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Original Research Article

Simultaneous analysis of allopurinol and oxypurinol using a validated liquid chromatography–tandem mass spectrometry method in human plasma

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A B S T R A C T

The present study describes a simple, reliable and reproducible liquid chromatography–tandem mass spectrometry method (LC–MS/MS) for the simultaneous determination of allopurinol and its active metabolite, oxypurinol in human plasma for a pharmacokinetic/bioequivalence study. After protein precipitation (PPT) of 100 µL plasma sample with 1.0% formic acid in acetonitrile, the recovery of the analytes and allopurinol-d2 as an internal standard ranged from 85.36% to 91.20%. The analytes were separated on Hypersil Gold (150 mm×4.6 mm, 5 µm) column using 0.1% formic acid-acetonitrile (98:2, v/v) as the mobile phase. Quantification was done using electrospray ionization in the positive mode. The calibration concentration range was established from 60.0 to 6000 ng/mL for allopurinol and 80.0–8000 ng/mL for oxypurinol. Matrix effect in human plasma, expressed as IS-normalized matrix factors ranged from 1.003 to 1.030 for both the analytes. The developed method was found suitable for a clinical study with 300 mg allopurinol tablet formulation in healthy subjects.

1. Introduction

Gout is a common medical illness associated with inflammatory arthritis due to increased levels of uric acid in blood, leading to monosodium urate monohydrate crystal formation in the joints [1]. Allopurinol (AP) is one of the most effective and widely used drugs for the treatment of hyperuricaemia and gout. Its main function is to inhibit xanthine oxidase which catalyzes the formation of xanthine from hypoxanthine and further to uric acid [2,3]. AP is rapidly metabolized by xanthine oxidase to its major active metabolite, oxypurinol (OP). After intravenous and oral administration, AP is recovered unchanged in the urine up to 12% and about 76% as OP, while OP is eliminated unchanged almost entirely in urine with 1.0%–3.0% as two riboside metabolites [4]. The mean oral bioavailability of unchanged AP is estimated to be 79.0% ± 20.0% with an apparent oral clearance of 15.8% ± 5.2%. OP has a much longer elimination half life (~23 h) compared to AP (approximately 1.2 h) and the hypouricaemic efficacy of AP is essentially due to this metabolite [2,5]. Despite the fact that OP is mainly responsible for the pharmacological effect, the parent drug is still used due to poor absorption of OP preparations [6]. As new and more efficacious AP formulations are being developed, it is essential to have more selective, sensitive and reliable methods to measure the plasma concentration of AP and OP for improved hypouricaemic response, to reduce risk of toxicity and for better patient compliance.

A survey of literature reveals several methods for the simultaneous determination of AP and OP in different biological samples like human plasma [3], human urine [7], human serum [8,9], rat plasma, intestinal wash and bile [10]. These methods are mainly based on high-performance liquid chromatography (HPLC) with UV or electrochemical detection, except for one report which utilizes capillary electrophoresis with end-column amperometric detection [7]. AP has also been estimated by micelle-stabilised room temperature phosphorescence in urine samples [11]. However, some of these methods involve lengthy extraction protocols [10], have chromatographic run time more than 10 min [8], are less sensitive [8,9] and employ large sample volume for processing [3]. Few methods report use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) technique for the

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determination of OP as a single analyte in human urine [12] and for the simultaneous estimation of AP and OP in human plasma [13–15] and urine [14]. The method described by Ayalasomayajula et al. [13] involved a combination of protein precipitation (PPT) and liquid-liquid extraction (LLE) for the extraction of the analytes; however, there was limited information on the method development as the aim was to study the effect of AP on the pharmacokinetics of aliskiren. Though the method reported by Liu et al. [14] is sensitive but employs two different mobile phases for eluting AP and OP separately and also different electrospray ionization (ESI) modes for mass spectrometric detection. Another LC–MS/MS method developed by Kasawara et al. [15] required large plasma volume for processing (500 μL) and both the analytes were detected in the negative ionization mode.

To the best of our knowledge, there are no reports on the pharmacokinetics of AP in the Indian subjects. Thus, in the present work we report a simple, sensitive and rugged LC–MS/MS assay for the simultaneous estimation of AP and OP from small plasma volume (100 μL). Both the analytes were detected in the positive ionization mode using a single mobile phase to afford baseline separation of the analytes with minimal matrix interference. The method was successfully applied for a clinical study involving healthy Indian subjects.

2. Experimental

2.1. Chemicals and materials

AP (99.76%), OP (99.60%) and allopurinol-d2 (AP-d2, 98.00%; isotopic purity, 99.2 atom% deuterium, 0.6% D_o) were purchased from Ipca Laboratories Ltd. (Mumbai, India), Vivan Life Sciences Pvt. Ltd. (Mumbai, India) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively. HPLC grade methanol (MeOH) and acetonitrile (ACN) were procured from J. T. Baker Inc. (Phillipsburg, NJ, USA). Analytical reagent grade formic acid (FA) and sodium hydroxide (NaOH) were purchased from Qualigens Fine Chemicals (Mumbai, India) and Merck Specialties Pvt. Ltd. (Mumbai, India), respectively. Water was purified using Milli-Q water purification system from Millipore (Bangalore, India). Blank human blood was collected with sodium heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 2061*g* at 10 °C and stored at −20 °C.

