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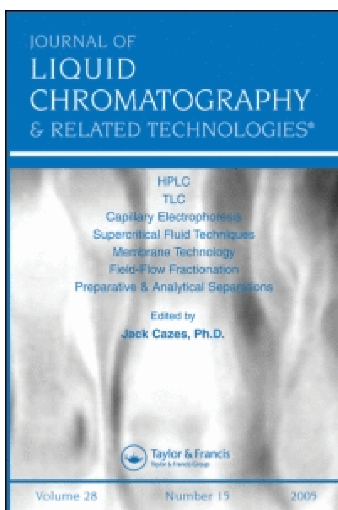

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VALIDATED RP-LC-MS/MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF ALLOPURINOL AND ITS MAJOR METABOLITE, OXYPURINOL, IN HUMAN PLASMA

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VALIDATED RP-LC-MS/MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF ALLOPURINOL AND ITS MAJOR METABOLITE, OXYPURINOL, IN HUMAN PLASMA

Gajanan Kasawar,[1] Mushtaq Razzak,[1] Zahid Zaheer,[2] and Mazahar Farooqui[1]

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□ *A rapid and highly sensitive LC-MS/MS method was developed and validated for the determination of allopurinol (AP) and its major metabolite, oxypurinol (OP) in human plasma using lamivudine as an internal standard (IS). The analytes were extracted from human plasma by protein precipitation using acetonitrile. The chromatographic separation was employed on "waters symmetry shield RP_8, 150 mm × 3.9 mm, 5 µm" columns using a mixture of 0.01% formic acid in water and acetonitrile in the ratio of 95:05 (v/v) as the mobile phase. Mass spectrometer in the selected reaction-monitoring mode with negative electro spray was used for the detection and quantification of the analyte. Recovery study was performed showing the accuracy at upper and lower limits of quantification. The stability study was also carried out. The described method was found to be linear over a range of 0.01–10 $\mu g\,mL^{-1}$ with a lower limit of quantification of 0.01 $\mu g\,mL^{-1}$ for both AP and OP. The coefficient of variation for the assay precision was found <6.94, and the accuracy was found >96.03. The extraction recoveries for AP and OP were found to be in between 70 and 80%, the accuracy was found to be in between 95 and 106% in human plasma. The dilution integrity test, hemolysis and anticoagulant effect, and matrix effect studies were reported.*

Keywords allopurinol, human plasma, LC-MS/MS, oxypurinol, validation

INTRODUCTION

AP, 1,2-dihydropyrazolo[4,3-e]pyrimidin-4-one (Figure 1) is a structural isomer of hypoxanthine (a naturally occurring purine in the human body) used primarily to treat hyperuricemia (excess uric acid in blood plasma) and its complications, including chronic gout; and it is an enzyme inhibitor

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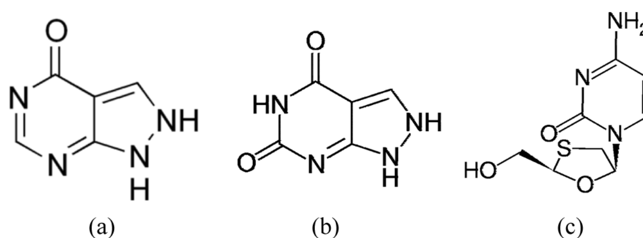

FIGURE 1 Chemical structures of (a) Allopurinol, (b) oxypurinol, and (c) lamivudine (IS).

of xanthine oxidase.[1] Xanthine oxidase is responsible for the successive oxidation of hypoxanthine and xanthine resulting in the production of uric acid, which is the product of human purine metabolism.[2] In addition to blocking uric acid production, inhibition of xanthine oxidase causes an increase in hypoxanthine and xanthine, which are converted to closely related purine ribotides adenosine and guanosine monophosphates. Increased levels of these ribotides causes feedback inhibition of amidophosphoribosyltransferase, which is the first and rate-limiting enzyme of purine biosynthesis. AP, therefore, decreases both uric acid formation and purine synthesis.

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About 90% of oral dosage of AP is rapidly absorbed from the gastrointestinal tract (its plasma half life is about 1 to 2 hr).[3] AP's major metabolite is OP (alloxanthine), which is also an inhibitor of xanthine oxidase with a plasma half -life of about 18 to 40 hr in patients with normal renal function. Although this is greatly prolonged by renal impairment, AP and OP are not bound to plasma proteins. Excretion is mainly through the kidney, but it is slow since OP undergoes tubular re-absorption.

