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Measurement of urinary oxypurinol by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Oxypurinol is the active metabolite of allopurinol which is used to treat hyperuricaemia associated with gout. Both oxypurinol and allopurinol inhibit xanthine oxidase which forms uric acid from xanthine and hypoxanthine. Plasma oxypurinol concentrations vary substantially between individuals and the source of this variability remains unclear. The aim of this study was to develop an HPLC-tandem mass spectrometry method to measure oxypurinol in urine to facilitate the study of the renal elimination of oxypurinol in patients with gout. Urine samples (50 μL) were prepared by dilution with a solution of acetonitrile/methanol/water (95/2/3, v/v; 2 mL) that contained the internal standard (8-methylxanthine; 1.5 mg/L), followed by centrifugation. An aliquot (2 μL) was injected. Chromatography was performed on an Atlantis HILIC Silica column (3 μm, 100 mm × 2.1 mm, Waters) at 30 °C, using a mobile phase comprised of acetonitrile/methanol/50 mM ammonium acetate in 0.2% formic acid (95/2/3, v/v). Using a flow rate of 0.35 mL/min, the analysis time was 6.0 min. Mass spectrometric detection was by selected reactant monitoring (oxypurinol: m/z 150.8 → 108.0; internal standard: m/z 164.9 → 121.8) in negative electrospray ionization mode. Calibration curves were prepared in drug-free urine across the range 10–200 mg/L and fitted using quadratic regression with a weighting factor of $1/x$ ($r^2 > 0.997$, $n = 7$). Quality control samples (20, 80, 150 and 300 mg/L) were used to determine intra-day ($n = 5$) and inter-day ($n = 7$) accuracy and imprecision. The inter-day accuracy and imprecision was 96.1–104% and <11.2%, respectively. Urinary oxypurinol samples were stable when subjected to 3 freeze–thaw cycles and when stored at room temperature for up to 6 h. Samples collected from 10 patients, not receiving allopurinol therapy, were screened and showed no significant interferences. The method was suitable for the quantification of oxypurinol in the urine of patients ($n = 34$) participating in a clinical trial to optimize therapy of gout with allopurinol.

1. Introduction

Allopurinol is the most frequently prescribed urate lowering agent, due to its clinical efficacy, low side-effect profile and convenient, once a day dosing regimen [1]. A structural analogue of hypoxanthine, allopurinol is a substrate for, and a competitive inhibitor of, the oxidised form of xanthine oxidase, thereby preventing urate synthesis [2]. Allopurinol is rapidly metabolised to its active metabolite, oxypurinol. Oxypurinol is an inhibitor of the reduced form of xanthine oxidase, is primarily excreted by the kidneys and has a considerably longer elimination half-life than allopurinol [3]. Therefore, oxypurinol is responsible for most of the pharmacologic activity of allopurinol [4].

Many patients fail to achieve target plasma urate concentrations when treated with this allopurinol [5–7]. The reason for treatment failure remains unclear; however, a lack of adherence to therapy [8–12] and/or the large inter-patient variability in oxypurinol pharmacokinetics may be contributing factors. The ability to determine oxypurinol concentrations in urine, as well as plasma, should help elucidate the pharmacokinetics of oxypurinol and, in turn, the factors which may contribute to the inter-individual variability in response to allopurinol.

Allopurinol and oxypurinol have been measured in both plasma and urine by HPLC with ultra-violet detection [13–16]. However,

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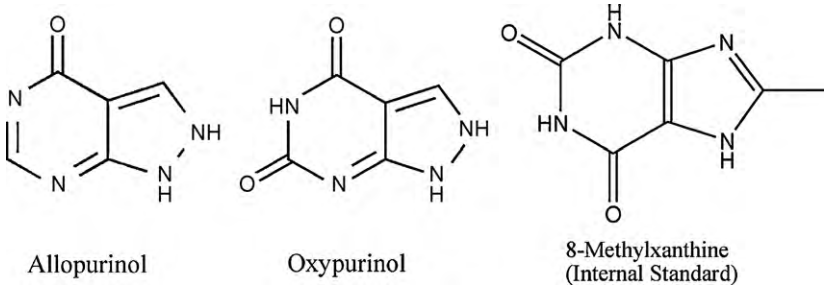

