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Simultaneous determination of azathioprine and its metabolite 6-mercaptopurine in human plasma using solid phase extraction-evaporation and liquid chromatography–positive electrospray tandem mass spectrometry

Mokkaisamy Jegadeesh Raja[1], Jegadeesh Raja Kavitha[1], Kothamasu Pavan Kumar[2], *Thangavel Sivakumar[2]

[1]*Department of Pharmaceutical Analysis, Karpagam University, Coimbatore, TamilNadu, India-641021*
[2]*Department of Pharmaceutical Analysis, Nandha College of Pharmacy and Research Institute, Koorapalayam Piruvu, Erode, Tamil Nadu, India-638052*

ABSTRACT

A simple, rapid, specific, sensitive and liquid chromatography coupled with tandem mass spectrophotometric method was developed and validated for the estimation of azathioprine and its metabolite 6-mercaptopurine in human plasma by using lamivudine and 6-mercaptopurine D3 as the internal standard. Azathioprine and 6-mercaptopurine were extracted from human plasma by solid-phase extraction (SPE)-Evaporation method, using Oasis MCX cartridge for cleaning procedure. The stationary phase was chromatographed on a ZORBAX SB CN, (75X50 mm, 5 µ) column where as mobile phase constitutes of acetonitrile: 2mM ammonium acetate (70:30 v/v) at a flow rate of 0.800 ml/min. The detection was performed with an Applied Biosystems Sciex API 4000 mass spectrometer by multiple reaction monitoring (MRM). The method validation proofs were carried out as per the USFDA guidelines as described, showing a linearity system ($r^2 > 0.99$) over a range of 2.455 ng/mL to 106.568 ng/mL for azathioprine and 1.165 ng/mL to 101.143 ng/mL concentrations for 6-mercaptopurine and a recovery shows 99.36% and 100.44% for azathioprine and 6-mercaptopurine respectively. The results show that this proposed approach is effective and can be applied to the extraction and analysis of other pharmaceutical compounds.

***Key Words*:** Human plasma, multiple reaction monitoring, cartridge, linearity, quality control.

INTRODUCTION

Azathioprine (6-[(1-Methyl-4-nitro-1H-imidazol-5-yl)thio]-1H-purine) (Figure 1) is an immunosuppressant used in organ transplantation and autoimmune disease such as rheumatoid arthritis or inflammatory bowel disease or Crohn's disease (Anstey and Lear, 1988). It is a safe and effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation (Oellerich *et al.*, 1997). Azathioprine acts to inhibit purine synthesis necessary for the proliferation of cells, especially leukocytes and lymphocytes. Azathioprine is a prodrug and more than 80% of azathioprine is converted to 6-Mercaptopurine (6-MP) (1, 7-Dihydro–6H-purine–6–thione monohydrate) (Figure 2) in the blood by the enzyme glutathione-s-transferase (GST) (Thierry and Roselyne, 1998). Azathioprine and the metabolite 6-MP are moderately bound to serum proteins (30%). Azathioprine and 6-MP are structurally very similar, differing only in that azathioprine has a methyl–nitro-imidazolyl group attached to the sulfur atom at the 6-position of the purine ring of 6-MP (Janine and Friedman, 2002, William and Charles, 1975). On the average, 47% of an orally administered dose of azathioprine is available to the systemic circulation. A number of analytical methods have been used to determine the azathioprine with 6-mercaptopurine. It involves mainly high performance liquid chromatography (Van Os *et al.*, 1996, Fell and Plag, 1979), gas chromatography (Wypior *et al.*, 1982) and very few liquid chromatography tandem mass spectrometry methods (Hofmann *et al.*, 2012, Tibor *et al.*, 2010) have been developed for the quantification in biological samples of plasma (El-Yazigi and Wahab,

***Corresponding Author**:
Dr. Thangavel Sivakumar, M.Pharm, PhD
Principal, Department of Pharmaceutical Analysis
Nandha College of Pharmacy and Research Institute
Erode-638052, Tamil Nadu, India
E-mail: *sivaecp@yahoo.com*
Contact No.: 9842660400

