

**RAPID AND SENSITIVE LC-MS/MS METHOD FOR THE ANALYSIS OF ALLOPURINOL IN HUMAN PLASMA****V.V.RAMI REDDY*¹ AND K. HUSSAIN REDDY²**¹*Bioanalytical Dept. Apotex Research Pvt. Ltd, Bangalore- 560099, India*²*Department of Chemistry, Sri Krishna Devaraya University, Anantapuramu-515003, India.***ABSTRACT**

A rapid and highly sensitive liquid chromatography/tandem mass spectrometric (LC/MS/MS) method for determination allopurinol (AP) in human plasma was developed. The analyte and internal standard (IS), Ganciclovir-d₅, were extracted from human plasma via liquid/liquid extraction (LLE) with ethyl acetate (2.5mL), supernatant was dried and reconstituted in 200 µL of 10mM ammonium acetate-methanol (80:20 V/V) solution and separated on an Waters Symmetry Shield, RP8, 3.5µm, 4.6 x 100mm within 4 min. Quantification was by multiple reaction monitoring (MRM), where the acquired mass for allopurinol and IS are m/z 136.7→110.2 and 261.1→151.8 respectively. The assay was validated with linear range of 50–3000 ng/mL. The intra- and interday precisions (RSD %) were within 1.71 to 10.34% and 3.79 to 7.43% respectively. This method is suitable for the quantification of AP in human plasma for real sample analysis.

KEYWORDS: LC-MS/MS method, allopurinol, ganciclovir-d₅, liquid- Liquid extraction**V.V.RAMI REDDY**

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INTRODUCTION

Allopurinol (AP) is known chemically as 1, 5-dihydro-4*H*-pyrazolo [3, 4-*d*] pyrimidin-4-one. It is used to treat the hyperuricemia (excess uric acid in blood plasma) and its complications. It is commonly used as prophylaxis with chemotherapeutic treatments, which can rapidly produce severe hyperuricemia. AP is approximately 90% absorbed from the gastrointestinal tract. Peak plasma levels generally occur at 1.5 hours and 4.5 hours. The elimination of the substance is fast, with a half-life of approximately 2.0 h. AP acts on purine catabolism, without disrupting the biosynthesis of purines. It reduces the production of uric acid by inhibiting the biochemical reactions immediately preceding its formation¹. Allopurinol has gained widespread favor in the treatment of gout and other hyperuricemic states. Although it is conventional to prescribe allopurinol in multiple divided doses each day, the drug is metabolized in man in such a way as to suggest that a single daily dose may prove equally effective² and commonly used in the treatment of chronic gout or of hyperuricaemia associated with leukaemia, radiotherapy, anti-neoplastic agents and treatment with diuretics³. In the light of the above, a rapid and highly sensitive LC-MS/MS method is 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 are extracted from human plasma by protein precipitation using acetonitrile⁴. A new assay method is developed for the simultaneous determination of allopurinol and oxypurinol in plasma and urine using ultrafiltration and ion exchange purification steps for plasma and urine, respectively. Reversed-phase high-performance liquid chromatography with ultraviolet detection is applied for the separation and quantitation of both compounds⁵. A rapid and highly sensitive LC/MS/MS method for the simultaneous determination of AP in human plasma is reported in this article. The application of this method is demonstrated for the quantification of AP in plasma. A high performance liquid chromatographic (HPLC) assay is described for allopurinol and oxypurinol determination in

human plasma and urine. The procedure involves addition of trichloroacetic acid to samples, followed by centrifugation. The supernatant is then neutralized and analyzed by reversed-phase HPLC. Characteristics of the method are reported, and data are presented on its application to the pharmacokinetics studies. Separation is optimal with an octadecylsilane (ODS) stationary phase and a sodium acetate mobile phase adjusted to pH 7.2 for plasma and pH 5 for urine⁶. Another method is based on high-performance ion-exchange chromatography following an efficient sample purification step using Chelex-100 resin in the Cu²⁺-form⁷. Allopurinol, oxypurinol and uric acid have been determined in human serum and urine by liquid chromatography with electrochemical detection. In particular the use of a polarographic detector operating in the oxidative mode, whose principle of detection is based on the property of allopurinol, oxypurinol and uric acid to form insoluble anodic films on mercury. The performance of such a detector is compared with that of a glassy carbon wall-jet detector⁸.