Fig. 1. Chemical structures of allopurinol, oxypurinol (its major active metabolite) and 8-methylxanthine (internal standard).

the often complex and time-consuming sample preparation and limited sensitivity make the clinical application of these methods impractical. Initial attempts in our laboratory to use HPLC with ultra-violet detection for urinary oxypurinol quantification suffered from chromatographic interference. The purpose of this study was to develop a simple, yet selective, method for the determination of oxypurinol in urine using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS).

2. Experimental

2.1. Chemicals and reagents

Oxypurinol, allopurinol and 8-methylxanthine (internal standard; Fig. 1) were purchased from Sigma–Aldrich (Sydney, Australia). HPLC grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Blank urine was obtained from patients not receiving allopurinol therapy and healthy anonymous donors not receiving medicines.

2.2. Standards and quality controls

Stock solutions of oxypurinol (1 g/L) were prepared in sodium hydroxide solution (0.1 M). To overcome any potential instability of oxypurinol in the sodium hydroxide solution, standard and calibrators were made from freshly prepared stock solutions. Stock solutions of the internal standard, 8-methylxanthine (1.5 mg/L) were prepared in an acetonitrile, methanol and water mixture (95/2/3, v/v). Standards were prepared by supplementing urine, obtained from subjects not receiving allopurinol treatment, with oxypurinol (10, 25, 50, 75, 100 and 200 mg/L). Quality controls were prepared, in the same manner as the standards, at concentrations of 20, 80, 150 and 300 mg/L. The oxypurinol standards and quality controls were stored at −20 °C.

2.3. Sample preparation

Urine standards, quality controls and patient samples (50 μL) were prepared by dilution with a solution of acetonitrile/methanol/water (95/2/3, v/v; 2 mL) containing the internal standard (8-methylxanthine; 1.5 mg/L). The samples were vortex mixed (10 s), centrifuged (600 × *g*; 3 min) and an aliquot of the supernatant (2 μL) was injected onto the column. Samples were analysed in singlicate. Patient samples with measured concentrations exceeding the upper limit of the standard range (i.e. >200 mg/L) were diluted (1:2 with blank urine) and re-assayed.

2.4. Instrumentation and operating conditions

A Waters Alliance HT 2795 liquid chromatography system (Waters Corp, Milford, MA, USA) consisting of a degasser, pump, column oven and an autosampler was used for solvent and sample delivery. Chromatographic separation was achieved on an Atlantis HILIC Silica column (100 mm × 2.1 mm 3 μm, Waters). The analytical column was maintained at a temperature of 30 °C. The mobile phase consisted of acetonitrile, methanol and 50 mM ammonium acetate in 0.2% formic acid (95/2/3, v/v). A flow rate of 0.35 mL/min was used. The total chromatographic run time was 6.0 min per sample.

Mass spectrometric detection was performed by selected reaction monitoring on a Quattro Premier triple quadrupole mass spectrometer equipped with an electrospray ionization interface (Waters). Ions were generated in negative ionization mode (−2500 V). The compound specific operating parameters of cone voltage and collision energy were −30 V for both compounds and −22 eV for oxypurinol and −17 eV for the internal standard, respectively. The monitored mass transitions for oxypurinol and the internal standard were m/z 150.8 → 108.0 and m/z 164.9 → 121.8, respectively. The HPLC–MS/MS was controlled and data processed using MassLynx version 4.1 (Waters).

2.5. Assay validation

The analytical range was assessed by analysing standards (10–200 mg/L) on 7 days. Standard curves were fitted using quadratic regression with a $1/x$ weighting factor. Performance of fitted curves is presented as the coefficient of determination (r^2). Selectivity was examined by analyzing blank urine ($n = 10$) samples from different patients (not receiving allopurinol therapy) and evaluating the extent of interference from potential co-eluting compounds at the retention times of oxypurinol and 8-methylxanthine.