2.2. Liquid chromatographic and mass spectrometric conditions

A Shimadzu HPLC system (Kyoto, Japan) with a Hypersil Gold (150 mm×4.6 mm, 5 μm) analytical column from Thermo Scientific (Cheshire, UK) was used for chromatographic separation of analytes. The column temperature was maintained at 40 °C. The mobile phase consisted of 0.1% (v/v) FA in water-ACN (98:2, v/v). For isocratic elution, the flow rate of the mobile phase was set at 0.5 mL/min. The total eluant from the column was split in 70:30 (v/v) ratio; flow directed to the ion spray interface was equivalent to 150 μL/min. The autosampler temperature was maintained at 5 °C, injection volume was kept at 2 μL, and the pressure of the system was maintained at 1100 psi. The LC system was connected to a triple quadrupole mass spectrometer MDS SCIEX API-5500 (Toronto, Canada), equipped with electro spray ionization and operated in positive ionization mode. The multiple reaction monitoring (MRM) transitions were monitored at *m/z* 137.0/109.9, 153.1/136.0 and 139.0/111.9 for AP, OP and AP-d2, respectively. The optimized mass parameters for quantitation of analytes and internal standard (IS) are presented in Supplementary Table 1. Analyst classic software version 1.5.2 was used to control all parameters of LC and MS.

2.3. Preparation of standard stock, calibration standards and quality control samples

Stock solutions of AP (1000 μg/mL) and OP (1000 μg/mL) were prepared by dissolving their requisite amounts in methanol (MeOH):1.0% (m/v) NaOH in water (90:10, v/v). Their combined intermediate solution containing 300.0 μg/mL of AP and 400.0 μg/mL of OP was prepared in MeOH:water (60:40, v/v). Calibration standards (CSs) and quality control (QC) samples were made by spiking blank plasma with appropriate volumes of working solutions prepared from intermediate stock solutions for both the analytes. The CS concentrations were 60.0, 120.0, 300.0, 600.0, 1200, 2400, 3600, 4800 and 6000 ng/mL for AP and 80.0, 160.0, 400.0, 800.0, 1600, 3200, 4800, 6400 and 8000 ng/mL for OP. The QC samples were prepared at four concentration levels, lower limit of quantification quality control (LLOQ QC): 60.0/80.0 ng/mL, low quality control (LQC): 180.0/240.0 ng/mL, medium quality control (MQC): 1920/2560 ng/mL and high quality control (HQC): 4620/6160 ng/mL for AP/OP, respectively. The stock solution of AP-d2 (100.0 μg/mL) was prepared by dissolving accurately weighed amount in methanol: 1.0% (m/v) NaOH in water (90:10, v/v). Its working solution (10.00 μg/mL) was prepared in MeOH: water (60:40, v/v). Standard stock and working solutions used for spiking were stored at 2–8 °C, while CSs and QC samples in plasma were kept at −20 °C until use.

2.4. Sample preparation

Prior to analysis, all frozen subject samples, CSs and QC samples were adequately thawed and allowed to equilibrate at room temperature. To an aliquot of 100 μL of spiked plasma sample/ subject sample, 25 μL of AP-d2 (10.00 ng/mL) was added and vortexed for 30 s. Further, 500 μL of 1.0% FA in ACN was added and again vortexed for 30 s to precipitate the proteins. The sample was centrifuged at 13,148*g* for 10 min at 10 °C and the supernatant was collected in a prelabeled radioimmunoassay vial. The supernatant was then evaporated to dryness at 50 °C under nitrogen. Thereafter, the sample was reconstituted with 500 μL of 1.0% FA in water, vortexed for 30 s and 2 μL was used for injection into the chromatographic system.

2.5. Method validation procedures

The method was validated for system suitability, selectivity, carryover, sensitivity, linearity, accuracy and precision, recovery, matrix effect, stability, ruggedness and dilution reliability as per the current regulatory requirements [16]. The experimental details of the parameters studied were similar to our previous work [17] and are briefly described in Supplementary material.

2.6. Bioequivalence study and incurred sample reanalysis

The aim of the study was to determine the bioequivalence of a single dose of 300 mg AP tablet (Generic Company, India) with a reference tablet, ZYLOPRIM™ (Allopurinol, 300 mg) from Aspen Pharma Pty Ltd. (Australia). The design was an open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover study with 44 healthy Indian subjects under fasting. Each subject was checked to be healthy through medical history, physical examination and routine laboratory tests. All the subjects were informed about the objectives and possible risks of the study and a written consent was obtained. The work was subject to review by an Independent Ethics Committee constituted as per Indian Council of Medical Research (ICMR), India, which approved the study protocol. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines [18]. The subjects were orally administered a single dose of test and reference formulations with 240 mL of water after recommended wash out period of 10 days. Blood samples were collected at 0.00 (pre-dose), 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 24.00, 36.00, 48.00, 72.00 and 96.00 h after oral administration of test and reference formulation in labeled sodium heparin-vacuettes. After thorough mixing, the plasma was separated by centrifugation at 1811*g* and kept frozen at

−20 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of AP and OP were estimated using SAS software version 9.2.