MATERIALS AND METHODS

Allopurinol was supplied by Creative Organics (Bangalore, India). Ganaciclovir-d5 (Internal standard, IS) was obtained from Apotex Inc., Canada. Methanol and ethyl acetate of HPLC grade from JT Bakers and Labscan make respectively were used. Ammonium acetate and formic acid were procured from Merck and Milli-Q purification system from Millipore (Bangalore, India). Control human plasma with K₂-EDTA as an anticoagulant was procured from (Dr. Pathological Lab, Meerpet, Hyderabad, India) and was stored at -30°C. Symmetry Shield, RP8, 3.5µm, 4.6 x 100 mm was purchased from Waters (Bangalore, India).

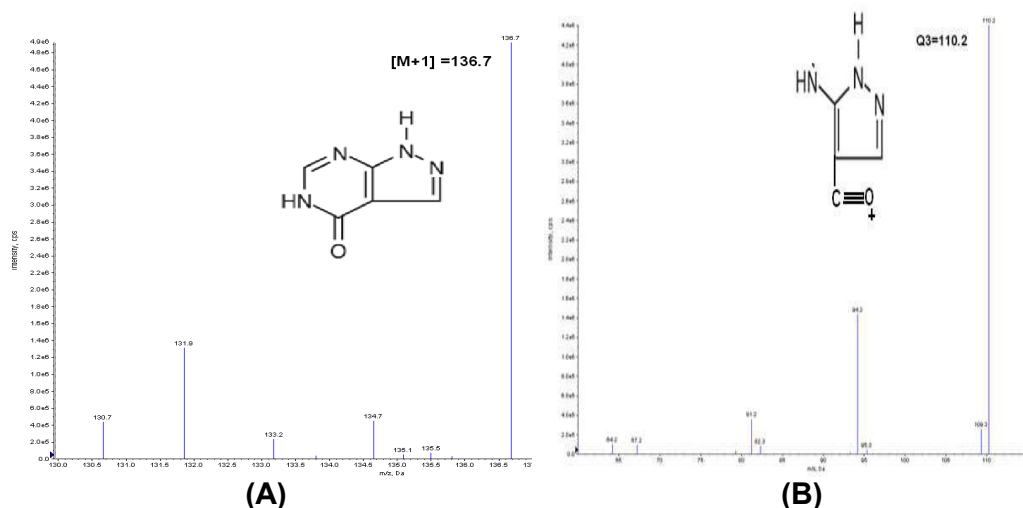
Instrumentation and conditions

A Sonicator model 3510 from Bronson, centrifuge 5810 R from Eppendorf, Heidolph vibramax 110 and IKA Vortex GENIUS 3 were used. A series 1200 HPLC system (Agilent, USA) consists of a quaternary pump, an

autosampler and an online degasser and connected to PAL CTC auto sampler. The analyte and IS are separated on Waters Symmetry Shield, RP8 (3.5 μ m, 4.6 x 100mm) with the mobile phase, 10mM ammonium acetate and methanol (80:20 (v/v)), at a flow rate of 0.6 mL/min. The autosampler temperature was maintained at 40°C and the injection volume was 10 μ L. The total LC run

time was 4 min. Detection of analytes and IS was performed on API 4000 tandem quadrupole mass spectrometer (Applied Biosystems, USA). Turbo Ion spray (API) Positive Mode MRM was used for the detection. Multiple reaction monitoring (MRM) was used to monitor precursor to product ion transition of 136.7 \rightarrow 110.2 for AP and m/z 261.1 \rightarrow 151.8 for IS (Figure 1)¹

Figure 1
Mass spectra from infusion of (A) Parent ion and (B) Product ion for AP carried with reconstitution solution.



All parameters of LC and MS were controlled by analyst software version 1.5.1. For AP and IS the source parameters were ion spray voltage (ISV) 5500V, turbo heater temperature (TEM) 550 °C, collision activation dissociation (CAD) 7psi, curtain gas (CUR) 20 psi. The compound dependent parameters like declustering potential (DP) and collision energy (CE) were optimized at 60 volts and 30 eV for AP and 45 volts and 20 eV for IS, respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 300 ms for both the analyte and IS.