Quality control samples (20, 80, 150 and 300 mg/L) were assayed to determine intra-day ($n = 5$) and inter-day ($n = 7$) accuracy and imprecision. The out of analytical range quality control (300 mg/L) was diluted 1/6 with blank urine. Accuracy was determined as: (measured concentration – nominal concentration)/nominal concentration × 100%. Imprecision was expressed as co-efficient of variation (CV). The acceptance criteria for the lower limit of quantification were an accuracy of 100 ± 20% and imprecision of less than 20%.

The process efficiency (representing the combination of matrix effects and recovery of the analyte from the sample by the extraction process) of the method was determined by comparing the peak area response for oxypurinol (80 mg/L) and the internal standard (60 mg/L) obtained for urine samples with water samples ($n = 3$) [17,18]. Inter-subject variability was assessed by supplementing oxypurinol (80 mg/L) into urine from 6 individuals not receiving allopurinol therapy and the concentration measured. The stability of oxypurinol in urine was determined, after one, two and three freeze–thaw cycles, using quality control samples (20, 80 and 150 mg/L). Stability of oxypurinol, over 6 h at ambient temperature, was evaluated using quality control samples (20, 80 and 150 mg/L). Samples were assayed in triplicate for both these stability experiments.

2.6. Clinical study

Hospitalised patients at St. Vincent's Hospital, Sydney, Australia who were receiving regular allopurinol (i.e. at steady-state) for the treatment of gout and/or hyperuricaemia were recruited for this study between April 2008 and August 2009. Ethical approval for this study was obtained from the St. Vincent's Hospital Human Research Ethic Committee, Sydney (H06-107). All study participants provided informed and written consent. Urine samples were collected over a 2-h period at least 5–7 h after administration of allopurinol. A midpoint blood sample was also collected to allow estimation of the renal clearance of oxypurinol. Plasma oxypurinol concentrations were determined by a previously reported HPLC method [13]. Patient samples were stored at −20 °C until analysis.

2.7. Data analysis

Descriptive data are presented a mean and 95% confidence intervals. Comparison of the renal clearance of oxypurinol between patient sub-groups was assessed using an unpaired *t*-test and a probability value of less than 0.05 was considered significant. Statistical analyses were conducted using GraphPad version 5 software (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Method validation

The standard curves gave excellent quadratic fit over the range of 10–200 mg/L ($r^2 > 0.997$, $n = 7$). The accuracy and imprecision for the back calculated standard concentrations were 97.6–103% and <5.4%, respectively (Table 1). Representative chromatograms of a blank urine sample and a urine standard (10 mg/L) are presented in Fig. 2. The total chromatographic analysis time was 6.0 min; with the retention times for oxypurinol and the internal standard being 1.6 and 2.7 min, respectively. The chromatographic run time was extended beyond the retention time of the internal standard as in some samples late eluting peaks were observed, out to 6 min, in either or both mass transitions. The use of a HILIC column provided sufficient retention of the hydrophilic analytes at a relatively high organic solvent component in the mobile phase. The high organic composition favours desolvation under electrospray conditions and thus excellent ionization of the analytes. No significant interferences were observed at the retention times of oxypurinol or the internal standard in urine samples from subjects not receiving allopurinol therapy ($n = 10$). Any peaks, at similar retention times to

Table 1
The inter-day accuracy and imprecision of back-calculated standard results ($n = 7$).

	Nominal concentration (mg/L)					
	10	25	50	75	100	200
Mean	9.8	25.7	50.1	73.8	100.6	200.4
Standard deviation	0.3	1.4	2.1	2.2	3.3	2.2
Accuracy (%)[a]	97.6	103	100	98.4	101	100
Imprecision (%)[b]	3.0	5.3	4.2	3.0	3.3	1.1

[a] Expressed as [(measured concentration − nominal concentration)/nominal concentration] × 100%.
[b] Expressed as coefficient of variation.

