Practice research in the field of gout - clinical pharmacology of antihyperuricemic drugs

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Publication date:
2008

Chapter 4

Therapeutic drug monitoring of allopurinol treatment

Chapter 4.1

A simple method for quantification of allopurinol and oxipurinol in human serum by high-performance liquid chromatography with UV-detection

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Abstract

Objectives

Allopurinol is an antihyperuricemic drug used in the treatment of gout and the prevention of tumor lysis syndrome. Allopurinol and its active metabolite oxipurinol inhibit xanthine oxidase, which forms uric acid from xanthine and hypoxanthine. Therapeutic drug monitoring is an important option for evaluation and optimisation of allopurinol treatment in case of renal impairment, interaction with uricosuric drugs or to verify patient adherence. In this study we developed and validated a simple quantitative assay using reversed-phase high-performance liquid chromatography (HPLC) with UV-detection as a method for quantification of allopurinol and oxipurinol in human serum in the presence of different frequently used drugs.

Methods

The HPLC-UV method uses a mobile phase consisting of sodium acetate (0.02 M; pH 4.5), at a flow rate of 1.0 ml/min. Allopurinol and oxipurinol are detected by UV-absorption at 254 nm with a retention time of 9.9 min for oxipurinol and 12.3 min for allopurinol. Aciclovir is used as internal standard.

Results

Validation showed for allopurinol lower and upper limits of quantification of 0.5 and 10 mg/l and for oxipurinol 1 and 40 mg/l, respectively. The assay was linear over the concentration range of 0.5-10 mg/l (allopurinol) and 1-40 mg/l (oxipurinol). Intra- and inter-day precision showed coefficients of variation <15% over the complete concentration range; accuracy was within 5% for allopurinol and oxipurinol. Endogenous purine-like compounds were separated from allopurinol, oxipurinol and aciclovir with a resolution factor >1.5. Exogenous purine-like compounds and co-medication frequently used by gout patients did not hinder the analysis due to the dichloromethane washing step or to low UV-absorpion at 253 nm. Serum levels of 66 patients prescribed allopurinol 300 mg/day were determined using this HPLC-UV method. Measured serum allopurinol and oxipurinol concentrations in clinical practice showed large variability with a range of <0.5-4.3 mg/l for allopurinol and <1.0-39.2 mg/l for oxipurinol, respectively.

Conclusion

We developed an easy-to-operate and validated HPLC-UV method for the quantification of allopurinol and oxipurinol in human serum. This method was proven valid for samples of gout patients frequently using concomitant medications.

Introduction

Allopurinol is worldwide the mainstay of modern treatment of gout and prevention of tumor lysis syndrome. Allopurinol - an isomer of hypoxanthine - and its active metabolite oxipurinol (alloxanthine) act by inhibiting xanthine oxidase, an enzyme that forms uric acid (urate) from xanthine and hypoxanthine.

Allopurinol can be administered either orally or intravenously. The oral bioavailability is about 67% to 90% with a peak plasma concentration occurring within one hour; the volume of distribution is approximately 1.6 l/kg [1]. Allopurinol is principally metabolised by aldehyde oxidase to the active compound oxipurinol [2]. The peak plasma concentration of oxipurinol occurs within 3-5 h. Mean elimination plasma half lives range from 0.7 to 1.5 h for allopurinol and from 18 to 40 h for oxipurinol [1].

Allopurinol is excreted in urine for less than 10% unchanged and for 70% as oxipurinol; 20% is excreted in feces. In patients with renal impairment (creatinine clearance <80 ml/min [3]), the maintenance dosage of allopurinol must be reduced to prevent toxic effects related to increased oxipurinol serum levels [3-4]. When renal impairment is present, the initial allopurinol dosage can be calculated based on the estimated creatinine clearance (Table 1) [3]. Optimisation of individual allopurinol dosage can be done by targeting of the oxipurinol - steady state - serum concentrations [5-9] as advised in the product information of allopurinol [5]. Reference serum oxipurinol values that are considered therapeutic, range from 5 to 15 mg/l [9].

The renal excretion of oxipurinol is increased by co-administration of uricosuric drugs (e.g. probenecid and benzbromarone) that are also used to decrease serum urate levels, presumably by interaction at the URAT-1 transporter [10-11]. Combination of these drugs with allopurinol is frequently used in patients with severe gout, although, optimisation of allopurinol dosage by measuring oxipurinol serum levels might be necessary. Another indication for therapeutic drug monitoring (TDM) is to verify a patient's adherence to the use of allopurinol, which in general is reported to be a point of concern [12-13].