Thorough literature survey reveals that few methods were reported for the estimation of AP and OP in human serum using HPLC,[4–9] CE,[10,11] electrochemical detection,[12–14] ion-exchange chromatography,[15,16] polarographic and voltammetric anodic detection,[17] and micelle-stabilized room-temperature phosphorescence in real samples.[18] However, these published methods have several limitations. 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 for AP and OP in serum under refrigerated conditions. To the best of our knowledge, no LC-MS/MS method was reported in any literature. Hence, we focused on developing a rapid and sensitive LC-MS/MS method for the simultaneous determination of AP and OP in human plasma. The objective of the present study was to develop a rapid and sensitive method for the determination of AP and its metabolite OP

using LC-MS/MS which enables quantitation of AP and OP in representative plasma samples obtained from daily clinical study.

EXPERIMENTAL

Reagents and Materials

AP, OP, and lamivudine (Internal standard IS) working standard were procured from Adura Labs, Aurangabad, India. The purity of AP, OP, and Lamivudine standards were 98.9, 98.4, and 99.7%, respectively. Lamivudine can be efficiently extracted by protein precipitation with AP and OP and can ionize in negative mode, as it is acidic in nature. Also, the structure of Lamivudine is similar to AP and OP. Hence, Lamivudine was selected as an internal standard. Drug free human heparinized plasma was obtained from the Marathwada Blood Bank, Aurangabad, India. Analytical reagent grade formic acid and dimethyl formamide, HPLC grade acetonitrile, and methanol were obtained from Merck Limited, Mumbai, India. High purity deionized water was obtained from Milli-Q purification system, Millipore, Bedford, MA, USA.

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Preparation of Standards and Quality Controls (QCs)

Stock Solution of AP and OP

A stock solution containing $1\,mg\,mL^{-1}$ each of AP and OP was prepared in dimethyl formamide.

Internal Standard (IS) Solution

Solution containing $1\,mg\,mL^{-1}$ of IS was prepared in methanol and, subsequently, diluted with methanol and water in the ratio of 80:20 (v/v) to get a concentration of $100\,\mu g\,mL^{-1}$.

Calibration Solution

The stock solutions of AP and OP were serially diluted and $10\,\mu L$ each of these diluted solutions were added, along with $25\,\mu L$ of IS solution to drug free human plasma in order to produce final concentrations of 0.10, 0.20, 0.75, 1.75, 5.00, 6.25, 8.00, and $10.00\,\mu g\,mL^{-1}$ for AP and OP. Calibration curves for AP and OP in human plasma were derived from the peak area ratio of AP or OP to the IS using linear regression with 1/x as a weighting factor. Quality control samples were analyzed along with each batch of plasma samples.

The QC samples were prepared in $490\,\mu L$ of blank human plasma by adding $10\,\mu L$ of the diluted stock solutions to give AP and OP concentrations

of 0.3, 4.5, and 8.5 μg mL^{-1}. The QC samples were used to evaluate the intra- and inter-day precision and accuracy of the method. All prepared plasma samples stored at −70°C (HFU 486 Basic, Heraeus) and stock solutions were stored at 2 to 10°C (refrigerator).

Sample Preparation

A 500 μL aliquot of plasma sample was spiked with 25 μL of IS, and 1 mL of acetonitrile was added to this solution. This mixture was vortexed for about 5 min for complete protein precipitation. Samples were centrifuged at 15000 rpm for 5 min at 10°C to get a clear supernatant solution. About 200 μL of this supernatant solution was mixed with 800 μL of mobile phase and injected directly into the LC-MS/MS system.

Analytical System

AP and OP in plasma were quantified using a SCIEX API 4000 LC-MS/MS system (Applied Biosystems, USA) equipped with an electrospray ionization interface to generate negative ions $[M-H]^-$. Shimadzu SIL-HTc series HPLC system was used throughout the analysis. The chromatographic separation was achieved on a Symmetry Shield RP18, 150 mm × 3.9 mm, 5 μm column (Waters Corporation, Milford, USA). The mobile phase involved an isocratic composition of 0.01% formic acid and acetonitrile in the ratio of 95:05 (v/v) and was pumped through the column with a flow rate of 0.8 mL min^{-1}. The column compartment was thermostats at 40°C and auto sampler temperature was kept at 10°C.