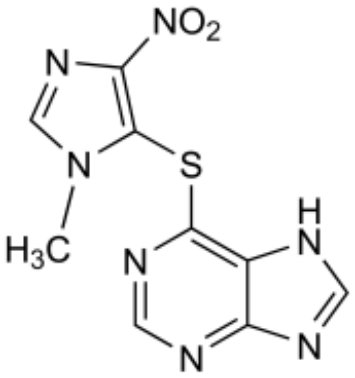

Figure 1 : Structure of Azathioprine.

1992, Albertioni *et al.*, 1995, Maddocks, 1979, Teck and Leslie, 1979) and serum (Sahnoun *et al.*, 1990, Torsten *et al.*, 1996, Tsutsumi *et al.*, 1982). The combination liquid chromatography/mass spectrometry is currently acknowledged as being a powerful means of determining organic molecules from complex biological matrices. The selectivity and sensitivity of LC–MS/MS has allowed for analysis times to be reduced, such that sample preparation time often exceeds the analysis time of samples. Protein precipitation, LLE (Liquid-Liquid Extraction) and SPE (Solid Phase Extraction) are the sample preparation techniques most commonly used to process plasma (Prashant *et al.*, 2011). Protein precipitation has the potential to be significantly less time consuming, while SPE is the technique most amenable and can also be adapted for direct injection. LLE generally provides cleaner extracts than the techniques mentioned previously, as evidenced by reduced matrix effect and less tendency for backpressure build-up in the chromatographic column as more samples are injected.

MATERIAL AND METHOD

Chemicals and reagents

Working standards of Azathioprine, Lamivudine, 6-Mercaptopurine and 6-Mercaptopurine D3 were procured from Clearsynth Labs and Vivan Life Sciences, Mumbai, India. HPLC grade Acetonitrile was purchased from JT Baker chemicals, Mumbai, India. Ammonium acetate GR grade and Ortho phosphoric acid GR grade were supplied from Merck chemicals, Mumbai, India. The solid phase extraction (SPE) cartridges of Oasis MCX (1 ml, 30 mg) were obtained by Waters (Milford, MA, sUSA). All aqueous solutions and buffers were prepared

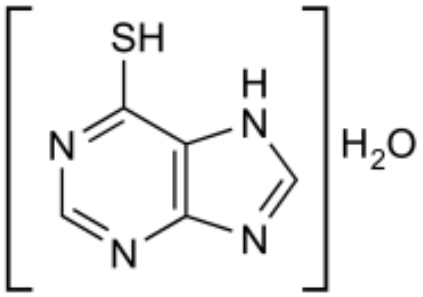

Figure 2: Structure of 6-Mercaptopurine.

using deionized and doubly distilled water (resistivity>18 MO cm) from a Milli-Q-System obtained from In-House were used through out the experiment.

Chromatographic conditions

The LC system (SHIMADZU) (SILHTP) consists of isocratic pump (LC-20AD Prominence liquid chromatography pump), autosampler (SIL-HTc) and column thermostat. The mobile phase consists of acetonitrile: 2mM ammonium acetate (70:30 v/v) and chromatographic separation was performed at 40°C temperature with flow rate 0.800 ml/min by using ZORBAX SB CN, 75X50 mm, 5µ column.

Mass Spectrometry conditions

Mass detection was carried out on a (API 4000, AB SCIEX, USA) equipped with a source of electrospray ionization. The LC-MS/MS detector was operated at unit resolution in Multiple Reaction Monitoring (MRM) mode. The data were acquired using the Analyst 1.5 software. The transitions of molecular ions were found azathioprine- 278.20/142.00, Lamivudine- 230.10/112.20, 6MP- 152.9/119.1 and 6-MP D3-156.0/122.0.