Several methods are described for the analysis of allopurinol and oxipurinol in human serum. However, these published methods, using reversed-phase high-performance liquid chromatography, might have several limitations [14-22]. For example, (1) lack of information on chromatographic interference on detection and quantification of the analytes by concomitant medications frequently used by gout patients; (2) upper limits of quantification not covering the complete concentration range as observed in clinical practice; and (3) absence of stability data of allopurinol and oxipurinol in serum kept under refrigerated conditions.

The objective of the present study was to develop and validate a new analytical method that enables measurement of allopurinol and oxipurinol in representative serum samples obtained from daily clinical practice.

Table 1. Maintenance dosage of allopurinol (for adults) based on individual creatinine clearance measurements [3, 8]

Creatinine clearance (ml/min)	Maintenance dosage of allopurinol
0-20	100 mg daily
20-60	200 mg daily
60-100	300 mg daily

Methods

Equipment

The chromatographic system consisted of a Merck-Hitachi L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Series 200 autosampler (Perkin-Elmer, Wellesley, MA, USA), and a Spectroflow 757 variable absorbance detector (Kratos Analytical, Manchester, UK). Isocratic chromatographic separation was performed on a reversed-phase LiChrospher 100 RP-18 column (5 µm; 250×4 mm; Merck, Darmstadt, Germany) connected to a precolumn (LiChroCart Guard column 4-4 packed with Lichrospher 100 RP-18, 5 µm, 15 mm; Merck, Darmstadt, Germany). The column temperature was maintained at 32.5 °C (Column heater 530; Alltech, Breda, The Netherlands).

The mobile phase consisted of a 0.02 M sodium acetate solution adjusted with acetic acid 30% to pH 4.5. All samples and standard solutions were chromatographed using this mobile phase (flow rate 1.0 ml/min), an injection volume of 40 µl, and UV-detection at 254 nm. Time between injections was 25 min.

Data from each chromatographic run were processed using TotalChrom version 6.2 (Perkin-Elmer). Concentrations were calculated from the peak height ratios in relation to the internal standard.

Preparation of the mobile phase

The mobile phase was prepared by dissolving 2.72 g $NaCH_3COO \cdot 3H_2O$ in 1,000 ml distilled water (Versol Sterile water for irrigation, Laboratoire Aguettant, Lyon, France) and correcting the pH to 4.5 with acetic acid 30%. The mobile phase was filtered and degassed before use.

Preparation of standard solutions and samples

Stock standard solution of allopurinol (0.25 mg/ml) and oxipurinol (0.25 mg/ml) were prepared in distilled water. For dissolution, 1% (v/v) 0.1 M sodium hydroxide was added. Aciclovir was dissolved in distilled water (0.1 mg/ml) and used as an internal standard.

Stock solutions, stability samples and patient samples were stored at –20 °C for 5 months.

Patient serum samples were prepared by adding aciclovir solution (10 µl) as an internal standard to serum (100 µl) followed by mixing with 10% perchloric acid solution (50 µl).

After cooling in the refrigerator for 10 min, dichloromethane (200 µl) was added. After shaking the mixture for 30 s, it was centrifuged at 4,000 rpm for 5 min. The aqueous supernatant solution was analysed with HPLC-UV.

Quality control standards and samples for stability testing were prepared by spiking blank pooled human serum with stock standard solutions and further processed as patient serum samples.

Method validation

We used a method validation program based on the criteria developed by the European Agency for the Evaluation of Medical Products [23]. The limit of detection (LOD) was calculated using $S/N = 3$ and the lower limit of quantification (LLOQ) was calculated using $S/N = 10$ ($S/N =$ signal-to-noise ratio). Peak height was used for quantification. Linearity was examined over the complete concentration range of allopurinol (0.5-10 mg/l) and oxipurinol (1-40 mg/l) by testing nine different concentrations in duplo. Linearity was analysed using the lack of fit test and the goodness of fit test.