The precursor ions $[M-H]^-$ and the pattern of fragmentation were monitored using the negative ion mode. The major peaks observed in MS/MS scan were used to quantify AP, OP, and IS. The ion spray voltage and temperature were set at −4500 V and 500°C, respectively. The typical ion source parameters, viz, declustering potential, collision energy, entrance potential, and collision cell exit potential were −31, −23, −5, and −5 V, respectively, for AP; −20, −32, −5, and −5 V, respectively, for OP; and −58, −20, −10, and −10 V, respectively, for the IS. Nitrogen gas was used as the nebulizer gas, curtain gas, and collision-induced dissociation (CID) gas, and the gas pressure was set at 50, 20, and 80 psi, respectively. Quantification of analytes was performed by selected reaction monitoring of the deprotonated precursor ion and the related product ion for AP and OP. The mass transitions used for AP, OP, and IS where m/z 135.0 → 92.1, 151.0 → 42.0, and 228.0 → 133.9, respectively, and a dwell time of 200 ms per transition. Quadrupoles Q_1 and Q_3 were set on unit resolution. The analytical data were processed by analyst software (Version 1.4.1; Applied Biosystems).

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Method Validation

The optimized LC-MS/MS method was validated according to the US Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods with respect to selectivity, injector carry over test, linearity, sensitivity, accuracy, precision, dilution integrity, re-injection reproducibility, anticoagulant, hemolysis, matrix effects, recovery, and stability in human plasma.

Selectivity

Selectivity of the method was studied by comparing the chromatograms of six different lots of human heparin plasma, two lots of EDTA plasma, and one each of lipemic and hemolyzed plasma samples, with the plasma samples spiked with AP, OP, and IS.

Injector Carry over Test

Injector carry over test was performed by injecting the higher concentration of AP or OP with the IS followed by a blank plasma sample. Calibration curves were prepared using standard plasma samples of AP and OP concentrations ranging from $0.1-10\,\mu g\,mL^{-1}$.

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Linearity

The plots of peak area ratio of AP or OP to the IS were under the curve (AUC) of the peak responses of the analytes against their corresponding concentrations; they fitted straight lines responding to equations. The calibration curves were constructed by weight (1/x) least squares linear regression. The y-intercepts were close to zero with their confidence intervals containing the origin. The correlation coefficients (r) exceeded 0.98, the acceptance threshold suggested for linearity of procedures. Furthermore, the plot of residuals exhibited random patterns with the residuals passing the normal distribution test ($p < 0.05$), all of which evidenced that the method is linear in the tested range.

Limit of Quantification

The lower limit of quantification (LLOQ) for AP and OP in human plasma was defined using the signal-to-noise ratio method and is estimated as ten.

Accuracy and Precision

Accuracy was evaluated by the simultaneous determination of analytes in solution prepared by the standard addition method. The experiment was carried out by adding known amounts of each components corresponding to three concentration levels of 80%, 100%, and 120% of the target

analyte concentration. Accuracy and precision was verified by the analysis of 10 replicates.

Within and between-batch accuracy and precision for this method was determined at three different concentration levels on four different occasions and, on each occasion, six replicates were analyzed using independently prepared calibration curves. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) × 100 and precision as the relative standard deviation.

Dilution Integrity Test

The dilution integrity test for AP or OP was performed by diluting the plasma samples spiked with 20 μg mL^{-1} for 5 and 10 times with blank plasma. Six replicates for each dilution were analyzed and concentrations were obtained by multiplying the respective dilution factor. Six replicate samples spiked with 0.3 and 8.5 μg mL^{-1} of AP or OP, respectively, were kept in an auto sampler after analysis for precision and accuracy determination at 10°C and re-injected after a definite time interval.

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Hemolysis and Anticoagulant Effect

Hemolysis and anticoagulant effects were determined at two different concentration levels in hemolyzed plasma and in different anticoagulants (EDTA). These samples were analyzed with freshly processed calibration (processed in blank human heparin plasma) sets.

Effect of Matrix

To evaluate the matrix effect of AP and OP at the ionization of the analyte, i.e., the potential ion suppression or enhancement caused by the matrix components, plasma from six different sources was used. The matrix effect and extraction recovery values are calculated as follows.

$$\text{Matrix effect } (\%) = \frac{B}{A} \times 100$$

$$\text{Extraction recovery } (\%) = \frac{C}{B} \times 100$$

Where:

A: Peak area of solvent standard solution
B: Peak areas for solvent standard solutions spiked after extraction into plasma extraction
C: Peak areas for solvent standard solutions spiked before plasma extraction into plasma extraction.