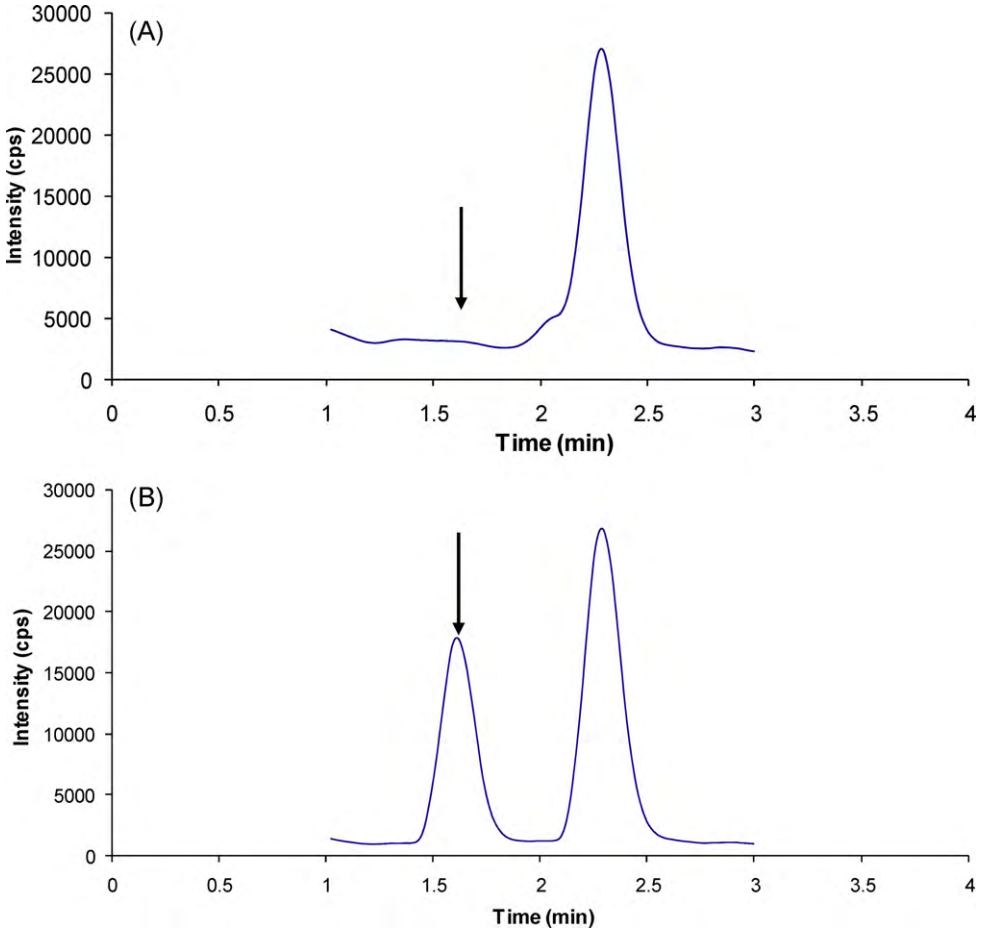

Fig. 2. Representative chromatograms of the oxypurinol mass transition (m/z 150.8 → 108.0) for (A) a urine sample from a patient not taking allopurinol therapy and (B) a urine sample supplemented with 10 mg/L of oxypurinol (i.e. the lower limit of quantification). The arrows represent the retention time of oxypurinol.