Selectivity was examined by studying the possible interference of endogenous peaks and various co-medications with the determination of allopurinol, oxipurinol and the internal standard aciclovir. Acceptance criterion for lack of interference was a resolution factor (Rs) between the peaks of allopurinol, oxipurinol and internal standard and the peak of each potentially interfering substance greater than 1.5. Substances were evaluated by preparing test solutions. Drugs and endogenous substances studied were non-steroidal anti-inflammatory drugs (acetylsalicylic acid, diclofenac, etoricoxib, ibuprofen, indomethacine, naproxen, piroxicam, propyphenazone, salicylic acid), cardiovascular drugs (bisoprolol, furosemide, hydrochlorothiazide, lisinopril, losartan, metoprolol), endogenous purines and xanthines test-solution (adenine, adenosine, creatinine, hippuric acid, hypoxanthine, guanine, guanosine, inosine, orotate, pseudo-uridine, thymine, uracil, urate, xanthine, xanthosine), xanthine derivatives (caffeine, paraxanthine, theobromine, theofylline), acetaminophen, colchicine, metformine, omeprazole and simva-statine.

The intra-day reproducibility was examined by analyzing four independent preparations of each standard concentration, each injected five times, on the same day. Inter-day reproducibility was examined by analyzing four independent preparations of each concentration, each injected five times, on three different days within a period of two weeks. For each day, freshly prepared standard solutions were made. All standard solutions were prepared from an independent standard stock solution. The calibration curve used for calculating patient samples was the calibration curve determined from all data of the four separate repetitions of intra-day reproducibility testing. These data were fitted using least sum of squares analysis. Criteria for acceptance for each separate calibration curve were a regression coefficient of >0.99 and a back-calculated concentration within 15% of the respective target concentration.

Stability testing was based on the guidance of the Food and Drug Administration [24]. Stability of allopurinol 0.5 and 8 mg/l and oxipurinol 1 and 30 mg/l in human serum was studied by repeated, duplicate analysis. The solution was divided into 3 ml aliquots and kept frozen at –20 °C until the moment of analysis. Samples were analysed at week 0, 1, 2, 4, 10 and 22. Furthermore, stability after two freeze-thaw cycles and after 24 h storage at room temperature was tested. Also, the stability of stock solutions kept frozen at –20 °C for 5 months was verified.

Chemicals

Dichloromethane and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Allopurinol was purchased from Bufa (Uitgeest, The Netherlands) and oxipurinol from Sigma-Aldrich (St. Louis, USA). Aciclovir was purchased from Mayne Pharma (Melbourne, Australia). Perchloric acid was purchased from Mallinckrodt Baker (Phillipsburg, USA).
Human serum was purchased from a pool of anonymous and unpaid, healthy volunteers.

Patient samples

All consecutive patients visiting our outpatient department of rheumatology from June 2005 to June 2006 were asked to participate in a study for evaluation of allopurinol 300 mg/day (ISRCTN21473387, http://www.controlled-trials.com). After eight weeks of treatment a single venous blood sample in anticoagulant free containers was taken for determination of allopurinol and oxipurinol concentrations. After sampling, blood was directly centrifuged and serum was kept frozen at –20 °C. Directly prior to analysis, samples were processed as described above.
In case of serum concentrations of allopurinol or oxipurinol above the upper limit of quantification, samples were diluted 1:1 with blank human serum and reanalysed. All patient samples were injected twice, comparable to standard and control samples.
The Medical Ethical Committee of both hospitals approved the study.

Results

Method validation

With the described method, Rs >1.5 of allopurinol, oxipurinol and the internal standard (aciclovir) was achieved (Figure 1). The retention times of oxipurinol, allopurinol and aciclovir are 9.9, 12.3 and 17.7 min, respectively. Chromatographic performance depended on column temperature. Because no cooling device and air conditioning was available, we used a column heater and evaluated column temperatures 30-40 °C. Best results were obtained with a column temperature of 32.5 °C. Recovery after deproteinising and washing with dichloromethane was estimated at 65% for allopurinol and 75% for oxipurinol.
Chromatogram of blank human pooled serum sample is displayed in Figure 1a. Testing endogenous purine- and xanthine-like compounds showed several peaks (which were not further identified); important peaks for validation eluted at 8.9 min, 11.8 min and 19.4 min.

Exogenous purine- and xanthine-like compounds and co-medication frequently used by gout patients did not show any peaks (due to the dichloromethane washing step or low UV-absorpion at 253 nm).

The LOD was calculated as 0.1 mg/l (4 ng) for allopurinol and 0.2 mg/l (8 ng) for oxipurinol and the LLOQ was calculated as 0.4 mg/l (16 ng) for allopurinol and 0.6 mg/l (24 ng) for oxipurinol. The calibration curve for allopurinol and oxipurinol was linear over the full concentration ranges of allopurinol 0.5-10 mg/l and oxipurinol 1.0-40 mg/l.