Preparation of working standard/quality control (QC) solutions

The stock solution preparation of azathioprine and 6-MP were prepared in basified methanol at concentration of 1000 µg/mL each. The mixed calibration curve of working solutions were prepared from above stock solutions using the diluent of Methanol : Water (50:50 v/v) and ranges from 49.100 ng/mL to 2131.360 ng/mL concentrations range for azathioprine and 23.300 ng/mL to 2022.860 ng/mL concentrations range for 6-MP. The quality control samples for azathioprine were prepared at concentrations of 49.640 ng/mL (LLOQQC), 141.740 ng/mL (LQC), 506.240 ng/mL (MQC1), 843.740

Table 1: Concentration Response Data of Batches: Linearity, Precision and Accuracy of Azathioprine.

	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8
	2.455	**4.911**	**12.277**	**24.553**	**40.922**	**68.204**	**85.254**	**106.568**
Reading 1	2.530	4.394	13.483	25.164	42.582	65.858	81.975	104.821
Reading 2	2.400	4.923	13.899	23.118	42.931	68.117	83.513	97.905
Reading 3	2.425	4.915	13.545	22.237	40.902	70.470	84.988	103.657
Mean	2.4517	4.7440	13.6423	23.5063	42.1383	68.1483	83.4920	102.1277
S.D. (+ / -)	0.06898	0.30314	0.22443	1.50164	1.08482	2.30616	1.50661	3.70296
C.V. (%)	2.81	6.39	1.65	6.39	2.57	3.38	1.80	3.63
% Nominal	99.86	96.60	111.12	95.74	102.97	99.92	97.93	95.83

Table 2: Concentration Response Data of Batches: Linearity, Precision and Accuracy of 6-MP.

	Nominal Concentration (ng/mL)							
	CC1	**CC2**	**CC3**	**CC4**	**CC5**	**CC6**	**CC7**	**CC8**
	1.165	**2.33**	**4.661**	**11.652**	**23.303**	**38.839**	**80.914**	**101.143**
Reading 1	1.16	2.374	4.683	10.774	22.672	41.15	81.688	102.505
Reading 2	1.147	2.426	4.656	10.381	25.795	40.39	75.181	101.944
Reading 3	1.123	2.557	4.48	10.988	24.392	40.198	78.61	99.235
Mean	1.1433	2.4523	4.6063	10.7143	24.2863	40.5793	78.493	101.228
S.D. (+ / -)	0.01877	0.0943	0.11024	0.30787	1.56418	0.50345	3.25508	1.74863
C.V. (%)	1.64	3.85	2.39	2.87	6.44	1.24	4.15	1.73
% Nominal	98.14	105.25	98.83	91.95	104.22	104.48	97.01	100.08

ng/mL (MQC) and 1687.500 ng/mL (HQC) respectively using the diluents [Methanol : Water (50:50 v/v)]. The quality control samples for 6-MP were prepared at concentrations of 23.740 ng/mL (LLOQQC), 66.800 ng/mL (LQC), 477.120 ng/mL (MQC1), 795.180 ng/mL (MQC) and 1590.360 ng/mL (HQC) respectively using diluent [Methanol : Water (50:50 v/v)]. All solution was stored at 2-8 °C and was brought to room temperature as when required. The Lamivudine and 6-MP D3 stock solutions (1 mg/ml each) were used to prepare IS mixture to achieve concentration 100000.000 ng/mL for Lamivudine and 320000.000 ng/mL for 6-Mercaptopurine D3 using diluent [Methanol : Water (50:50 v/v)]. All solution was stored at 2-8°C and was brought to room temperature as when required.