Preparation of standard stock solution & plasma samples

The standard stock solution was prepared by dissolving 1mg of AP in methanol. The IS standard stock solution was prepared by dissolving 5.00 mg of ganciclovir-d5 in 10 mL of 10% methanol in 0.1% formic acid. The working solution was prepared by dilution with 50% methanol to the final concentration of 25

μ g/mL. All the solutions were stored at refrigerated conditions (2 - 8 °C). Drug free plasma (blank plasma) was stored in the freezer and allowed to completely thaw before use. The calibration standards and quality control (QC) samples were prepared by spiking blank plasma with combined working solutions. Calibration standards were made at 50.0, 100.0, 200.0, 500.0, 1000.0, 1200.0, 1800.0, 2400.0 and 3000.0 ng/mL and final AP quality control concentrations were 50.0, 150.0 900.0 and 2100.0 ng/mL. The spiked plasma samples were stored at -30 °C for validation.

Procedure of sample extraction

Allopurinol (AP) is sensitive to light; therefore, samples were prepared under gold light. Liquid-liquid extraction was used to isolate AP from human plasma. In a 5mL polypropylene centrifuge tube an aliquots of 200 μ L plasma, mixed with 100 μ L working solution of IS (25 μ g/mL) and 2.5 mL of ethyl acetate

solvent, was vortexed for 10 min, followed by centrifugation for 10 min at 4000 rpm, at 20 °C temperature. 2.5mL of supernatant was transferred into another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue, dissolved into 200 µL reconstitution solution, was vortexed for 1 min and injected into the LC/MS/MS system for analysis^{9, 10}.

RESULTS AND DISCUSSION

Method Validation

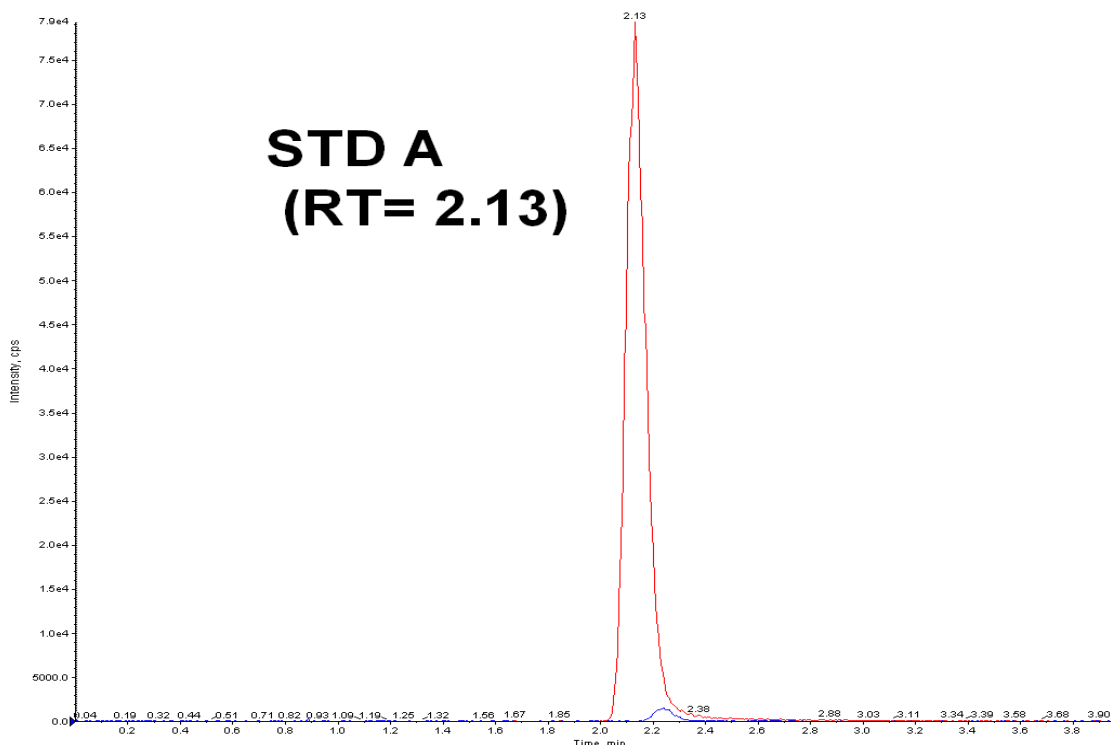
Analytical method validation was carried out for AP in human plasma. The method was validated for selectivity, sensitivity, carry over check, linearity, precision and accuracy, reproducibility, recovery, matrix effect, stability studies in matrix i.e. Bench Top stability, Prepared samples stability, Freeze/Thaw cycle and Long term stability and stabilities in

