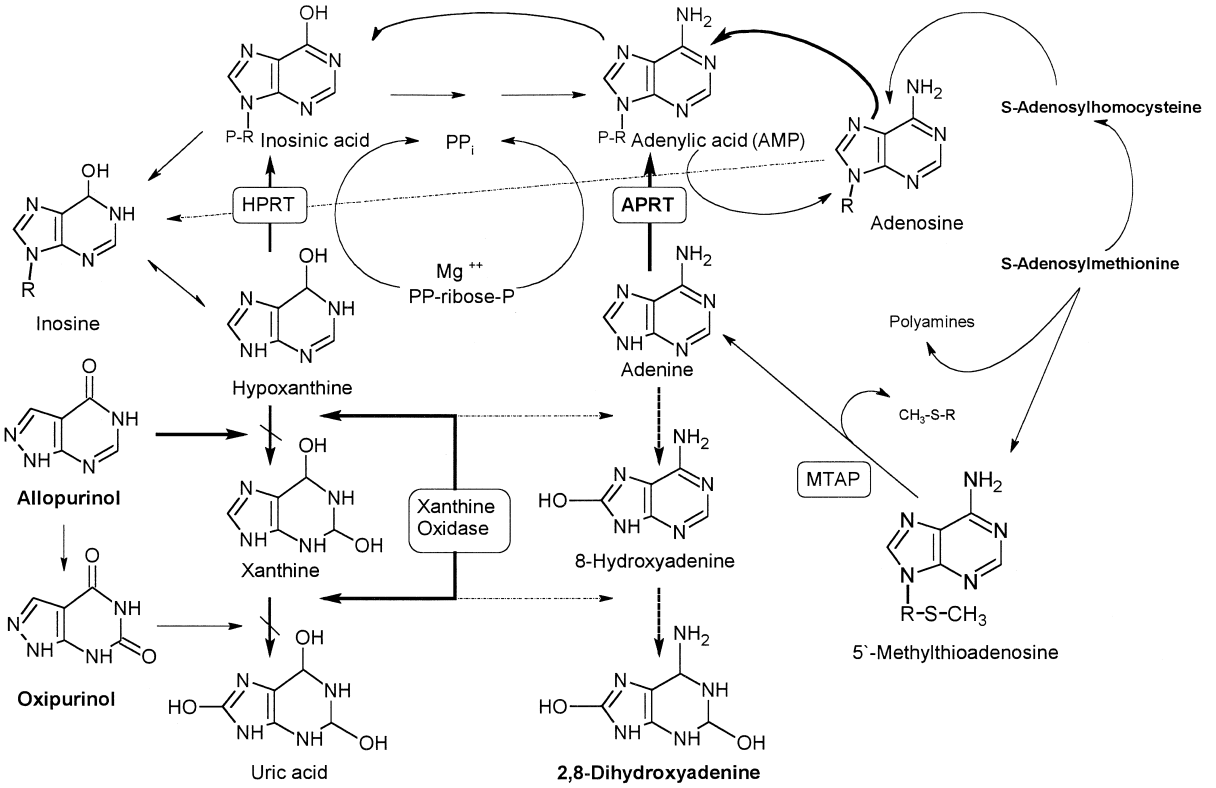

Fig. 1. Diagram showing the metabolic pathways for the formation and disposal of adenine, adenosine and hypoxanthine in humans. Adenine is normally converted by APRT into adenylic acid (AMP). In the absence of APRT it is oxidised by xanthine oxidase via the 8-hydroxy intermediate to 2,8-dihydroxyadenine (2,8-DHA). Allopurinol and its main metabolite oxypurinol block xanthine oxidase.

from urine samples with varying ionic strength. CE is a very promising technique for the determination of drugs in biological fluids. In comparison to HPLC, the method shows a higher separation power and is more insensitive to endogenous compounds from the biological matrix [5].

2. Experimental

2.1. Chemicals

2,8-DHA, allopurinol, oxypurinol, adenine, hippuric acid, hypoxanthine, xanthine, 6-methylmercaptopurine (6-MMP) and etofylline were all purchased from Sigma (St. Louis, MO, USA). Water used for all buffers and stock solutions was purified with a Milli-Q-Plus unit (Millipore, Eschborn, Germany). All chemicals used in this study were of analytical grade and commercially available.