Table 2 shows the results of the intra-day and inter-day reproducibility, respectively. Coefficients of variation and accuracy for intra-day and inter-day reproducibility are within 15% over the concentration ranges of allopurinol 0.5-10 mg/l and oxipurinol 1.0-40 mg/l. Based on these results, using this method the lower and upper limits of quantification of allopurinol are 0.5 and 10 mg/l, respectively, and the lower and upper limits of quantification of oxipurinol are 1.0 and 40 mg/l, respectively.

Results from the assays for studying long-term stability, freeze-and-thaw stability and autosampler stability of allopurinol and oxipurinol are shown in Table 3. Samples show acceptable long-term stability for at least 5 months, with differences between the baseline concentration and concentration at follow-up <20% for the samples at the lower limit of quantification and <15% for samples at the higher limit of quantification. Good stability of allopurinol and oxipurinol was found after two freeze-thaw cycles and after 24 h storage at room temperature. Furthermore, good stability of stock solutions was found for at least five months (data not shown).

Table 2. Intra- en inter-day reproducibility for assay of allopurinol and oxipurinol in human serum by high-performance liquid chromatography

Drug	Spiked (mg/l)	Intra-day [1]			Inter-day [2]		
		Measured [3] (mg/l)	CV (%)	Accuracy [4] (%)	Measured [3] (mg/l)	CV (%)	Accuracy [4] (%)
Allopurinol	0.52	0.53 ± 0.01	2.1	1.4	0.53 ± 0.05	10.1	2.4
	2.6	2.53 ± 0.11	4.2	–2.2	2.49 ± 0.10	3.9	–3.7
	7.8	7.96 ± 0.11	1.3	2.5	7.74 ± 0.22	2.9	–0.4
Oxipurinol	1.0	1.02 ± 0.04	3.6	0.3	1.07 ± 0.07	6.6	4.7
	10.2	10.2 ± 0.14	1.4	–0.1	10.2 ± 0.28	2.7	–0.4
	30.7	31.3 ± 0.32	1.0	1.8	30.5 ± 1.2	3.8	–0.6

Legend: CV = coefficient of variation; [1] five repetitions each; [2] three separate days, five repetitions each day; [3] mean ± standard deviation; [4] relative deviation (%).