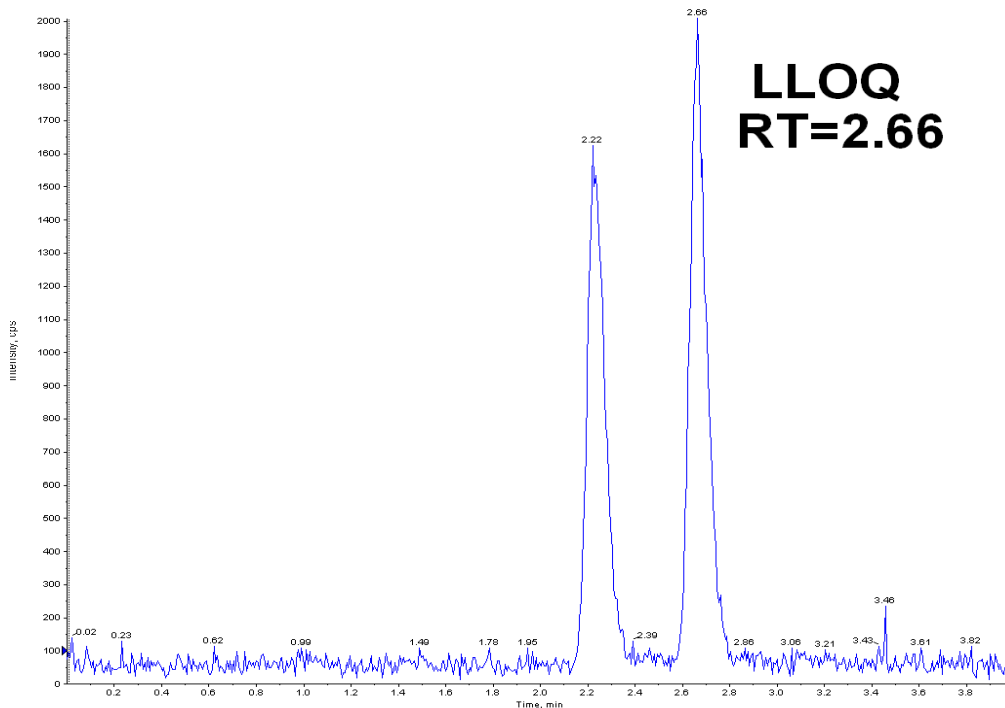
neat. i.e Master stocks, IS spiking solutions and neat standards¹⁰.

Selectivity and Sensitivity (LLOQ)

Ten different lots of plasma (obtained from The Doctor's Pathological Lab's, Merrpet, Hyderabad-36, Andhra Pradesh, India) were extracted along with the lowest analytical standard STD B (50.0 ng/mL in plasma) and blank with internal standard (STD A) chromatographed as per the analytical method. Results showed that there is no significant interference from endogenous plasma components. The liquid-liquid extraction methodology in combination with mass spectrometry detection gave very good selectivity for AP and IS. Figure 2 shows that the total ion chromatography (TIC) of AP and IS. The retention times were 2.69 min and 2.13 min for AP and IS respectively.