2.2. Capillary electrophoresis

A Beckman P/ACE 5510 system (Beckman Instruments, Munich, Germany) equipped with a UV detector and a diode array detector, a 254/300 nm filter (2,8-DHA, 300 nm; all others, 254 nm) and a 37 cm (effective length 30 cm)×50 μm I.D. uncoated fused-silica capillary was used for analysis. Samples were introduced into the capillary by pressure injection with 0.5 p.s.i. (1 p.s.i.=6895 Pa) for 10 s (2,8-DHA) or 4 s (all others). The running buffer formulations were prepared as follows: (a) allopurinol, adenine and hypoxanthine, 80 m*M* sodium dodecyl sulfate (SDS) was dissolved in 60 m*M* sodium tetraborate buffer (pH 8.7); (b) hippuric acid, oxypurinol and xanthine, 60 m*M* sodium tetraborate

buffer (pH 8.7); and (c) 2,8-DHA, 60 m*M* sodium tetraborate buffer (pH 9.7). The pH was adjusted with 1 *M* NaOH or 1 *M* HCl to the desired value. All buffers were degassed with sonication and filtered through 0.45 μm pore size membrane filters (Minisart RC25, Sartorius, Göttingen, Germany) prior to use. After each run the capillary was flushed with 0.1 *M* NaOH and the electrophoresis buffer for 2 min each in a high-pressure rinse (20 p.s.i.). All electrophoresis was carried out at 20°C and the applied constant currents were 2.16 μA/cm (~480 kV/cm) (a and b) and 2.08 μA/cm (~400 kV/cm) (c).

2.3. Peak identification

Both retention time and UV spectra were used for peak identification. The UV spectra of chromatographic peaks in patient samples with retention times corresponding to authentic standards were compared with the UV spectra of standards to confirm peak identity.

2.4. Sample preparation

For the examinations we used either fresh urine samples from the same day or samples not older than 2 weeks. All samples were stored at +4°C. A special preparation procedure is not necessary and only 25 μL of a solution of the internal standard (etofylline, 7 μg/μL or 6-methylmercaptopurine, 7.2 μg/μL) has to be added to 1500 μL of the urine sample (allopurinol, oxypurinol, hippuric acid, hypoxanthine, xanthine and adenine: etofylline; 2,8-DHA: 6-methylmercaptopurine).

3. Results and discussion

3.1. Capillary electrophoresis

For the detection of the purines of interest, several HPLC methods have been described over the past decades [4,6–9]. However, for the purpose of a TDM in urine CE with its high separation efficiency, flexibility and high sample throughput can serve as an excellent tool [10]. Initially, for the routine clinical examination of urine levels of 2,8-DHA, we modified a method originally developed for the determination of 6-mercaptopurine in urine. We used the same buffer conditions of 60 μ*M* sodium tetraborate and pH 9.7 and attained a sufficient and rapid separation. It is well known that purines and pyrimidines in plasma and urine can be separated under these conditions [10,11]. In contrast to the findings of Sevcik et al. [12] we found no interference from other purines or the background analytes at this pH and the limit of quantification is 100-fold lower. This limit is necessary to control 2,8-DHA excretion of the patient under allopurinol therapy. Another possibility for the identification of 2,8-DHA is the characteristic long-wavelength absorbance maximum at 304 nm [12].