TABLE 1 Stability Results of Allopurinol and Oxipurinol in Human Plasma

	Allopurinol		Oxypurinol	
Long-term stability				
Reference (n = 6)*	0.297 μg/mL	8.406 μg/mL	0.300 μg/mL	8.492 μg/mL
38 days (−70°C) (n = 6)[a]	−2.72%	1.02%	−4.63%	1.59%
CV (%)	4.27%	4.19%	−2.42%	3.07%
Freeze-thaw stability				
Reference (n = 6)*	0.297 μg/mL	8.406 μg/mL	0.300 μg/mL	8.492 μg/mL
After 3 cycles (n = 6)[a]	2.10%	−2.09%	9.98%	1.16%
CV (%)	4.24%	2.56%	4.54%	6.96%
Autosampler stability				
Reference (n = 6)*	0.297 μg/mL	8.406 μg/mL	0.300 μg/mL	8.492 μg/mL
52 h (n = 6)[a]	2.53%	2.53%	3.46%	−1.39%
CV (%)	4.26%	5.57%	2.56%	5.75%
Coolant stability (24 hr in dry ice)				
Reference (n = 6)*	0.297 μg/mL	8.406 μg/mL	0.300 μg/mL	8.492 μg/mL
24 hr (n = 6)[a]	4.20%	−8.12%	8.68%	−6.22%
CV (%)	5.72%	10.90%	7.83%	10.84%
Bench top stability (12 hr at room temp)				
Reference (n = 6)*	0.297 μg/mL	8.406 μg/mL	0.300 μg/mL	8.492 μg/mL
12 hr (n = 6)[a]	−0.49%	−4.74%	3.01%	−4.80%
CV (%)	0.44%	9.71%	3.85%	9.25%

CV = Coefficient of Variation; *Nominal Concentration; [a]% change.

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The matrix effect and extraction recovery of the IS were evaluated using the same method. All assays were performed by using six replicates at concentrations of 0.3 and 8.5 μg mL^{-1} of AP and OP, respectively.

Stability in Human Plasma

The stability of AP or OP in human plasma was assessed by analyzing six replicate samples spiked with 0.3 and 8.5 μg mL^{-1}, respectively, of AP or OP under four different conditions. Storage at room temperature for 12 hr (short term storage), storage in dry ice for 24 hr, storage at −70°C for 38 days (long term storage), and freeze–thaw three cycles and sample preparation for 52 hr at 10°C (autosampler temperature). All these stability samples were analyzed with freshly processed calibration and comparison samples. Concentrations were calculated to determine % change over time. The stability data is represented in Table 1.

RESULTS AND DISCUSSION

Optimization of Mass Spectrometry and Chromatographic Conditions

LC/MS/MS, Triple Quad, Sciex, API 4000 coupled with ESI source was employed for the analysis. MS conditions were optimized by tuning the

compound dependent parameter for analyte, metabolite, and internal standard, respectively, for stable and intense peak. Initially, the source temperature was set at 400°C and gas pressure, such as nebulizer gas, curtain gas, and collision-induced dissociation gas were set at 30, 15, and 60 psi, respectively. During method development, both polarity (i.e., positive and negative) were tried, but a more stable and more intense molecular and fragment ion was found in the negative mode with better signal to noise ratio with only one fragment. As AP and OP are both weak acids, they get well ionized in the negative mode; hence, we have selected negative mode only. In the negative ion mode, both AP and OP gave deprotonated molecules, $[M\text{-}H],^{-}$ as the major ionic species.

It is clear from Figure 1 that all three components are pharmaceutical organic compounds and have a slight polarity; hence, for the better chromatography, middle polarity stationary phase (C_8) (i.e., Waters Symmetry $C_{8,}$ 50 × 4.6 mm, 5μ column) was employed. A mixture of acetonitrile and 0.2% acetic acid in water in the ratio of 85:15 (v/v) was used as the mobile phase and pumped through the column at a 0.4 mL min^{-1} flow rate. All three components were separated well within two min. But, in the blank plasma sample, interference was observed at the retention time of allopurinol. To separate the interference, Waters Symmetry Shield RP18, 150 × 3.9 m, 5μ column was used. The mobile phase consisted of 0.01% formic acid and acetonitrile in the ratio of 95:5 (v/v). To reduce the run time, the flow rate was increased to 0.8 mL min^{-1}, and all the three peaks were eluted well within 5 min. The final methodology utilized is already discussed in the Analytical System section.