Figure 2
Chromatograms of STD A sample and LLOQ sample





Standard Curve

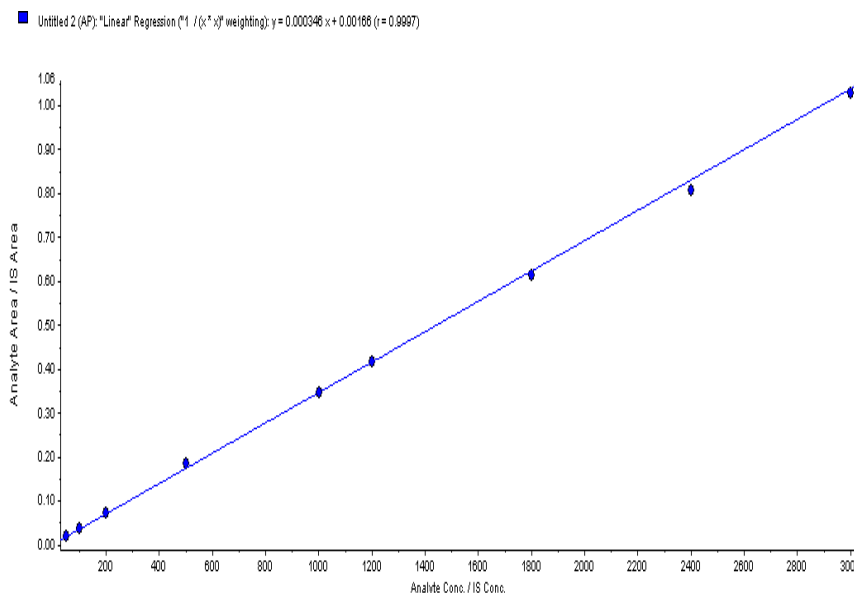
All calibration curves are linear (Figure 3) over the concentration range 50-3000ng/mL. A weighted $1/\text{conc}^2$, linear regression model describes the standard curve for AP with correlation coefficient $r^2 \geq 0.9993$. A straight line fit was made through the data points by least square regression analysis to give the mean linear equation:

$$Y = mX + C$$

Where m = slope,
 C = intercept,
 Y = peak area ratio
 X = standard concentration

Unknown Concentration $X = (Y-C)/m$

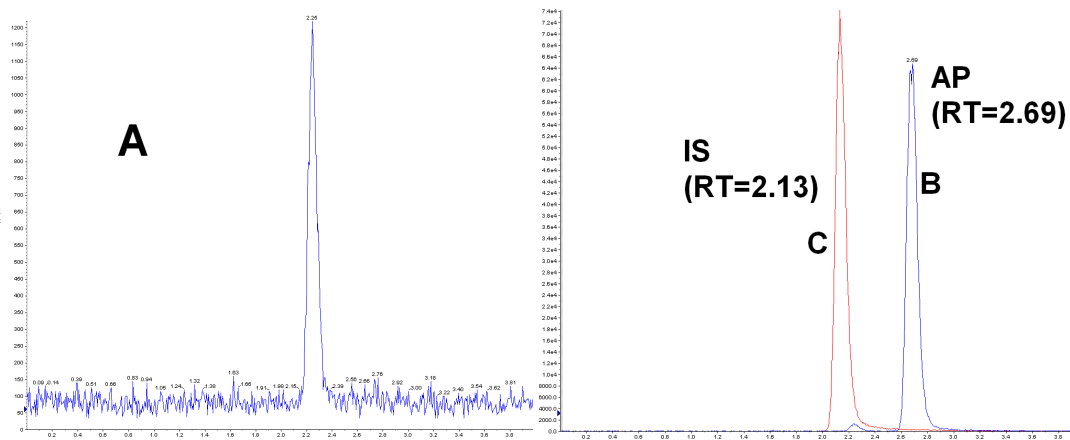
Figure 3
Linearity graph for AP over the concentration range 50-3000 ng/mL



Carry-over

Carry-over test was performed to ensure that it does not affect the accuracy and precision of the proposed method. There is no significant area count was observed in blank run after ULQ which suggests no carry-over of the analyte in subsequent runs (Figure 4)¹¹.

Figure 4
Chromatograms of (A) Blank , (B) AP and (C) IS



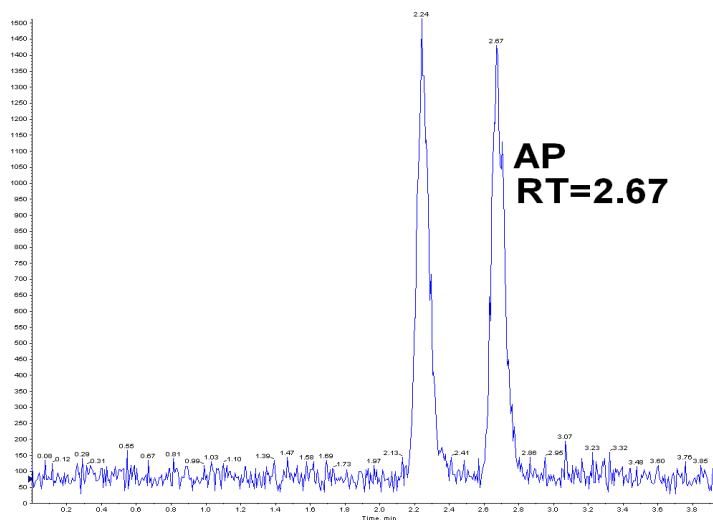
Chromatograms show that no carry-over of the analyte in subsequent run.