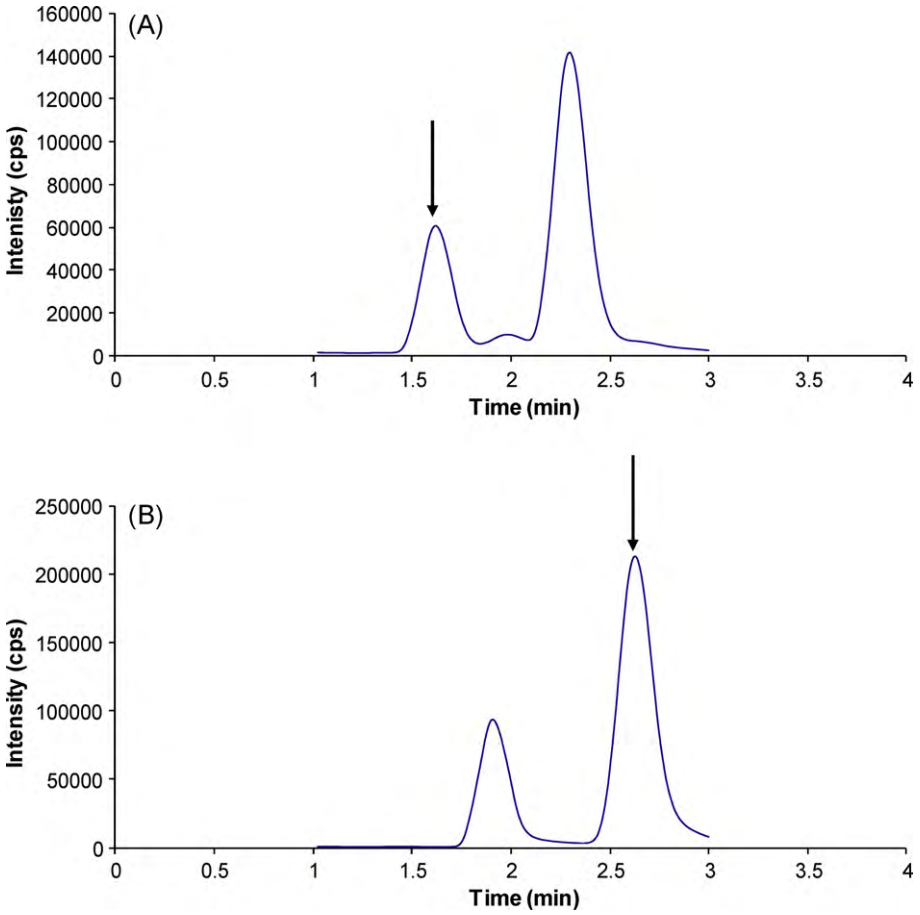

Fig. 3. A representative chromatogram of a patient receiving allopurinol therapy: (A) oxypurinol (m/z 150.8 → 108.0) and (B) the internal standard (m/z 164.9 → 121.8). The measure oxypurinol concentration was 39 mg/L. The arrows represent the retention times of oxypurinol and the internal standard in their respective chromatogram.

oxypurinol and the internal standard, were typically 1% of the oxypurinol response for the lower limit of quantification (10 mg/L). Fig. 3 shows a chromatogram of a patient urine sample with a measured oxypurinol concentration of 39 mg/L. Additional peaks were seen in some patient samples. These interferences were not observed at the retention times of oxypurinol or the internal standard (Fig. 3).

Table 2
The intra- and inter-day accuracy and imprecision of the urinary oxypurinol HPLC–MS/MS method as determined by quality control samples (n = 5).

Nominal concentration (mg/L)	Measured concentration (mean ± SD) (mg/L)	Accuracy (%)[a]	Imprecision (%)[b]
Intra-day			
10	11.1 ± 0.5	111	4.6
20	20.0 ± 1.5	100	7.2
80	78.2 ± 1.3	97.7	1.7
150	150.8 ± 5.7	101	3.8
300[c]	49.4 ± 1.5	98.8	2.9
Inter-day			
10	10.4 ± 1.9	104	18.5
20	20.8 ± 2.3	104	11.1
80	76.9 ± 4.0	96.1	5.2
150	146.7 ± 10.5	97.8	7.2
300[c]	49.5 ± 1.1	98.9	2.3

[a] Expressed as [(measured concentration − nominal concentration)/nominal concentration] × 100%.
[b] Expressed as coefficient of variation.
[c] Out of range quality control sample diluted 1:6 with blank urine.