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Method Validation

The specificity of the method was established by injecting the blank plasma and spiked blank plasma. It was found that there is no interference at the retention time of AP, (4.03 min), OP, (3.70 min), and IS, (1.80 min). Typical chromatogram of blank plasma spiked with 100 μg mL^{-1} of IS and 0.1 μg mL^{-1} of AP and OP in plasma is shown in Figure 2. No significant injector carry over was observed. The calibration curves for human plasma provided reliable responses for AP and OP from 0.1 to 10 μg mL^{-1}. The best linear fit and least-squares residuals for the calibration curve were achieved with 1/x weighing factor. The mean correlation coefficient during the validation was found to be 0.9988 for AP and OP in human plasma. The LOQ for both AP and OP (0.1 μg mL^{-1}) was determined in human plasma.

The intra- and inter-day precisions and accuracy were determined by analyzing six replicates of QC samples at three concentrations on 4 different days and are listed in Table 1. The coefficients of variation for the

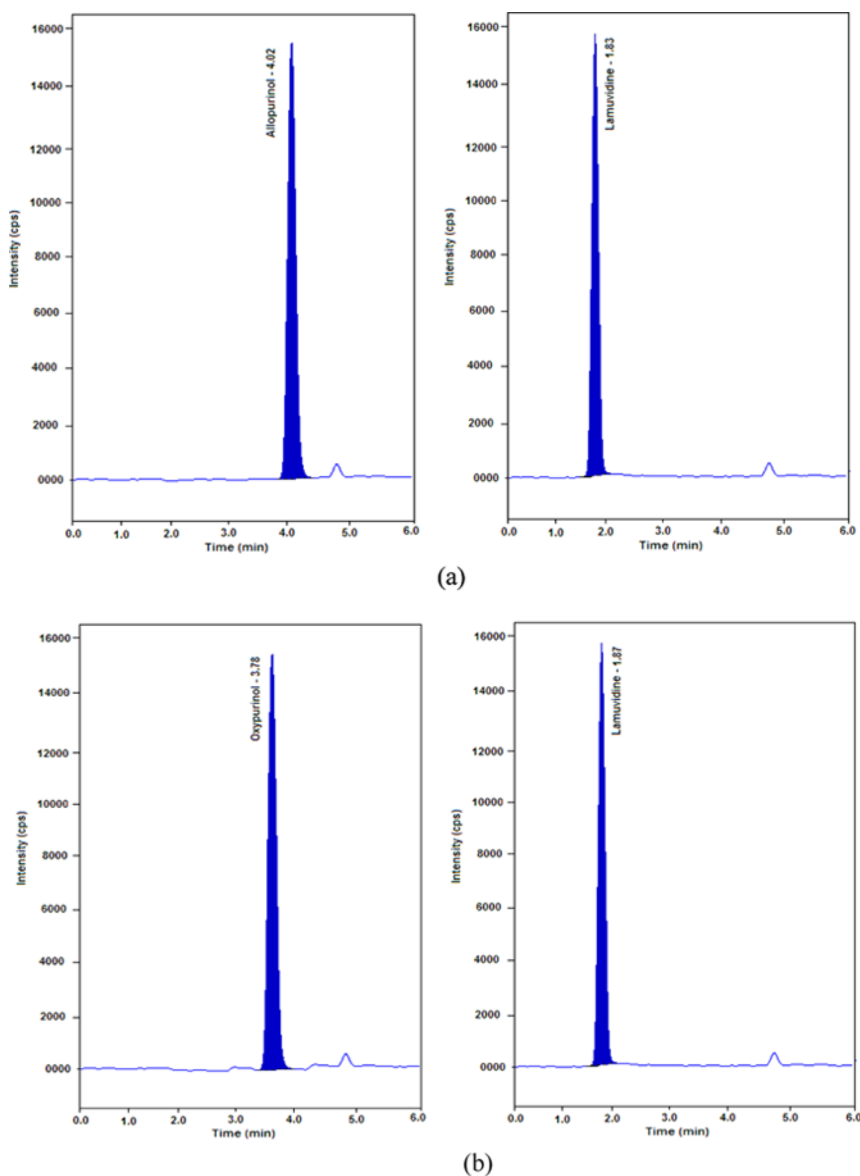

FIGURE 2 Extracted zero standard plasma spiked with (a) 0.1 μg mL^{-1}of Alopurinol and 100 μg mL^{-1} of lamivudine and (b) 0.1 μg mL^{-1} of oxypurinol and 100 μg mL^{-1} of lamivudine. (Figure available in color online.)

intra- and inter-day precision and percentage accuracy were found well within the acceptable limit and the results are tabulated in Table 2.