Limit of quantitation

The analytical lower limit of quantitation for AP was determined by six replicate assays of the LOQ QC (QC D, 50.0 ng/mL plasma concentration) within each assay. Figure 5

represents the LOQ chromatogram. The intra-assay precision (CV %) for the LOQ samples ranged from 2.33 to 8.19. The intra-assay accuracy (% Dev) ranged from 93.20 to 106.26. The inter-assay precision (CV %) and inter-assay accuracy (% Dev) for the LOQ samples were 8.19 and 102.29 for AP¹¹.

Figure 5
Chromatogram of lower limit of quantitation for AP



Precision and Accuracy

Accuracy and precision of the assay was evaluated on an inter-assay basis using prepared QC standards at three concentrations spanning the range of the

analytical standard curve. Accuracy and precision were evaluated at three QC levels LOW, MID and HIGH of concentration 150.0, 900.0 and 2100.0 ng/mL respectively (as discussed in the Preparation of standard stock

solution & plasma samples) in six replicates for AP. The precision is expressed as CV%, and accuracy is expressed as %Deviation (%Dev). The results are summarized in Table 1. As it can be seen inter-day precision varied

between 3.79% and 7.43%, and intra-day precision between 1.71% and 10.34%. Inter-day accuracy varied from 99.85% to 105.84% and intra-day accuracy from 95.53% to 113.47%¹⁰.

Table 1
Intra and Inter-day Precision and Accuracy of QCs

Quality control ID	Precision (CV %)		Accuracy (% dev.)	
	Inter	Intra	Inter	Intra
LOW	5.42	1.71 to 4.13	105.84	99.79 to 113.47
MID	3.79	2.02 to 5.10	104.04	101.48 to 105.84
HIGH	7.43	2.93 to 10.34	99.85	95.53 to 104.01

The precision (% Coefficient of variation (CV)) at each concentration level from nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85-115%¹¹.

Recovery

Recovery of AP and the internal standard (Ganciclovir-d5) was assessed by comparing the peak response of extracted spiked plasma against that of the neat standards. The QC standards covering the entire range of concentrations (i.e. 50.0, 900.0 and 2100.0ng/mL) used for the calibration standards were used for the testing. Results are presented in Table 2.

Table 2
Recovery of Allopurinol and the internal standard

Compound	% Recovery	
Allopurinol	LOW	46.98
	MID	46.77
	HIGH	49.56
	Mean	47.77
Ganciclovir-d5	LOW	29.00
	MID	29.88
	HIGH	29.62
	Mean	29.50

Matrix Factor

To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows:

$$\text{Matrix Factor} = \frac{\text{peak response ratio (analyte/IS) in the presence of extracted matrix}}{\text{Peak response ratio (analyte/IS) in the absence of extracted matrix (neat standards)}}$$