The results of the intra-day and inter-day accuracy and imprecision evaluation for quality control samples (20, 80, 150 and 300 mg/L) are presented in Table 2. The intra-day accuracy and imprecision was 97.7–101% and <7.3%, respectively. The inter-day accuracy and imprecision was 96.1–104% and <11.2%, respectively. The acceptable analytical performance for the out of analytical range quality control (300 mg/L) under a dilution protocol enabled the analysis of patient samples outside the analytical range. The intra- and inter-day accuracy and imprecision of the method at the lower limit of quantification (10 mg/L) was within acceptable limits (Table 2).

Process efficiency for oxypurinol (80 mg/L) and the internal standard (60 mg/L) was determined to be 92.1% and 80.1%, respectively (Table 3). As process efficiency represents the combination of

Table 3
Determination of the process efficiency for oxypurinol and 8-methylxanthine (n = 3).

	Oxypurinol (80 mg/L)	8-Methylxanthine (60 mg/L)
Water (peak area)		
Mean	25,281	122,551
Standard deviation	2074	10,435
CV (%)[a]	8.2	8.5
Urine (peak area)		
Mean	23,289	98,173
Standard deviation	1290	3385
CV (%)	5.5	3.5
Process efficiency (%)	92.1	80.1

[a] CV, coefficient of variation.

Table 4
The stability of oxypurinol in urine after various freeze–thaw cycles or storage at room temperature for 6 h.

Conditions	Nominal concentration (mg/L)					
	20		80		150	
	Observed mean (mg/L)	Accuracy (%)[a]	Observed mean (mg/L)	Accuracy (%)[a]	Observed mean (mg/L)	Accuracy (%)[a]
Fresh	19.0	104.70	69.5	86.9	134.5	89.7
Stability after one freeze–thaw cycle	18.9	94.20	73.0	91.2	125.0	83.3
Stability after three freeze–thaw cycles	21.1	105.30	75.7	94.6	144.5	96.3
Bench-top stability at room temperature for 6 h	21.3	106.30	71.2	89.0	133.5	89.0

[a] Expressed as [(measured concentration − nominal concentration)/nominal concentration] × 100%.

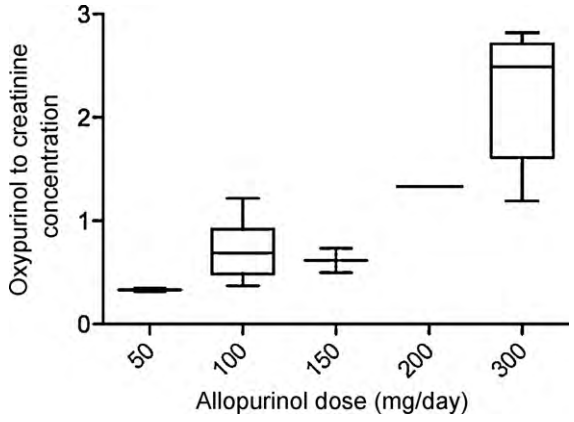

Fig. 4. The mean (95% CI) urinary concentrations of oxypurinol adjusted for the concentration of creatinine in gouty patients ($n = 34$) receiving a range of allopurinol doses.