Figure 1. Chromatograms of allopurinol and oxipurinol analysis in human serum
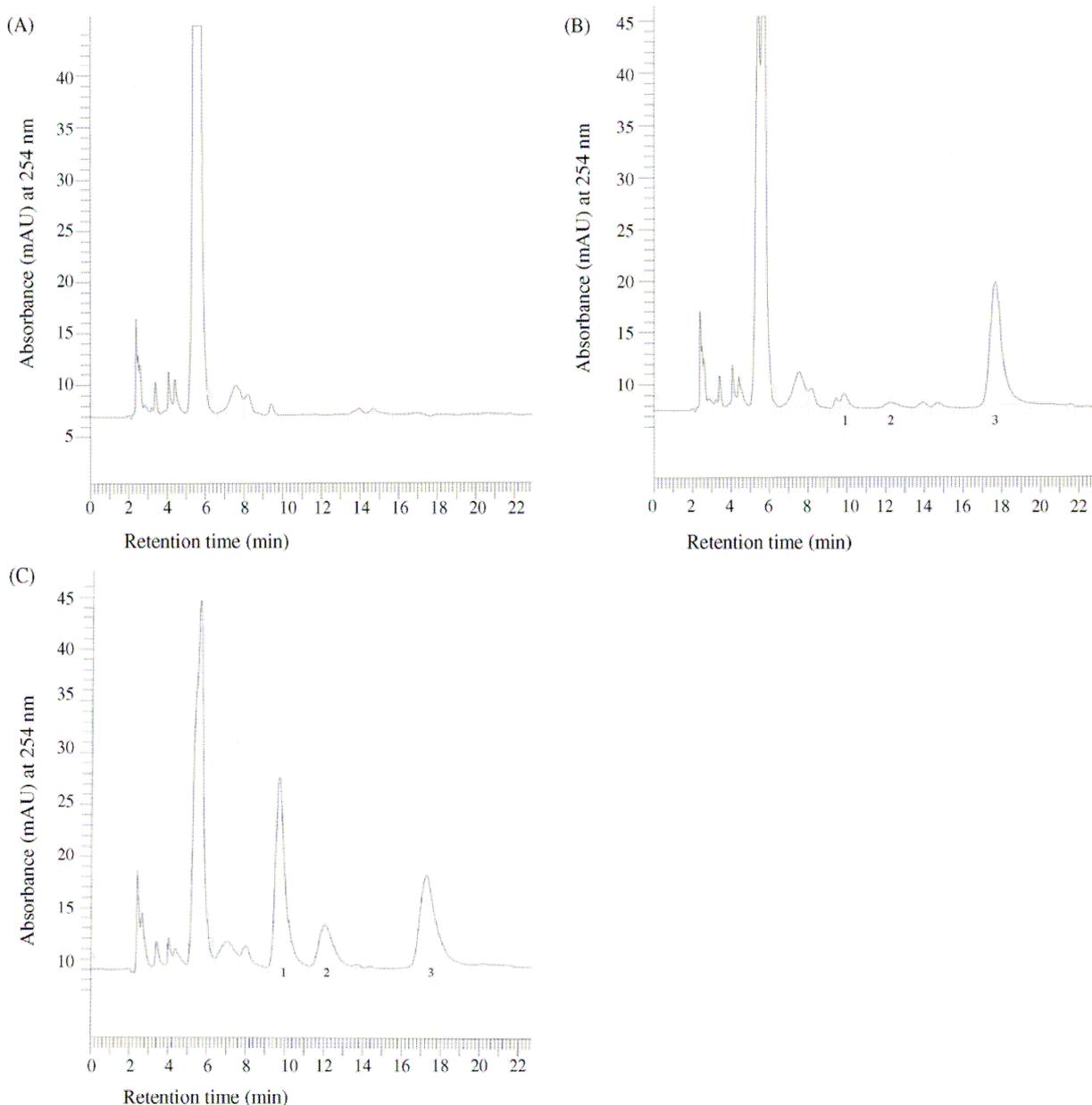
Retention times are 9.9 min for oxipurinol (1), 12.3 min for allopurinol (2), and 17.7 min for aciclovir (3; internal standard).
(A) Blank pooled human serum; (B) allopurinol 0.5 mg/l and oxipurinol 1.0 mg/l; (C) allopurinol 4.0 mg/l and oxipurinol 20 mg/l.

Patient samples

Sixty-six blood samples were taken for determination of allopurinol and oxipurinol concentrations. Patient characteristics are given in Table 4. Measured serum concentrations show large variability, ranging from <0.5 to 4.3 mg/l for allopurinol and from <1.0 to 39.2 mg/l for oxipurinol. Median concentrations are <0.5 mg/l for allopurinol and 11.5 mg/l for oxipurinol (43 patients with allopurinol serum concentration <0.5 mg/l).

Table 3. Stability results of allopurinol and oxipurinol in human serum. Test samples were measured in 2-fold; reference samples were measured in 5-fold. [1]

		Allopurinol		Oxipurinol	
Long-term stability (–20 °C)					
reference (n=5)	mean	0.52 mg/l	7.89 mg/l	0.98 mg/l	30.7 mg/l
1 week (n=2)	deviation	–2.4%	–6.4%	–0.7%	–2.9%
2 weeks (n=2)	deviation	–15.1%	–1.7%	–4.9%	0.6%
4 weeks (n=2)	deviation	–0.8%	–1.9%	5.8%	–1.9%
10 weeks (n=2)	deviation	–4.6%	–1.1%	7.4%	–0.1%
22 weeks (n=2)	deviation	13.9%	–5.4%	1.1%	0.6%
Freeze-and-thaw stability					
reference (n=4)	mean	0.58 mg/l	8.52 mg/l	1.09 mg/l	31.3 mg/l
after 2 cycles (n=4)	deviation	–1.3%	–2.6%	–0.0%	–1.4%
	CV	2.9%	1.6%	2.0%	1.5%
Autosampler stability					
reference (n=5)	mean	2.87 mg/l		10.6 mg/l	
24 h (n=5)	deviation	0.0%		2.2%	
	CV	3.0%		1.4%	

Legend: CV= coefficient of variation.

[1] Stability results stated as relative deviation from reference (%);

Table 4. Characteristics of patients included for determination of allopurinol and oxipurinol concentrations. [1]

Characteristics		TDM population
Number of patients		66
Age (y)	mean [± SD]	58 [± 13]
Male gender (%)		91%
Body mass index (kg/m^2)	mean	29 [± 4.7]
	range	20.3-48.9
Calculated creatinine clearance [1]	50-80 ml/min	30%
	>80 ml/min	70%
Prescribed daily allopurinol dose (mg)		300

Legend: SD = standard devation.