Subsequently, we quantified the other relevant substances for this individual TDM: allopurinol and its main metabolite oxypurinol, adenine, hippuric acid, hypoxanthine and xanthine. Other CE methods developed for their detection did not fit our requirements, e.g. they were not validated for urine or they showed no satisfactory baseline separation, the validated range of quantification was too small or they cannot quantify all relevant substances [10,11,13–16]. Thus, we first modified our 2,8-DHA method. An optimised separation was achieved with the same buffer at pH 8.7 and at 254 nm. To prevent peak splitting of allopurinol, as reported by Shihabi et al. [13], we added 80 μ*M* of micellar additives (SDS). However, despite intensive method variations, it was not possible to determine all relevant substances reproducibly and with full baseline separation in one run (data not shown), e.g. due to problems with different capillary charges. Because of the natural presence of several substances of interest and their high concentration in pooled urine it was necessary to dissolve the pure compounds for the standards in deionized water, with the exception of 2,8-DHA. Another important aim was to develop a method, contrary to previous methods, reproducible over a wide concentration range without dilution and with minimal sample preparation. This mark was hit with these two methods: both with 60 μ*M* sodium tetraborate buffer at pH 8.7, in the first step to resolve allopurinol by micellar electrokinetic capillary electrophoresis with SDS (MEKC), in the second step by simple capillary zone electrophoresis (CZE).

Table 1
Regression parameters for calibration curves spiked with substance standards[a]

	Slope (area) (mean)	±SD	γ-Intercept (area) (mean)	±SD	Correlation coefficient (mean)	±SD
2,8-DHA	0.00143	0.000233	0.00421	0.00521	0.9997	0.00037
Allopurinol	0.00619	0.000116	0.00125	0.01814	0.9984	0.00083
Oxypurinol	0.00959	0.000105	0.00581	0.00716	0.9990	0.00064
Adenine	0.00955	0.000233	0.01099	0.00705	0.9966	0.00071
Hippuric acid	0.00269	0.000069	0.00254	0.00432	0.9995	0.00010
Hypoxanthine	0.00591	0.000134	0.00261	0.00932	0.9993	0.00015
Xanthine	0.00493	0.000123	0.00388	0.00335	0.9999	0.00008

[a] Calibration curves were obtained from four consecutive runs performed over several weeks.

3.2. Reproducibility of the assay

3.2.1. Linearity

Table 1 shows calibration curves obtained from four consecutive runs performed over a period of several weeks. For the calibration curves, aqueous solutions (except for 2,8-DHA: urine) were spiked with six different concentrations and 38 μ*M* MMP, respectively 31 μ*M* etofylline of the internal standard (I.S.). A linear correlation was found between the peak-area ratios of all substances and the I.S. and the concentrations in the range: 2,8-DHA, 40–1100 μ*M*; allopurinol, 24–950 μ*M*; oxypurinol, 36–1800 μ*M*; adenine, 22–950 μ*M*; hippuric acid, 16–1800 μ*M*; hypoxanthine, 18–950 μ*M*; xanthine, 13–1500 μ*M*. Without the I.S. the linearity was not sufficient ($r \leq 0.99$) due to deviation, e.g. from the injection onto the capillary. Additionally, Table 2 shows the calibrations factors of the four runs.

For all calculations the internal standard method with a weighted regression of the corrected peak areas was used.

3.2.2. Precision and accuracy

The intra-day ($n = 6$) and inter-day ($n = 6$) precision and accuracy of the assay are summarised in Tables 3 and 4. RSD for intra- and inter-day was $<14\%$ for all of the compounds [except allopurinol at the limit of quantitation (LOQ): 16.7%] and the accuracy ranged from 87 to 113% at known low and high concentrations of all seven analytes.

The precision and accuracy should always be within ±15% for bioanalytical methods except at the lower limit of quantification, where they should not deviate by more than 20% [17]. Variations in the migration times are a common problem in CE. As pointed out by Thormann et al. [5], detection times are unreliable parameters for the identification of peaks because different matrices lead to different detection times. The relative standard deviations of the migration times from day to day are shown in Table 5. Due to matrix effects and different charges of capillaries, deviations of up to 5% are common. However, using the relative migration times calculated from the I.S. and comparing the substances with UV spectra, identification of the substance peaks is possible.

Table 2
Regression parameters for calibration curves spiked with substance standards

	Conc. added (μmol/L)	Correlation factor (mean)	±SD	RSD (%)
2,8-DHA[a]	1031	1.244	0.053	4.3
	40	0.055	0.010	18.7
Allopurinol	954	6.218	0.180	2.9
	24	0.140	0.018	13.1
Oxypurinol	1795	10.900	0.149	1.4
	36	0.217	0.015	6.8
Adenine	956	9.529	0.173	1.8
	22	0.198	0.008	4.2
Hippuric acid	1885	5.179	0.081	1.6
	16	0.045	0.005	11.7
Hypoxanthine	923	9.292	0.206	2.2
	18	0.187	0.004	1.9
Xanthine	1507	7.491	0.106	1.4
	13	0.066	0.004	6.3

[a] Pooled urine.