Preparation of calibration curve standards and quality control samples

The calibration curve standards and quality control samples were prepared by spiking blank plasma with above working solutions at 5% to preserve the integrity of plasma sample. The mixed calibration curve standards were prepared at concentration of 2.455 ng/mL to 106.568 ng/mL for azathioprine and 1.165 ng/mL to 101.143 ng/mL for 6-MP. The quality control samples for azathioprine were prepared at concentrations of 2.482 ng/mL (LLOQQC), 7.087 ng/mL (LQC), 25.312 ng/mL (MQC1), 42.187 ng/mL (MQC) and 84.375 ng/mL (HQC) respectively using the diluent [Methanol : Water (50:50 v/v)]. The quality control samples for 6-MP were prepared at concentrations of 1.187 ng/mL (LLOQQC), 3.340 ng/mL (LQC), 23.856 ng/mL (MQC1), 39.759 ng/mL (MQC) and 79.518 ng/mL (HQC) respectively using diluent [Methanol : Water (50:50 v/v)]. All aliquots of spiked plasma were transferred to RIA tube and stored at below -70±5°C.

Sample Preparation and Extraction

Aliquot 500 µL of spiked Plasma sample in RIA tube, add 50 µL (6-Mercaptopurine Labeled (6MP-D3) + Lamivudine), internal standard solution (Except calibration blank) and vortex it for 10 seconds. Add 500 µL of 2% O-Phosphoric acid and vortex for 30 seconds. Condition Oasis MCX cartridges with 1.0 mL of methanol and equilibrate with 1.0 mL of 2% O-Phosphoric acid, load the sample in SPE manifold and wash SPE cartridges with 1.0 mL of 2% O-Phosphoric acid and1.0 mL of acidified Methanol (pH: 2.3). Elute the cartridges with 2.0 mL of 5% ammonia in methanol and evaporate the sample at

Table 3: Intra-Run Precision and Accuracy (PA) (Batch 01, 02 and 03) of Azathioprine.

Nominal Concentration (ng/mL)	LLOQ QC 2.482	LQC 7.087	MQC1 25.312	MQC 42.187	HQC 84.375
PA BATCH-01					
Mean	2.4077	6.8788	26.3325	42.9318	88.0992
S.D. (+ / -)	0.10763	0.83238	1.95318	2.70443	10.54774
C.V. (%)	4.47	12.1	7.42	6.3	11.97
% Nominal	97.01	97.06	104.03	101.77	104.41
PA BATCH-02					
Mean	2.3188	6.8653	26.7838	42.1197	87.1977
S.D. (+ / -)	0.09098	0.7238	2.64589	1.78127	11.72285
C.V. (%)	3.92	10.54	9.88	4.23	13.44
% Nominal	93.43	96.87	105.81	99.84	103.35
PA BATCH-03					
Mean	2.4218	6.2945	24.4197	40.0023	86.2287
S.D. (+ / -)	0.10946	0.40974	1.37439	2.75257	10.48678
C.V. (%)	4.52	6.51	5.63	6.88	12.16
% Nominal	97.58	88.82	96.47	94.82	102.2

Table 4: Intra-Run Precision and Accuracy (PA Batch-01, 02 and 03) of 6-Mercaptopurine.

Nominal Concentration (ng/mL)	LLOQ QC 1.187	LQC 3.34	MQC1 23.856	MQC 39.759	HQC 79.518
PA BATCH-01					
Mean	1.2185	3.2678	22.9697	41.5532	86.5722
S.D. (+ / -)	0.07725	0.13571	0.71807	1.13516	3.27848
C.V. (%)	6.34	4.15	3.13	2.73	3.79
% Nominal	102.65	97.84	96.28	104.51	108.87
PA BATCH-02					
Mean	1.1717	3.3932	22.6685	38.9742	88.0208
S.D. (+ / -)	0.05269	0.14143	0.39698	2.16538	2.27225
C.V. (%)	4.5	4.17	1.75	5.56	2.58
% Nominal	98.71	101.59	95.02	98.03	110.69
PA BATCH-03					
Mean	1.2053	3.4698	23.5697	41.2802	87.5738
S.D. (+ / -)	0.04476	0.23348	0.73211	2.23062	4.16565
C.V. (%)	3.71	6.73	3.11	5.4	4.76
% Nominal	101.54	103.89	98.8	103.83	110.13

50°C under nitrogen gas at 15 psi. Reconstitute the dried residue with 200 µL of Mobile phase [(acetonitrile: 2mM ammonium Acetate (70:30 v/v)] and inject 20µL of volume into LC-MS/MS.