[1] Calculated by Cockcroft and Gault formula.

Discussion

Our HPLC-UV method for the quantification of allopurinol and oxipurinol in human serum samples is easy-to-operate, valid, and advantageous over other methods described in literature. Our method shows acceptable intra- and inter-day accuracy and precision over the allopurinol concentration range 0.5-10 mg/l and oxipurinol concentration range 1-40 mg/l. We did not investigate concentrations allopurinol >10 mg/l and oxipurinol >40 mg/l, because we considered it not clinically relevant. Because allopurinol, and to a lesser extent oxipurinol, are soluble in dichloromethane, no complete recovery was found after deproteinising and washing. Endogenous serum peaks and concomitant medication frequently used by gout patients, did not interfere with the detection and quantification of allopurinol and oxipurinol in the current HPLC-UV method. The method is not appicable to patients using aciclovir or valaciclovir due to the choice of internal standard.

Short-term stability of allopurinol and oxipurinol in human serum kept frozen at –20 ℃ is shown for at least 5 months until the moment of analysis, which enables collection of samples for periodical analysis in clinical practice.

Hyperuricemia is frequently accompanied by cardiovascular risk factors and the metabolic syndrome [25], which results in hypertension, hyperglycemia and dyslipidemia. Therefore, gout patients often have multiple drug use. Prior research had several major limitations regarding (1) information on interference of detection and quantification of allopurinol and oxipurinol by endogenous compounds and frequently used co-medication by gout patients; (2) an upper limit of quantification of oxipurinol that did not cover the complete concentration range expected to be obtained in patient samples; (3) information about the stability of the analyte [14-22]. In addition, we used a washing step with dichloromethane to extract relatively hydrophobic substances, thereby reducing the amount of possible interferences and late-eluting substances (e.g. caffeine).

Looking at the blank plasma chromatogram (Figure 1a), several peaks elute in the front. We did not identify these peaks individually, because we used a combined purine test-solution. However, giving these and previous results [16, 21], the front peaks might include uric acid, hypoxanthine and xanthine, which can be of interest when a method for biochemical or metabolic purpose is needed.

The samples injected are very acidic (pH 0.5). Previously, it was suggested to use ammonium sulfate for precipitation instead of a strong acid solution (perchloric acid as in our method or trichloroacetic acid as described by Kramer *et al.* [22]), because itwas found that the low pH of the injected samples could shorten column life [26]. Recently, Tada *et al.* did not find any column deterioration using acidic samples and a buffered mobile phase (100 mM potassium phosphate; pH 4.0) [14]. Using a buffered mobile phase, we injected more than 300 samples as described in the methods section. We did not experience any deterioration in the performance of analyzing allopurinol and oxipurinol (nor in the routine analysis of flecainide using the same column).

We used and validated this method with flow of 1.0 ml/min resulting in a runtime of 24 min, which was rapid enough for our purpose. When a more rapid method is preferred, a method might be set up with a flow of 2 ml/min, as described previously [14].

To study the applicability of the validated method in clinical practice, a series of patient serum samples were analysed for allopurinol and oxipurinol concentrations. The serum concentrations found are characterised by large inter-individual variation. This might be explained by differences in therapy adherence, sampling time compared to the last drug intake, variations in renal function and variations in body composition. The upper limit of quantification of our method covers all of the allopurinol and oxipurinol concentrations found in the patient samples in our study. The LLOQ of allopurinol and oxipurinol was based on the detection limit of our method. The LLOQ of oxipurinol is sufficient for use in clinical practice with regards to the therapeutic range (results below the lower limit of quantification questions patient's adherence to allopurinol). Because of the short elimination half-life of allopurinol of 0.5-2 h, serum trough concentrations found for allopurinol are predominantly below the LLOQ. However, since peak serum levels of allopurinol range from 1-5 mg/l, measuring serum allopurinol can provide additional information about the dosing-sampling interval and consequently puts the measured serum oxipurinol concentration in perspective.

Conclusion

We developed an easy-to-operate and validated HPLC-UV method for the quantification of allopurinol and oxipurinol in human serum for use in clinical practice. The method was shown to be employable for the assay of samples of gout patients frequently using concomitant medications.

Acknowledgements

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