The assay reproducibility was checked by reanalysis of 165 incurred samples near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results were compared with initial pharmacokinetic analysis using the same procedure. As per the acceptance criterion at least two-thirds of the original and repeat results should be within 20% of each other [19].

3. Results and discussion

3.1. LC–MS/MS method development

Few methods have reported use of liquid chromatography with mass spectrometric detection for the determination of AP and/or OP in human plasma [13–15] and urine [12,14]. In these methods OP has been detected in the negative ESI mode while AP was monitored either in the positive [14] or negative [12,13] ionization mode. While another method reported measurement in the negative mode for both the analytes [15]. As AP and OP are weakly acidic in nature with an ionization constant of 9.4 and 7.7, respectively [2], ionization was first tried in the negative mode; however, the response was too less for the deprotonated precursor ions of AP (*m/z* 135.1, 1.5e5 cps) as compared to OP (*m/z* 150.9, 4.2e7 cps). Thus, positive ionization mode was tested wherein the response for protonated precursor ions of AP (*m/z* 137.0, 1.9e7 cps) was high, while it was comparable for OP (*m/z* 153.1, 4.0e7 cps) in both the modes under the optimized mass conditions. Thus positive ESI mode was selected for both the analytes in the present work. This helped in curtailing time required for stabilization of high voltages during polarity switch. Moreover, the positive ESI mode provided better selectivity without significantly compromising the sensitivity, especially for OP. The most stable and consistent product ions in the Q3 mass spectra were found at *m/z* 109.9, 136.0 and 111.9 for AP, OP and AP-d2, respectively as shown in Fig. 1. These fragments were obtained after elimination of neutral species, HCN and NH_3 from AP/AP-d2 and OP precursor ions, respectively. Further, one qualifying transition was also monitored for the identity of the analytes at *m/z* 137.0 → 94.0 for AP and *m/z* 153.0 → 107.1 for OP. A dwell time of 200 ms was adequate to have 25 data points across the peaks for quantitative analysis. Cross-selectivity test was also performed to check for any possible conversion of AP to OP and vice versa during successive steps of analysis. No interfering peaks were found at the retention time of the analytes in their respective MRM windows.

After optimization of detection settings, the chromatographic conditions were suitably optimized through trials conducted on different reversed phase columns with different mobile phases to obtain adequate retention, response, peak shape and optimum elution time. The nature and composition of the mobile phase (organic and aqueous buffer ratio) had significant effect on analyte retention, signal and peak shapes with different reversed-phase columns tested, namely Symmetry Shield RP C_{18}, Hypersil Gold and Kromasil C_{18} columns with identical dimensions (150 mm×4.6 mm, 5.0 µm). Acidic buffer (formic acid-ammonium formate) in the pH range of 2.5–4.5 together with MeOH/ACN was tried as the mobile phase as reported previously [14]. It was observed that with higher proportion of organic diluent (> 70%) in the mobile phase there was inadequate retention of the analytes, especially OP which eluted within 1.50 min (capacity factor *k*, 0.3–0.5), with no significant change in the elution pattern by varying the flow rate from 0.8 to 1.0 mL/min on all the three columns. Consequently, the aqueous part was increased (up to 80%), which afforded better retention (above 2.0 min for both the analytes) but with partial separation (resolution factor, *Rs* 0.4–0.6). Use of 0.1% FA in place of the buffer solution helped in getting better response, while ACN provided better peak shape compared to MeOH and hence both of them were used for further optimization. These observations were similar on all the three columns studied. Nevertheless, the best balance with regards to peak shape, retention, run time and sensitivity was possible using 0.1% (v/v) FA in water-ACN (98:2, v/v) on Hypersil Gold column under isocratic conditions. By maintaining a flow rate of 0.5 mL/min the retention time and capacity factors for AP and OP were 7.20 min and 2.3 and 6.44 min and 2.1, respectively. Both the analytes were baseline separated within 9.0 min with a resolution factor of 1.7. The reinjection reproducibility expressed as coefficient of variation (CV) in determining the retention time for the analytes was ≤0.78% for more than 100 injections on the same column. The deuterated IS used in the study successfully monitored both the analytes with acceptable accuracy and precision for the method.