RESULTS AND DISCUSSION

The method here in presented is specific SPE-Evaporation protocol allowed a rapid method for the simultaneous extraction of azathioprine and 6-MP to be developed, since a large number of samples can be determined daily by using this approach (Dervieux and Boulieu, 1998, Kato *et al.*, 1991). As for the extraction itself, the selected method proved to be very simple and reproducible. The Solid Phase Extraction (SPE) procedure with Oasis MCX cartridges provided cleaner samples and good recovery (Angela *et al.*, 2008). The addition of 2% O-Phosphoric acid enhances the analyte interest retention other than plasma elements in SPE cartridges that influences the matrix effect (Matrinez *et al.*, 2003, Petrovic *et al.*, 2002). The addition of 2% O-Phosphoric acid and acidified methanol (pH-2.3) washing solution at washing step, decreased the presence of plasma elements upto >80%, when compared with the use of pure water washing twice (Miao *et al.*, 2002, Hilton and Thomas, 2003). However, even with this improvement, the final evaporation method for eluent aids further cleaner sample and improved sensitivity. The co-elution of plasma elements produced (despite the MRM) unexpected peaks when short C_8 and C_{18} columns (50 mm) were applied. The use of ZORBAX SB CN column, 75X50 mm, 5 µ increased the run time (3.0 min) but decreased the intensities of the peaks attributed to plasma elements. Furthermore, these peaks didn't appear in the respective retention times of azathioprine and 6-MP (Figure. 03, 04, 06 and 07). The S/N ratio was found to be >5 for azathioprine and 6-MP in the lower standard sample, which was set to be the LOQ, Since these peaks were determined using normal blank plasma, further experiments with hemolyzed and lipemic blank plasma were also performed, but the yield chromatograms for these did not exhibit any real differences from those for normal blank plasma (Figure. 05 & 08). Judging from our experience, there are very few hemolyzed and lipemic samples present among the hundreds obtained from a bioequivalence study. Therefore, the samples obtained from such studies are expected to show the same behavior with the spiked plasma samples produced during the pre-study validation. After the completion of the pre-study validation, the ESI source was quite clean, which is of great significance for SPE extraction protocols and evaporation. Therefore, it might be necessary to clean the source in the middle of a bioequivalence study (usually>1000 samples), especially if the signal intensity has decreased. We intend to provide studies dealing with the interference obtained from plasma elements in ESI when following an SPE-Evaporation

Table 5: Inter-Run Precision and Accuracy of Azathioprine & 6-Mercaptopurine.

	Azathioprine				
	LLOQ QC	LQC	MQC 1	MQC	HQC
Concentration (ng/mL)	2.482	7.087	25.312	42.187	84.375
Mean	2.3828	6.6796	25.8453	41.6846	87.1752
S.D. (+ / -)	0.10754	0.69697	2.20196	2.63201	10.30067
C.V. (%)	4.51	10.43	8.52	6.31	11.82
% Nominal	96	94.25	102.11	98.81	103.32
	6-Mercaptopurine				
Concentration (ng/mL)	LLOQ QC	LQC	MQC 1	MQC	HQC
	1.187	3.34	23.856	39.759	79.518
Mean	1.1985	3.3769	23.0693	40.6025	87.3889
S.D. (+ / -)	0.05977	0.18621	0.71008	2.15369	3.18936
C.V. (%)	4.99	5.51	3.08	5.3	3.65
% Nominal	100.97	47.65	91.14	96.24	103.57

Table 6: Stability data of Azathioprine and 6-Mercaptopurine.