Table 3
Precision and accuracy of standards in pooled urine and aqueous solutions spiked with known concentrations within 1 day ($n = 6$) (the lower concentration for each analyte represents the limit of quantification)

	Conc. added (μmol/L)	Conc. found (μmol/L)	Accuracy (%)	RSD (%)	*n*
2,8-DHA[a]	1118	1065	95.3	3.8	6
	44	50	113.6	4.5	6
Allopurinol	954	998	104.6	1.6	6
	24	27	112.5	16.7	6
Oxypurinol	1795	1885	104.2	2.5	6
	36	35	97.2	2.9	6
Adenine	956	996	104.2	2.7	6
	22	25	113.6	4.0	6
Hippuric acid	1885	1951	103.5	1.7	6
	16	14	87.5	7.1	6
Hypoxanthine	923	899	97.4	1.6	6
	18	21	116.7	4.8	6
Xanthine	1507	1548	102.7	3.0	6
	13	13	100.3	7.7	6

[a] Pooled urine.

3.2.3. Sensitivity

The limit of detection, defined as the concentration where the signal-to-noise ratio is 5, was found to be 5 μ*M* for all substances. The LOQ, defined as the lowest concentration which can be measured with acceptable precision and accuracy, was determined to be: 2,8-DHA 24 μ*M*, allopurinol 40 μ*M*, oxypurinol 36 μ*M*, adenine 22 μ*M*, hippuric acid 16 μ*M*, hypoxanthine 18 μ*M* and xanthine 13 μ*M*.

3.3. Application to patient urine samples

Fig. 2 shows a representative electropherogram of 2,8-DHA in a urine sample of a patient from the beginning of drug monitoring (June 1998). No compounds were observed to interfere with the analytes. Figs. 3 and 4 show electropherograms of the other substances from 24 h urine sampling (May 1999). Up to now, over a period of 1.5 years, 120

Table 4
Precision and accuracy of standards in pooled urine and aqueous solutions spiked with known concentrations on different days ($n = 6$)

	Conc. added (μmol/L)	Conc. found (μmol/L)	Accuracy (%)	RSD (%)	*n*
2,8-DHA[a]	1031	1034	100.3	2.7	6
	40	43	107.5	6.0	6
Allopurinol	954	991	103.9	2.4	6
	24	22	91.7	13.8	6
Oxypurinol	1795	1908	106.3	1.7	6
	36	37	102.8	6.1	6
Adenine	956	987	103.3	1.9	6
	22	21	95.5	9.7	6
Hippuric acid	1885	1983	105.2	1.2	6
	16	16	100.5	12.9	6
Hypoxanthine	923	934	101.2	2.5	6
	18	19	105.6	5.2	6
Xanthine	1507	1573	104.4	1.4	6
	13	14	107.7	11.9	6

[a] Pooled urine.

Table 5
Migration times on different days ($n = 6$)

	Retention time (min)	RSD (%)	n
2,8-DHA	10.34	5.67	18
I.S.[a]	9.69	6.06	18
Allopurinol	3.25	3.15	29
I.S.[b]	4.28	3.73	29
Oxypurinol	6.39	4.48	30
I.S.[b]	5.20	3.12	30
Adenine	3.35	3.30	29
I.S.[b]	4.28	3.73	29
Hippuric acid	6.08	0.26	30
I.S.[b]	5.20	3.12	30
Hypoxanthine	3.55	3.59	29
I.S.[b]	4.28	3.73	29
Xanthine	6.72	4.37	30
I.S.[b]	5.20	3.12	30

[a] 6-methylmercaptopurine (MMP).
[b] etofylline.

patient samples from eight 24 h collecting days and several samples from relatives have been analysed. The therapeutic target has been achieved to reduce 2,8-DHA production to very low levels. Intensified treatment with allopurinol produced a dramatic drop in 2,8-DHA excretion. Nocturnal maxima were removed by a shift of the last daily allopurinol dose to midnight. An attempt to achieve a urinary volume near 3 L/24 h prevented supersaturation. The next step is to determine the lowest possible allopurinol dose to prevent nephrotoxicity.