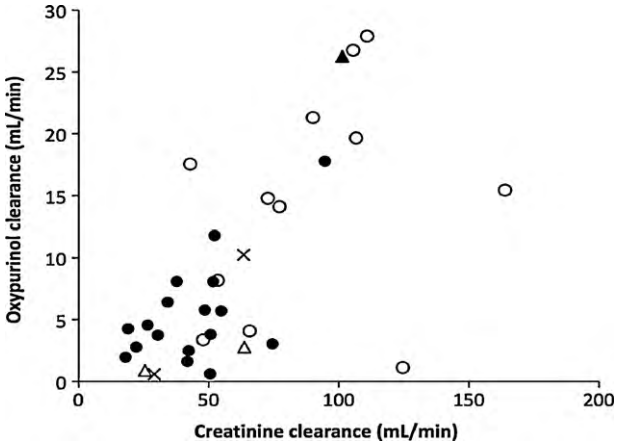

Fig. 5. The relationship between the renal clearance of oxypurinol and creatinine clearance ($n = 34$). The various doses of allopurinol are represented by different symbols; the open triangles 50 mg/daily, closed circles 100 mg/daily, crosses 150 mg/daily, closed triangles 200 mg/daily and the open circles 300 mg/daily. The renal clearance of oxypurinol was calculated by dividing the plasma concentration from a midpoint blood sample by the concentration in urine corrected for urine volume.

matrix effects and recovery of the analyte from the sample by the extraction process and the extraction process used in this method is dilution, it can be concluded that minimal signal suppression was observed (8% for oxypurinol and 20% for 8-methylxanthine). The absolute response (in terms of peak area) for the urine samples was reproducible for both oxypurinol (CV = 5.5%) and the internal standard (CV = 3.5%). The imprecision of the method when measuring different individuals' urine ($n = 6$), supplemented with oxypurinol (80 mg/L), was 6.2%. These data suggest that inter-subject variability and related matrix effects had minimal influence on results [18].

Oxypurinol urine samples were found to be stable at ambient temperature for at least 6 h (Table 4). Oxypurinol urine samples were stable after being taken through at least 3 freeze–thaw cycles (Table 4).

To the best of our knowledge, this is the first HPLC–MS/MS method for the quantification of oxypurinol in urine. Previously reported methods have employed reversed phase chromatography with UV detection [14–16], electrochemical detection [19], ion-pair chromatography [20] and capillary electrophoresis [19,21]. The disadvantages of these methods include more complex sample clean-up procedures, some using solid-phase extraction [15,22], and longer analysis times [15,20,22]. Further, the HPLC–MS/MS method presented here did not have the problematic interferences that were observed when establishing a HPLC with UV detection method. Therefore, the current method is an improvement on these previous methods; with simple sample preparation (dilution), excellent selectivity and relatively high-throughput (chromatographic run time of 6.0 min/sample).

3.2. Clinical study

The validated method was successfully applied to measure urinary concentrations of oxypurinol in patients with gout who were receiving allopurinol treatment (50–300 mg per day). In total 34 urine samples from gouty patients (9 female) receiving allopurinol were analysed. Most patients (79%) were receiving a number of concomitant medications, ranging from 1 to 8) medications. None of these concomitant medications appeared to interfere with the urinary oxypurinol assay.

The measured urinary concentrations of oxypurinol ranged from <10 to 437 mg/L (mean = 79 mg/L). The concentration of oxypurinol in the urine increased with increasing dose of allopurinol (Fig. 4). The observed urinary concentrations in our study were similar to those previously reported in healthy subjects [23].

The renal clearance of oxypurinol was positively correlated with creatinine clearance (Fig. 5). Female patients had a lower renal clearance of oxypurinol compared to male patients (mean, 95% CI; 4.3, 1.4–7.3 mL/min for females and 10.8, 7.2–14.4 mL/min for males; $p < 0.05$). Similarly, patients receiving concomitant therapy with a diuretic (including thiazides and frusemide) had lower renal clearances of oxypurinol (5.2, 2.9–7.5 mL/min) compared to those patients not taking a diuretic (15.3, 10.1–20.5 mL/min; $p < 0.05$).

4. Conclusions

An HPLC–MS/MS method for the determination of urinary oxypurinol was developed and validated. The present method met satisfactory performance criteria for analytical range, imprecision and accuracy as defined by FDA guidelines on method validation [24]. The practical utility of the assay has been demonstrated by the successful determination of oxypurinol excretion in patients with gout following the administration of allopurinol. The method

is simple, yet accurate and precise, and is suitable for the clinical setting where timely accurate test results are expected.

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