Stability	**Azathioprine**			**6-Mercaptopurine**		
	Nominal Concentration (ng mL^{-1})	**% Bias**	**% CV (n=6)**	**Nominal Concentration (ng mL^{-1})**	**% Bias**	**% CV (n=6)**
Bench Top	LQC-7.087	-4.14	5.82	LQC-3.340	4.13	6.26
	HQC-84.375	3.32	8.38	HQC-79.518	5.34	2.84
Freeze Thaw	LQC-7.087	1.35	8.06	LQC-3.340	-0.68	3.2
	HQC-84.375	-5.27	7.94	HQC-79.518	1.73	6.69
Long Term Stability in Matrix	LQC-7.087	6.69	11.12	LQC-3.340	-1.39	4.23
	HQC-84.375	8.34	5.5	HQC-79.518	0.5	5.6

protocol in an upcoming report. The retention times of all compounds and the column backpressure remained practically constant by the end of pre-study validation.

Method Validation

The complete validation was performed according to the guidelines of US Food and Drug Administration (USFDA) on bioanalytical method validation (Guidance for Industry, 2003, Guidance for Bioavailability and Bioequivalence Studies, 2005, Guidance for Industry, 2001, Chow and Liu, 1999).

Linearity

Linearity was used to confine the performance of the method. A linear least squares regression with a three calibration curves were prepared using eight non-zero standards ranging from 2.455 ng/mL to 106.568 ng/mL for azathioprine and 1.165 ng/mL to 101.143 ng/mL for 6-MP for each analytical run. Peak area ratios of each drug to IS were used for regression analysis. A linear regression model ($y=mx+c$) was evaluated, using $1/X^2$ as weighting factor, where X is the concentration of each drug and y corresponds to the areas ratio. The regression coefficients (r^2) for the three runs were greater than 0.98 for azathioprine and 6-MP. The standard curves obtained for the two molecules are presented in Table 1 and 2, respectively. Consequently, SPE-Evaporation procedure applied in this method was able to produce satisfactory concentration data for azathioprine and 6-MP standard samples.

Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were performed by measuring the analytical background response. The individual standard curve data from three runs met all of the preset criteria: i) <20% deviation from the nominal concentration at the limit of quantification (LOQ), which was defined as the lowest standard, ii) <15% deviation of standards from their back calculated concentration, other than LOQ from nominal concentrations, iii) at least six out of eight nonzero