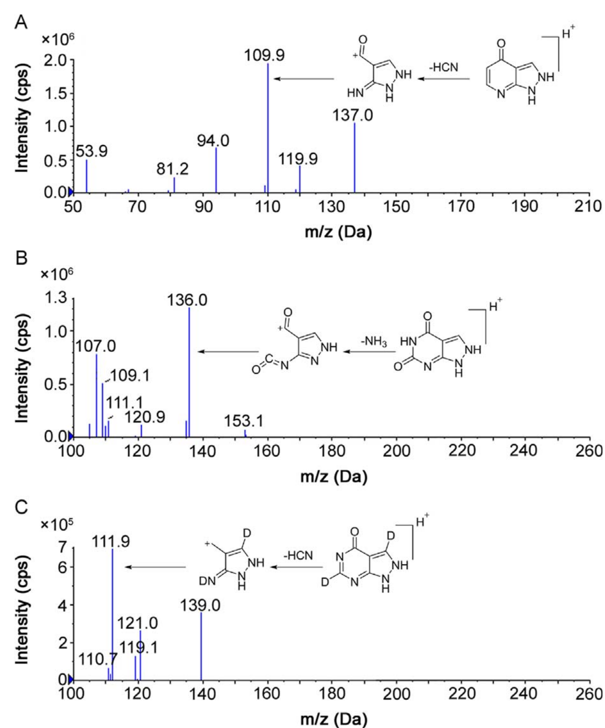

Fig. 1. Product ion mass spectra of (A) allopurinol (*m/z* 137.0 → 109.9, scan range 50–150 Da), (B) oxypurinol (*m/z* 153.1 → 136.0, scan range 100–200 Da) and (C) allopurinol-d2, IS (*m/z* 139.0 → 111.9, scan range 100–200 Da) in the positive ionization mode.

Generic PPT has been the technique of choice for quantitative recovery of AP and OP from biological samples as documented in several reports [3,9,20]. Two other methods report combined use of PPT and LLE [8,13]; however, the details of extraction procedure were not presented in one of the methods [13]. Further, Liu et al. [14] have recommended use of acidic conditions prior to LLE with ethyl acetate. The recoveries obtained for AP and OP were precise but not quantitative for PA (~55%), which may be due to large difference in the partition coefficient of AP (Log *P*, 0.28) and OP (Log *P*, 14) [2]. Thus, to have a simple and rapid method for sample preparation from plasma matrix, PPT was tested with common precipitants like acetonitrile, methanol and also with different acids like trichloroacetic acid (TCA), perchloric acid and FA. There have been some issues in published reports related to reduction in column efficiency due to direct injection of acidic supernatants, especially when using TCA as the precipitant and therefore it is recommended to neutralize the acid with ammonium sulfate [20]. Although the recovery was reduced to some extent due to neutralization, a similar approach was adopted after PPT with 20% TCA. Furthermore, two previous methods [8,9] have suggested use of buffered mobile phase with sodium acetate or potassium phosphate (pH 4.0–4.5) to prevent column deterioration when using perchloric acid or TCA for HPLC–UV based methods. In our initial trials with these two precipitants and FA, quantitative recovery was obtained in

Table 1.
Summary of chromatographic methods developed for the analysis of allopurinol and oxypurinol in biological matrices.

Sr. no.	Detection technique	Linear range (ng/mL)		Sample volume; extraction technique	Retention time (min) (AP/OP); run time (min)	Application	Ref.
		AP	OP				
1	HPLC–UV (260 nm)	500–5000	400–20,000	100 µL human serum; PPT with 10% TCA and filtration	4.58/3.99; 12.0	Pharmacokinetic study with 300 mg AP tablets in 2 healthy subjects	[9]
2	HPLC–UV (254 nm)	500–10,000	1000–40,000	100 µL human serum; PPT with 10% perchloric acid followed by LLE with DCM	12.3/9.9; 22.0	Measurement of AP and OP in 66 serum samples from patients undergoing AP therapy with 300 mg tablets	[8]
3[a]	LC–MS/MS Positive polarity for AP and negative for OP	50–5000 in plasma and 500–30,000 in urine	50–5000 in plasma and 1000–50,000 in urine	500 µL human plasma/ urine; Acidification of sample with 0.2 M HCl followed by LLE with EA	5.85/2.57; 7.0 for AP and 4.0 for OP	Pharmacokinetic study with 100, 200 and 300 mg injectable AP formulations in 36 healthy Chinese subjects	[14]
4	LC–MS/MS Negative polarity for AP and OP	100–10,000	100–10,000	500 µL human plasma; PPT with ACN	4.02/3.78; 6.0	–	[15]
5	LC–MS/MS Positive polarity for AP and OP	60.0–6000	80.0–8000	100 µL human plasma; PPT with 1.0% FA in ACN	7.20/6.44; 9.0	Bioequivalence study with 300 mg of AP tablet in 44 healthy Indian subjects and ISR study	PM

[a] Separate elution of AP and OP with different mobile phases and mass ionization modes; LLOQ: lower limit of quantification; AP: allopurinol; OP: oxypurinol; ACN: acetonitrile; MeOH: methanol; TCA: trichloroacetic acid; DCM: dichloromethane; EA: ethyl acetate; FA: formic acid; PPT: protein precipitation; LLE: liquid-liquid extraction; ISR: incurred sample reanalysis; PM: present method