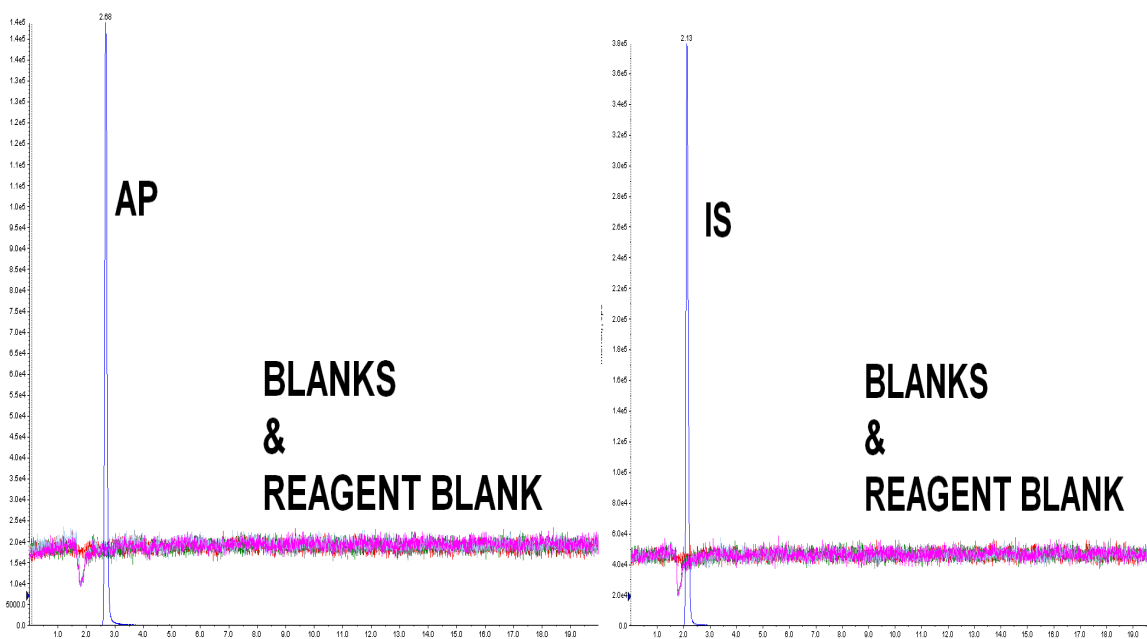
Matrix factor was determined at mid QC concentrations. Six lots of blank biological matrix were spiked each in triplicates with the neat standard at the mid QC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor will be expressed as coefficient of variation (CV %) and was determined to be 1.48 for AP^{12, 13}.A

matrix factor value higher than 100% indicates enhancement, whereas a lower one indicates suppression effects.

Infusion Matrix Effect

Ion suppression matrix effect was evaluated by injecting reagent blank, different lots of blank plasma (6 lots from K2 EDTA blank plasma) of extracted blank matrix while neat standard being infused. No significant matrix effect was observed (Figure 6).

Figure 6
Ion suppression matrix effect of AP and IS by Infusion method



Stability

AP in the blank biological matrix was stressed in three ways: by long-term storage in a -30°C set point freezer for 17days; by storage at room temperature for 24.5 hours; and by three cycles of freeze-thaw. Prepared samples were stressed by storage at refrigerated conditions for 29.0 hours. Stability comparing stressed samples with appropriate unstressed references illustrate AP is stable under the stress conditions applied. Master stock solutions were stressed by long-term storage at refrigerated conditions for 137 days for AP

and 31 days for IS combined with storage at room temperature for a minimum of 6 hours on the day of testing. Neat standard comprised of AP and ISTD was stressed by storage at refrigerated conditions for 26 days. Internal standard spiking solution was stressed at refrigerated conditions for 26 days. Stability comparing stressed samples with appropriate unstressed references illustrates AP and IS were stable under the stress conditions applied¹¹. Summary of stability experiments is given in Table 3.

Table 3
Summary of stability experiments

Stability	Test Mean ± SD
Plasma sample	24.5 hours at room temperature
freeze-thaw	Three cycles
Prepared sample	29.0 hours at refrigerated conditions
Master stock solutions	137 days for AP and 31 days for IS at refrigerated conditions
Neat standard	26 days at refrigerated conditions
Internal standard spiking solution	26 days at refrigerated conditions
long-term plasma sample	17days at -30°C set point freezer

Application of the method

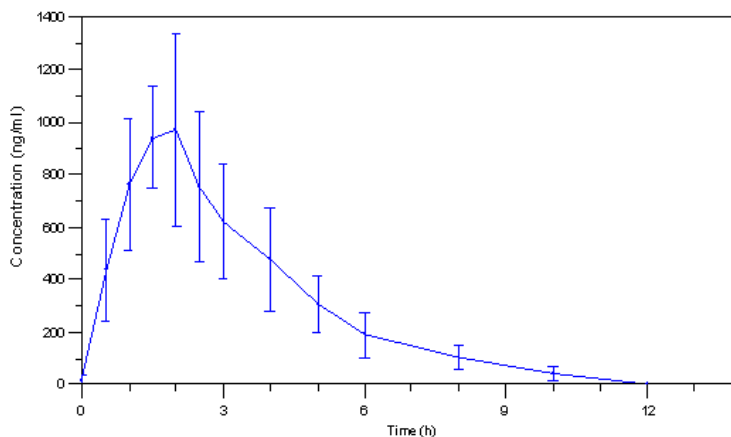
The method was applied to estimate the plasma concentration of AP in 6 healthy male volunteers received an oral dosage of allopurinol 100 mg (CIPLORIC 100 tab from CIPLA) under fasting condition. Blood was collected into K₂EDTA vacutainer tubes before drug administration (0.0h) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 12.0 h. After blood collection plasma was separated by centrifuge at 3000 rpm for 10