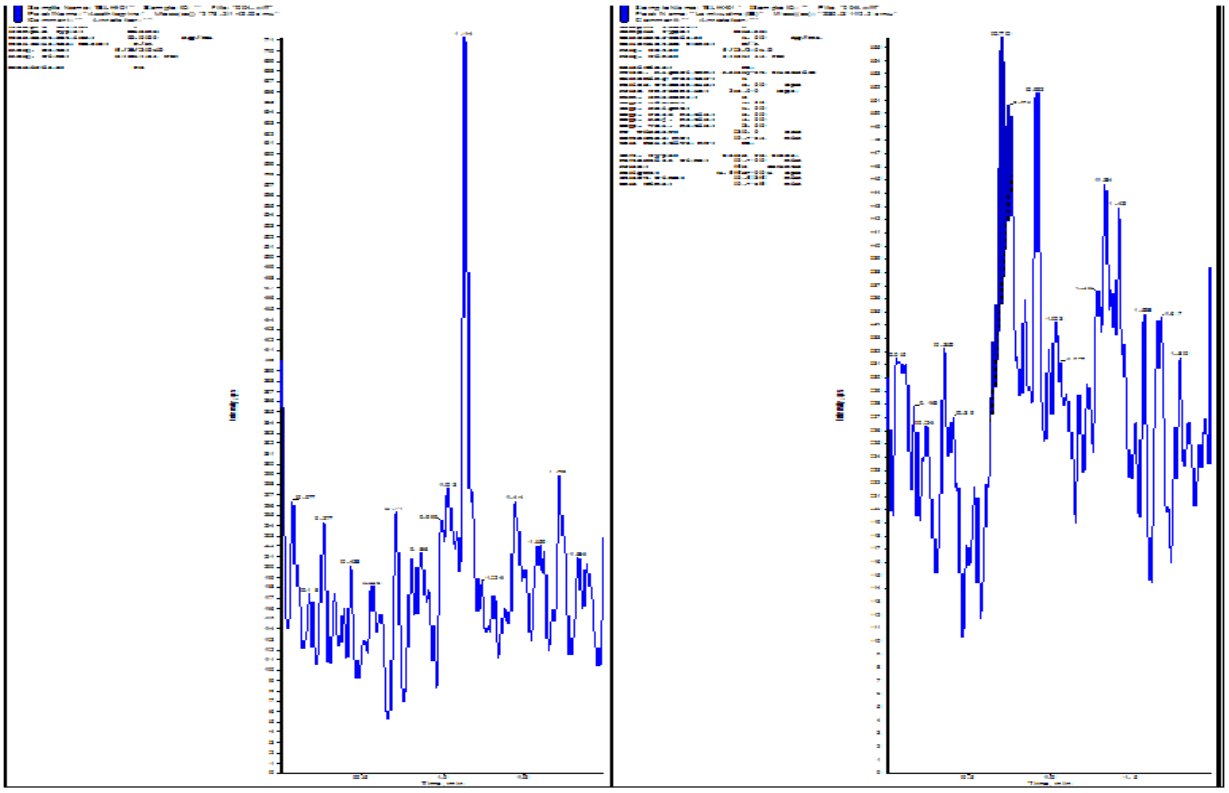

Figure 3: Blank of Azathioprine extracted from plasma.