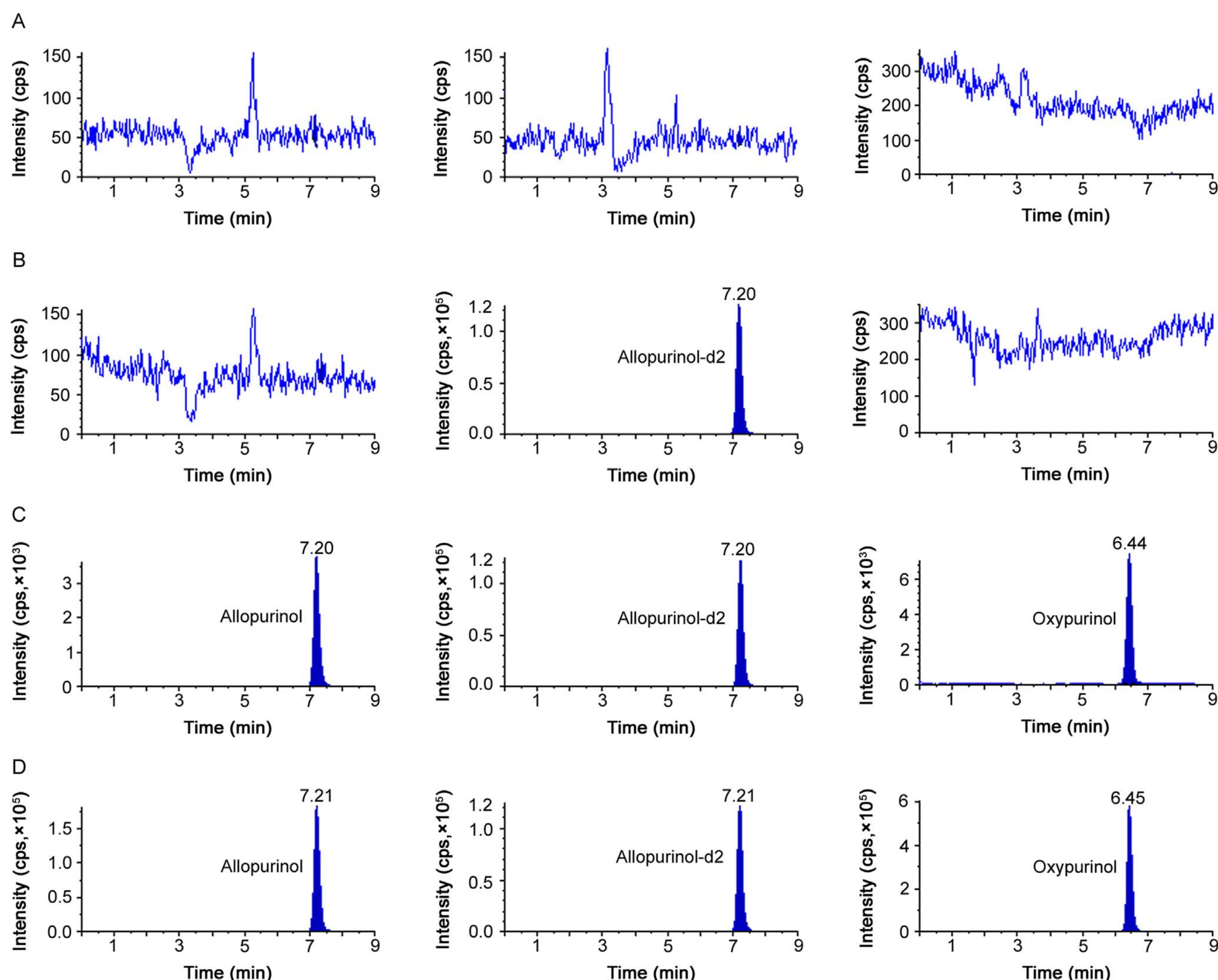

Fig. 2. MRM ion-chromatograms of (A) double blank plasma, without allopurinol-d2, IS, (B) blank plasma spiked with IS, (C) allopurinol, allopurinol-d2 and oxypurinol at lower limit of quantification and (D) a real subject sample at C_{max} after oral administration of 300 mg allopurinol tablet formulation.

Table 2.
Extraction recovery for allopurinol and oxypurinol (n=6).

Analyte and QC level	Area response		Extraction recovery (A/B) (%)
	Pre-extraction spiking (A)	Post-extraction spiking (B)	
Allopurinol			
LQC	187,006	219,067	85.36
MQC	2,043,446	2,336,715	87.45
HQC	5,347,462	6,013,475	88.92
Oxypurinol			
LQC	187,079	211,748	88.35
MQC	2,020,810	2,258,645	89.47
HQC	4,723,337	5,417,914	87.18
Allopurinol-d2			
LQC	1,891,768	2,105,005	89.87
MQC	1,951,733	2,140,058	91.20
HQC	1,890,315	2,083,451	90.73

LQC: low quality control; MQC: medium quality control; HQC: high quality control

the range of 78%–96%, while the extraction efficiency in ACN/MeOH was very less (46%–63%) for both the analytes. Nevertheless, to avoid any potential damage to the column and perform for large numbers of injections and at the same time to ensure quantitative and precise recovery, we used FA together with ACN/MeOH. Highly consistent and quantitative recoveries were obtained for AP, OP and AP-d2, ranging from 85.36% to 91.20% using 1.0% FA in ACN as the protein precipitant. No deterioration was found in the response even after 1000 injections on the same column.