4. Conclusion

Three simple, robust and easy-to-use CE methods for the determination of 2,8-DHA, allopurinol and its main metabolite oxypurinol, adenine, hippuric acid, hypoxanthine and xanthine in urine have been developed. The precision and accuracy are sufficient and urine concentrations can be monitored over a

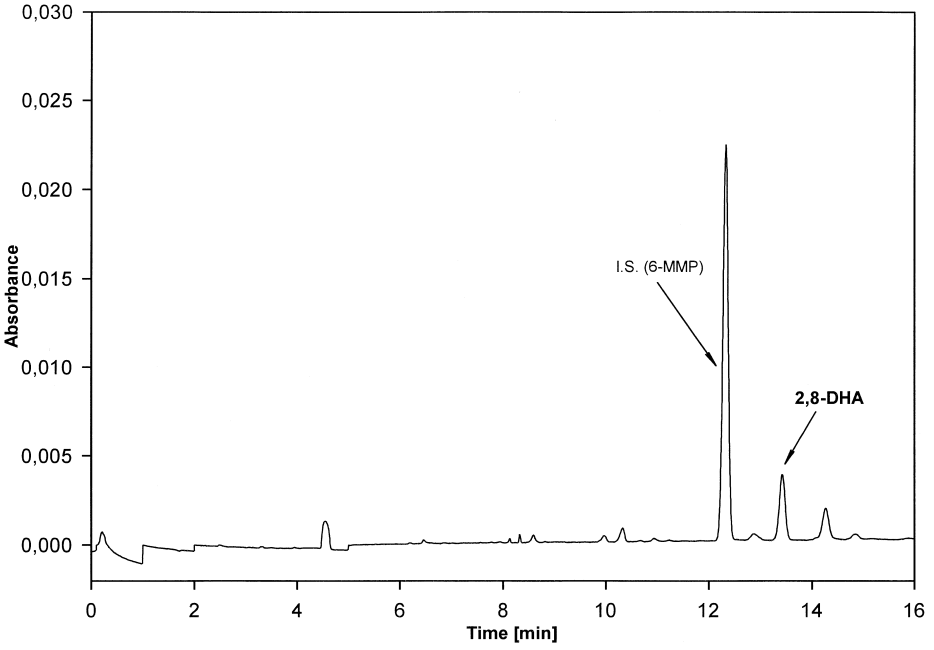

Fig. 2. Electropherogram of a urine sample (24 h sampling of 26 June 1998): capillary length 37 cm (effective length 30 cm), buffer pH 9.7, 77 μA; concentration 6-methylmercaptopurine (6-MMP) 38 μ*M*, 2,8-DHA 481 μ*M*.