mins at 5°C and plasma samples were stored at -30°C freezer until analysis. The concentration of AP in plasma has been measured by AP analytical method for 6 healthy male volunteers. The pharmacokinetic parameters were assessed for 6 volunteer's data and pharmacokinetic parameters are presented in Table 4 and the mean plasma concentration of AP versus time profile is represented in Figure 7¹⁴.

Table 4
Mean pharmacokinetic parameters of allopurinol after oral administration of 100 mg tablet formulation

Parameter	Test Mean ± SD
C _{max} (ng/mL)	1109.17 ± 293.05
T _{max} (h)	1.50 ± 0.45
t _{1/2} (h)	1.08 ± 0.25
AUG _{0-t} (h. ng/mL)	3747.29 ± 1127.31
AUG _{0-∞} (h. ng/mL)	3760.71 ± 1123.88
K _{el} (1/h)	0.67 ± 0.15

Figure 7
Represent the mean plasma concentration of AP versus time profile

**Incurred Sample Repeats**

Incurred samples were analyzed to support the precision and accuracy of the analytical method and validity of the study data. Results were met the specifications^{7,14}.

Specifications

No more than 33.0% of the ISR samples shall have a % difference greater than 20.0%. The validation results obtained in the present investigation are given in Table 5.

Table 5
Summary of the validation results

QC Intra-day precision range	LOW: 1.71 to 4.13% MID: 2.02 to 5.10% HIGH: 2.93 to 10.34%
QC Intra-day accuracy range	LOW: 99.79 to 113.47% MID: 101.48 to 105.84% HIGH: 95.53 to 104.01%
QC Inter-day precision range	3.79 to 7.43 %
QC Inter-day accuracy range	99.85 to 105.84 %
Bench-top stability	24.5 Hours at room temperature
Master Stocks stability	137 days at refrigerated for AP and 31days for ISTD
Processed stability	29.0 Hours at refrigerated conditions
Freeze-thaw stability	3 Cycles
Long-term storage stability	17 Days at -30°C set point freezer
Dilution integrity	Diluted 2 fold and 4 fold
Neat standards stability (refrigerated)	26 Days
Internal standard spiking solution stability(refrigerated):	26 Days

Sample analysis

Mean pharmacokinetic parameters of allopurinol after oral administration of 100 mg tablet formulation as follows 1109.17 ± 293.05 C_{max} (ng/mL), 1.50 ± 0.45 T_{max} (h), 1.08 ± 0.25 t_{1/2} (h), 3747.29 ± 1127.31 AUG_{0-t} (h. ng/mL), 3760.71 ± 1123.88 AUG_{0-∞} (h. ng/mL), 0.67 ± 0.15 K_{el} (1/h). No more than 33.0% of the ISR samples shall have a % difference greater than 20.0%.

CONCLUSION

A simple, selective, rapid and sensitive analytical method for determination of allopurinol in human plasma had been developed and validated using. The proposed LC/MS/MS method involves simple liquid-liquid extraction procedure and short run time that can increase sample throughput which is important for large sample batches. The

method provides excellent specificity and linearity with a limit of quantification of 50.0 ng/mL. The results obtained from the validation attest to the reliability of the analytical system, and the reliability and reproducibility of the assay. The assay is specific, results demonstrate freedom from any reasonably expected interferences. The analytes measured are shown to be stable under stress conditions that may be encountered during application of this method. It was concluded from these results that the analytical method as described is suitable for the assay of AP in human plasma. The current analytical method was developed and validated using sophisticated analytical technique viz. LC-MS/MS instrument with the sensitivity of 50ng/mL. This method is suitable for the quantification of AP in human plasma for real sample analysis.

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