The newly developed and validated method is better compared to several reported methodologies in terms of clinically relevant concentration range, sample processing volume, simple and straight forward extraction procedure and total analysis time. The method described by Liu et al. [14] is slightly more sensitive compared to the present method but utilizes fivefold higher sample volume for analysis and requires two different mobile phases for separate elution of AP and OP with run times of 7.0 and 4.0 min, respectively. Another LC–MS/MS method developed for simultaneous analysis of AP and OP has a shorter run time (6.0 min) but is less sensitive (100 ng/mL for both the analytes) and requires much higher sample volume for processing [15]. The other HPLC–UV based methods [8,9] involve higher chromatographic run times (≥12.0 min) and are less sensitive (500 ng/mL for AP and 400 or 1000 ng/mL for OP). A comparative summary of chromatographic methods published in the last two decades for AP and OP is presented in Table 1.

3.2. Method validation

3.2.1. Selectivity and carryover

No significant interference from endogenous plasma components was observed at the retention time of the analytes. Typical chromatograms of double blank plasma, blank plasma spiked with IS, spiked plasma sample at LLOQ concentration and clinical sample at C_{max} prove the selectivity of the method (Fig. 2). The experiment to evaluate autosampler and column carryover showed negligible carryover (less than 0.78%) in the extracted blank plasma after injection of the highest concentration of AP (6000 ng/mL) and OP (8000 ng/mL).

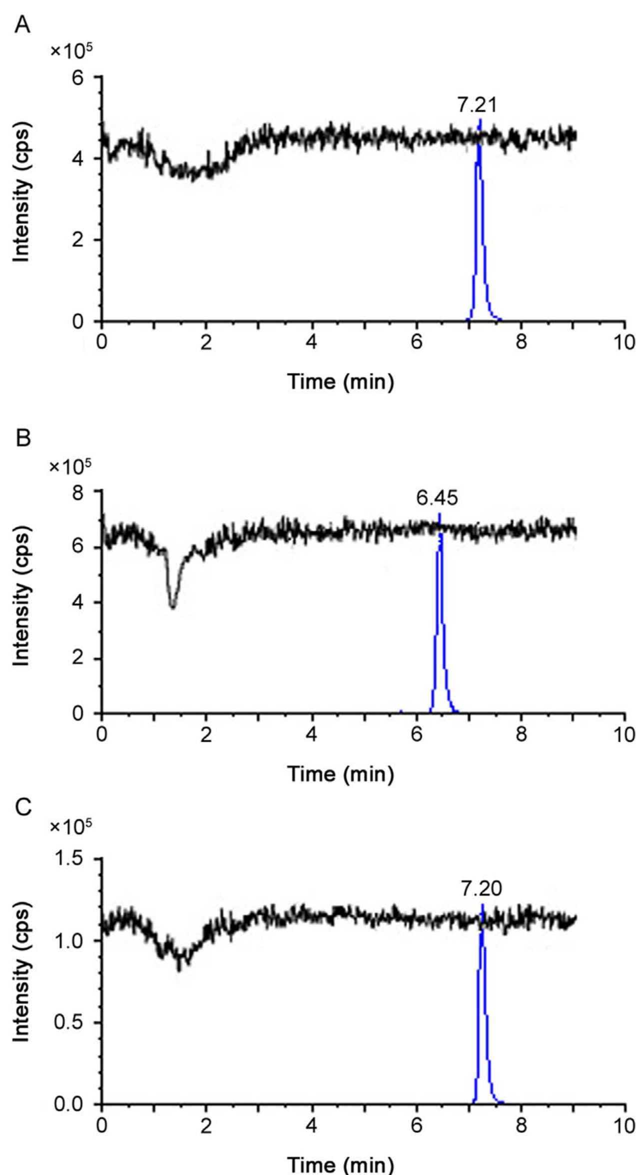

Fig. 3. Post-column analyte infusion chromatograms of (A) allopurinol, (B) oxypurinol and (C) allopurinol-d2 at upper limit of quantification while injecting extracted blank plasma.

3.2.2. Linearity and LLOQ

Calibration curves were obtained using nine different concentrations of the analytes by linear regression with $1/x^2$ weighting in the

Table 3.
Matrix effect for allopurinol and oxypurinol in human plasma (n=6).

Analytes	Mean area response				Matrix factor		IS-normalized matrix factor	
	Post-extraction spiking		Neat samples in mobile phase					
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Allopurinol	219,067	6,013,475	212,889	6,040,681	1.029	0.995	1.030	1.014
Oxypurinol	211,748	5,417,914	211,254	5,476,098	1.002	0.989	1.003	1.008
Allopurinol-d2	2,105,005	2,083,451	2,107,027	2,124,326	0.999	0.981	–	–

LQC: low quality control; HQC: high quality control.

Table 4.
Stability results for allopurinol and oxypurinol under different conditions (n=6).