The extraction recoveries of AP and OP were studied and the results are tabulated in Table 3. With a low matrix effect, consistent and reproducible recovery proved to be reliable for bioanalysis.

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TABLE 2 Precision and Accuracy Data of Allopurinol and Oxypurinol in Human Plasma

		Within Batch[a]			Between Batch[b]		
Compound	Amount Added Found ($\mu g\,mL^{-1}$)	Amount Found ($\mu g\,mL^{-1}$)[c]	CV (%)	Accuracy (%)[d]	Amount Found ($\mu g\,mL^{-1}$)[c]	CV (%)	Accuracy (%)[d]
AP	0.297	0.302 ± 0.012	4.12	101.63	0.298 ± 0.013	4.45	100.38
	4.450	4.623 ± 0.150	3.25	103.89	4.704 ± 0.242	5.15	105.70
	8.406	8.537 ± 0.317	3.72	101.56	8.105 ± 0.390	4.81	96.42
OP	0.300	0.317 ± 0.022	6.91	105.61	0.299 ± 0.021	6.94	99.71
	4.496	4.699 ± 0.147	3.12	104.52	4.805 ± 0.251	5.23	106.88
	8.492	8.571 ± 0.320	3.73	100.93	8.155 ± 0.468	5.73	96.03

$n = 6$.
CV = coefficient of variation;
[a]six repetitions each;
[b]four separate batch, six repetitions each batch;
[c]mean ± standard deviation;
[d]relative deviation (%).

In the dilution integrity experiment, the calculated concentrations for AP and OP, including the dilution factor for 1/10th and 1/5th, yielded coefficients of variation of <10.25 and <10.99, respectively. AP or OP was found stable in the autosampler and reproducible after reinjection. No hemolysis and anticoagulant effect was observed. In the percentage of the matrix effect, all the ratios (A/B × 100)% as defined in the Validation Procedure section were between 85 and 115%, which indicates that no significant matrix effect was found for AP, OP, and IS in this method.

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Stability Results of AP and OP in Human Plasma

The stability of solutions containing AP, OP, and IS were studied at different storage conditions, such as long term stability (−70°C) for 38 days, freeze-thaw stability after three cycles, coolant stability in dry ice for about 24 hr, and room temperature stability for about 12 hr. The solution was found stable in all storage conditions with coefficients of variation (CV)

TABLE 3 Extraction Recovery Data of Allopurinol and Oxypurinol in Human Plasma

	Allopurinol		Oxipurinol		Lamivudine (IS)	
Level	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
50%	73.93	1.58	71.39	1.75	68.56	1.63
100%	78.10	0.79	75.26	1.11	65.43	1.39
150%	76.40	0.78	73.85	1.81	67.93	0.98

$n = 3$.

of 3–10. No significant degradation of AP or OP in human plasma was found in any storage conditions.

CONCLUSION

The developed method is a rapid and sensitive LC–MS/MS method with protein precipitation for the simultaneous determination of AP and OP in human plasma over a concentration range of 0.1–10 μg mL^{-1}. Upper limit of quantification was covered in the recovery study, showing the accuracy at upper and lower levels. A stability study was carried out and an efficient LC-MS/MS method for the quantification of AP and its metabolite OP in human plasma was developed and validated. Validation experiments provided proof that the LC-MS/MS analytical method is linear in the proposed working range as well as accurate, precise (repeatability and intermediate precision levels), and specific. The extraction recoveries for AP and OP were found between 70 to 80% and accuracy was found between 95 to 106% in human plasma. Also, the dilution integrity test, hemolysis and anticoagulant effect, and matrix effect for any change in the assay of AP and OP were studied. The assay value for AP and OP was found well within the acceptable limit. Due to these characteristics, the method has stability, indicating the method evidences appropriate properties suitable for its intended purpose. Thus, it may find application for the routine quality control analysis of AP and OP in human plasma. This method requires only 0.5 mL of a biological sample and, owing to the simple sample preparation and short run time (5.5 min), it allows high sample throughput.

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