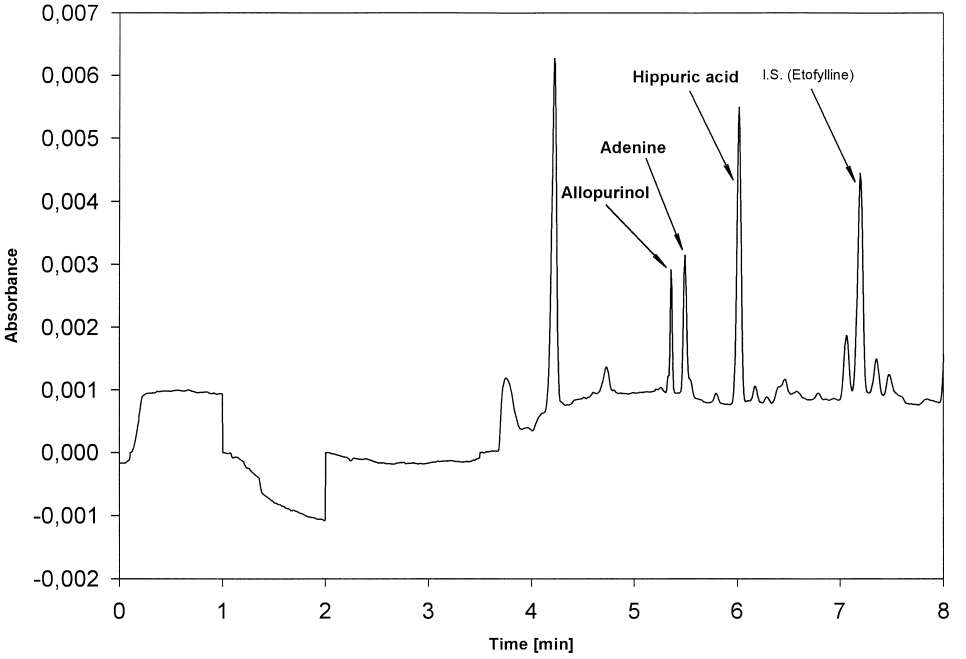

Fig. 3. Electropherogram of a urine sample (24 h sampling from 24 May 1999): capillary length 37 cm (effective length 30 cm), buffer pH 8.7, 80 m*M* SDS, 80 μA; concentration etofylline 31 μ*M*, allopurinol 54 μ*M*, adenine 72 μ*M*, hippuric acid 141 μ*M*.

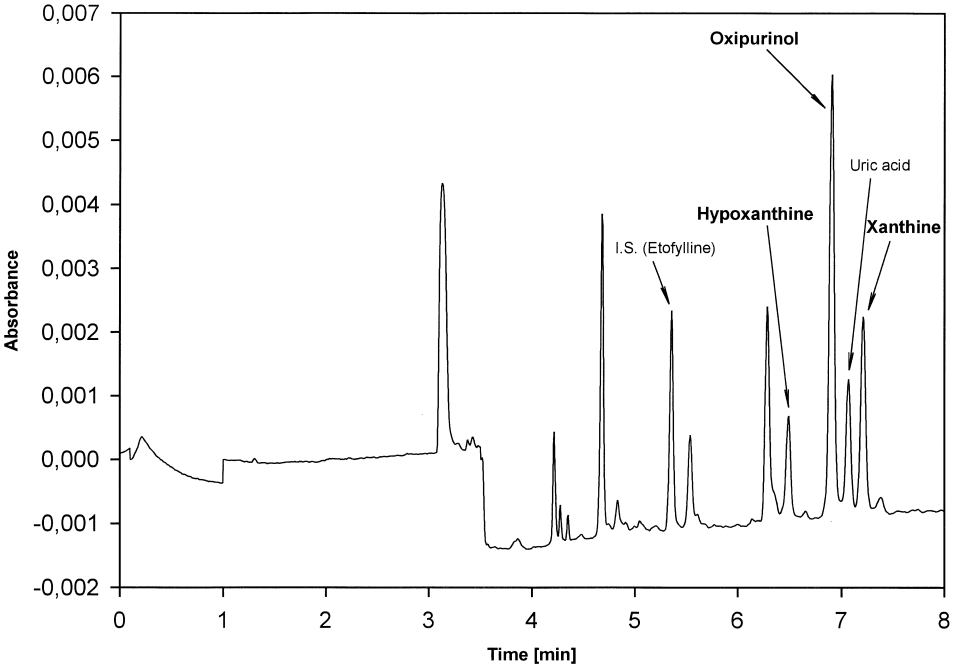

Fig. 4. Electropherogram of a urine sample (24 h sampling from 24 May 1999): capillary length 37 cm (effective length 30 cm), buffer pH 8.7, 80 μA; concentration etofylline 31 μ*M*, hypoxanthine 135 μ*M*, oxypurinol 379 μ*M*, xanthine 209 μ*M*.

wide range (20–2000 μM). Sample preparation or dilution is not necessary and the analysis of all analytes can be done within 30 min. The limit of detection is ~5 μM and is satisfactory for the purposes of this study. The methods have been applied to the drug monitoring of one patient with DHA lithiasis for 1.5 years.

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