Storage condition	QC level	Accuracy (%)		Precision (% CV)	
		Allopurinol	Oxypurinol	Allopurinol	Oxypurinol
Bench top stability	LQC	92.53	102.71	3.26	4.94
(28 h, 25 °C)	HQC	93.67	98.57	3.06	4.55
Freeze-thaw stability	LQC	91.63	99.38	5.96	5.32
(5 cycles,−20 °C)	HQC	95.30	95.62	1.40	3.04
Autosampler stability	LQC	92.80	96.84	5.48	2.39
(83 h, 5 °C)	HQC	93.83	91.47	1.98	6.67
Processed sample stability	LQC	94.29	89.67	2.15	2.53
(6 h, 25 °C)	HQC	97.94	95.52	0.60	1.95
Dry extract stability	LQC	90.31	100.89	2.18	4.15
(46 h, 5 °C)	HQC	90.94	99.54	1.12	4.36
Long-term stability in plasma	LQC	90.11	92.61	1.27	1.72
(112 days,−20 °C)	HQC	90.75	88.68	2.24	2.44

LQC: low quality control; HQC: high quality control; CV: coefficient of variation.

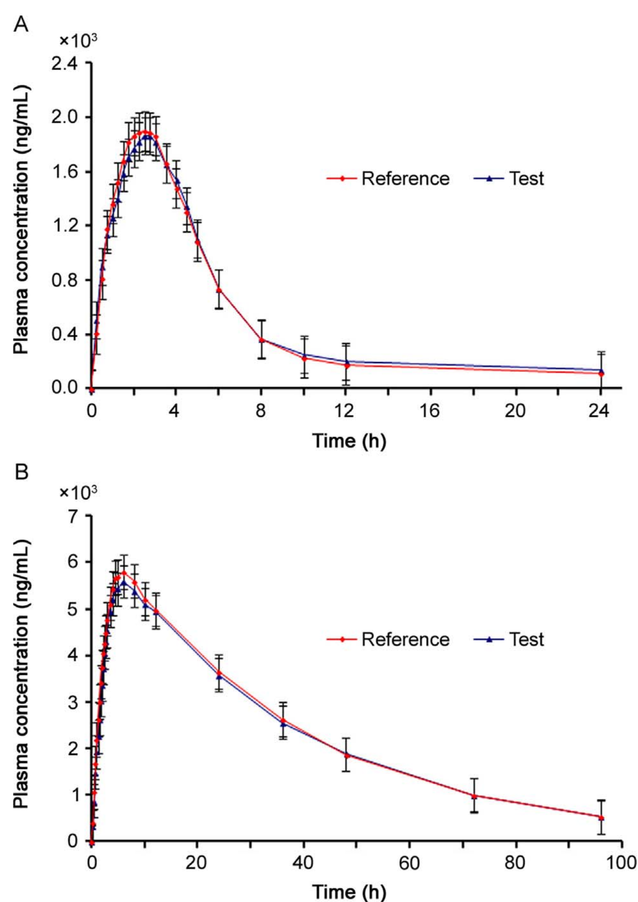

Fig. 4. Mean plasma concentration-time profile of (A) allopurinol and (B) oxypurinol after oral administration of 300 mg allopurinol tablet formulation (test) and reference formulation to 44 healthy Indian subjects. The plasma-time profile of allopurinol is presented up to 24 h as the concentration was below the limit of quantitation beyond this time. None of the subjects had a measurable concentration after this time point.

range of 60.0–6000 ng/mL for AP and 80.0–8000 ng/mL for OP with correlation coefficient (r^2)≥0.9952. The corresponding mean linear equations were y=(0.00063 ± 0.00007) x-(0.00137 ± 0.00308) and$y$=(0.00079 ± 0.00010) x+(0.00274 ± 0.000522) respectively. The accuracy and precision (CV) values for the calibration curve standards ranged from 97.7% to 102.3% and from 1.30% to 3.77% for both the analytes. The signal-to-noise (S/N) ratio was ≥28 for the established LLOQ of 60.0 ng/mL (accuracy, 98.29% and precision, 0.99 CV) for AP and 80.0 ng/mL for OP (accuracy, 98.63% and precision, 1.39 CV). The sensitivity of the developed method was superior to all existing methods for AP and OP except one report [14].

3.2.3. Intra-day and inter-day accuracy and precision

As summarized in Supplementary Table 2**,** the intra-day and inter-day precision ranged from 0.43% to 2.43% and from 1.23% to 6.42%, respectively across QC levels for both the analytes. Similarly, the intra-day and inter-day accuracy for the analytes varied from 94.74% to 97.03% and from 94.10% to 98.88%, respectively. All the values of precision and accuracy were within the acceptable range of ± 15% [16].

3.2.4. Recovery and matrix effects

The mean extraction recovery of the analytes from plasma samples ranged from 85.36% to 88.92% for AP and from 87.18% to 89.47% for OP, which indicates highly consistent recovery at different QC levels (Table 2). The mean recovery of AP-d2 was 90.60% with CV less than 2.0%. The matrix effect which results in ion suppression or enhancement of analyte signal due to co-eluting matrix components was assessed at two QC levels. The analytes and IS have almost identical matrix factors as shown in Table 3. The IS-normalized matrix factor for the analytes varied from 1.003 to 1.030. The precision (CV) in the measurement of analyte concentration for relative matrix effect in different plasma sources (six Na-heparinized, two haemolysed and two lipemic) was in the range of 1.63%–3.15% as shown in Supplementary Table 3. Fig. 3 shows the profiles obtained by injection of extracted blank plasma after post-column infusion of AP, OP and AP-d2 solutions at upper limit of quantitation (ULOQ) concentration. The results showed no ion suppression/enhancement in the response at the retention time of the analytes and IS.

3.2.5. Stability of the analytes and IS

The stability results obtained were within the United States Food and Drug Administration (US FDA) acceptance criteria required to establish the stability of analytes in plasma and stock/working solutions during storage, extraction and chromatographic analysis. The stock and working solutions kept for assessing short-term and long-term stability were stable for a minimum period of 31 h and 24 days, respectively. The bench top stability of the analytes in plasma was established up to 28 h. Processed sample stability was determined up to 6 h at 25 °C. Samples kept in an autosampler maintained at 5 °C were found stable for a period of 83 h, while the samples kept for ascertaining dry extract stability showed no appreciable change up to 46 h. Spiked plasma samples stored at −20 °C for assessing long-term stability of the analytes remained unaffected for a minimum period of 112 days. The detailed stability results are summarized in Table 4.

Table 5.
Mean pharmacokinetic parameters (± SD) after oral administration of allopurinol tablet formulation to 44 healthy Indian subjects under fasting.

Parameter	Allopurinol		Oxypurinol	
	Test	Reference	Test	Reference
C_{max} (ng/mL)	1904.54 ± 834.83	2012.07 ± 896.16	5878.05 ± 1714.36	6039.43 ± 1591.32
T_{max} (h)	2.32 ± 1.06	2.10 ± 0.96	6.32 ± 2.66	6.12 ± 2.45
$t_{1/2}$ (h)	1.24 ± 0.27	1.25 ± 0.25	25.76 ± 8.45	25.24 ± 7.15
$AUC_{0-96\ h}$ (h ng/mL)	9285.77 ± 5881.23	9392.12 ± 5231.11	221064.1 ± 67329.9	225876.9 ± 59360.5
$AUC_{0\text{-}inf}$ (h ng/mL)	9565.22 ± 5998.04	9676.56 ± 5310.63	245456.8 ± 78245.9	247859.1 ± 70277.3
K_{el} (1/h)	0.579 ± 0.103	0.572 ± 0.093	0.029 ± 0.008	0.029 ± 0.008

C_{max}: Maximum plasma concentration; T_{max}: Time point of maximum plasma concentration; $t_{1/2}$: Half life of drug elimination during the terminal phase; $AUC_{0-96\ h}$: Area under the plasma concentration vs time curve from zero hour to 96 h; $AUC_{0\text{-}inf}$: Area under the plasma concentration-time curve from zero hour to infinity; K_{el}: Elimination rate constant; SD: Standard deviation

3.2.6. Ruggedness and dilution integrity

The precision (CV) and accuracy values to establish method ruggedness with different columns and analysts ranged from 0.99% to 1.19% and from 91.72% to 96.79%, respectively for AP and OP across QC levels. Similarly, to ascertain the dilution integrity for 1/2 and 1/4th dilution, the accuracy and precision ranged from 94.3% to 97.2% and from 3.53% to 6.37%, respectively for both the analytes.

3.3. Comparative bioavailability study

To the best of our knowledge, this is the first report on the pharmacokinetics of AP in healthy Indian subjects. The purpose of the study was to evaluate the comparative bioavailability of a test and reference product in healthy subjects under fasting. The developed method was utilized to measure the plasma concentration of AP and OP following administration of a standard oral dose of 300 mg AP formulation to 44 young and healthy subjects (age range 25–45 years, BMI range 21.5–26.5 kg/m^2). Fig. 4 shows the time-plasma concentration profiles of AP and OP for both the formulations. The mean pharmacokinetic parameters evaluated from the plasma concentration-time curves of AP and OP are given in Table 5. As reported by Day et al. [2], AP is rapidly absorbed with peak plasma concentration (C_{max}) of about 2000 ng/mL at 2.32 h and then rapidly declines with half life ($t_{1/2}$) of 1.24 h, whereas the corresponding C_{max} and T_{max} values for OP were about 3 times higher than AP and the $t_{1/2}$ value was in the range of 25–26 h. This significantly higher elimination half life of OP allows AP to be administered once daily. Due to much shorter half life of AP together with the higher clinical significance of OP, the estimates of oral bioavailability between different formulations for bioequivalence assessment were mainly dependent on the plasma concentration of OP [2,3]. To assess the reproducibility of the method, 165 study samples near the C_{max} and the elimination phase in the pharmacokinetic profile of the drugs were reanalyzed. The results obtained demonstrate acceptable % change of ± 17% from the initial study results.

4. Conclusion

A present work relates to the development and validation of a simple, sensitive and a rugged LC–MS/MS method for the simultaneous estimation of AP and OP in human plasma and its successful implementation for a bioequivalence study in healthy subjects. The method involves detection and quantification of both the analytes under positive ESI mode and a single mobile phase for complete resolution unlike a previous study which employed different polarities and separate mobile phases for these analytes [14]. Further, the results for absolute and relative matrix effects show absence of matrix interference as evident from the IS-normalized matrix factors and in the measurement of AP and OP concentration from different plasma sources. The simple sample preparation step meets the high throughput requirement for clinical studies and is shown to be highly reproducible through the results of reanalyzed subject samples.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2016.05.005.

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