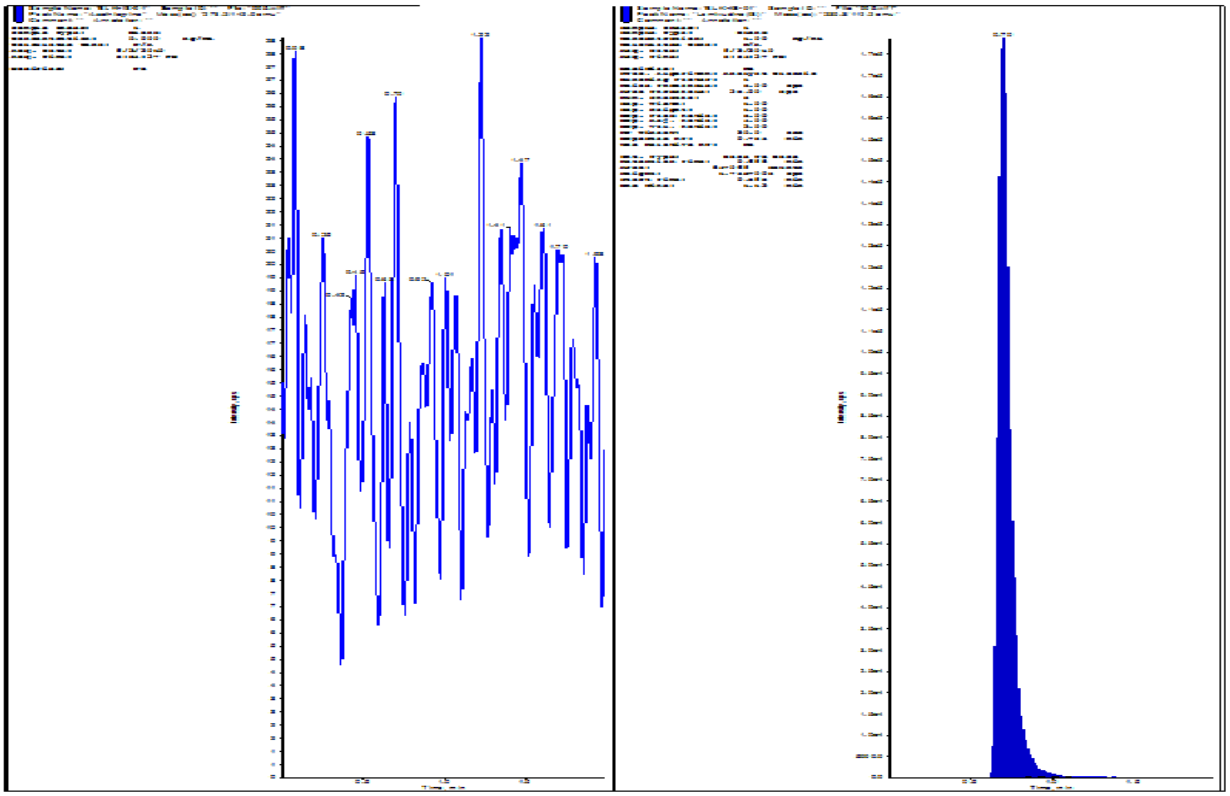

Figure 4: Chromatogram of Azathioprine with its internal standard.

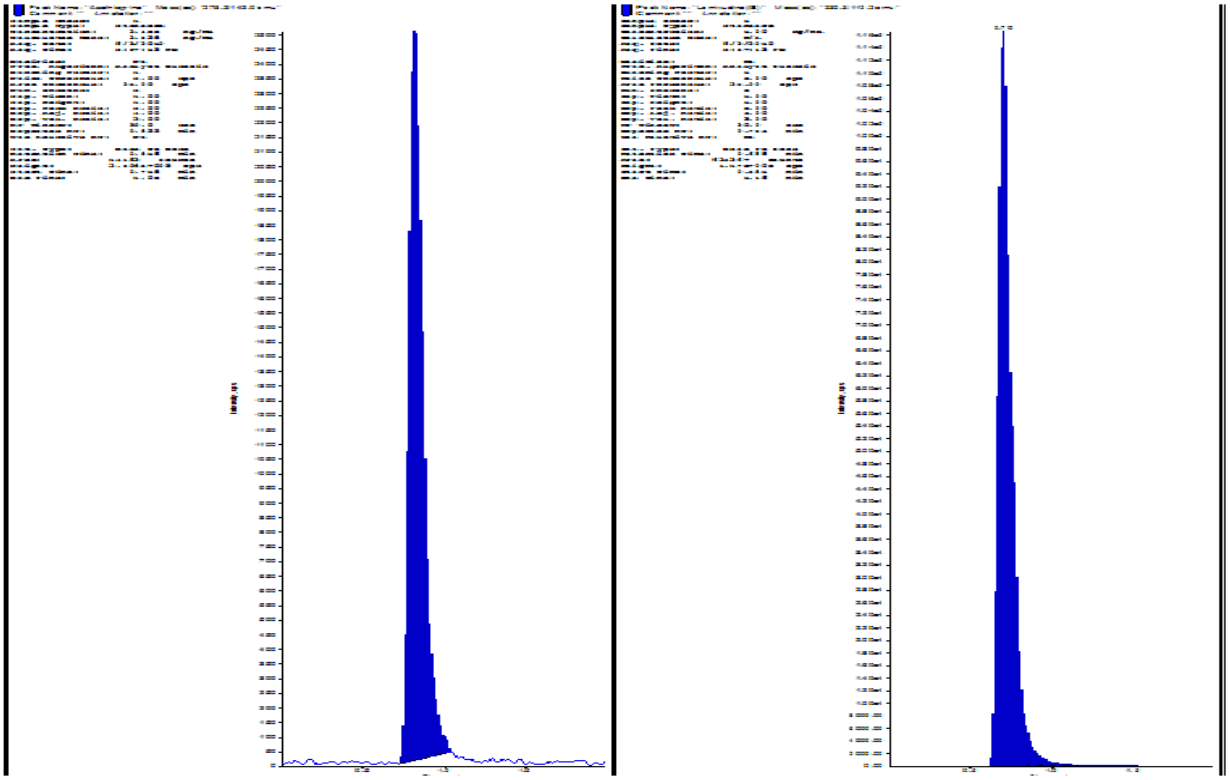

Figure 5: LLOQ Chromatogram of Azathioprine.

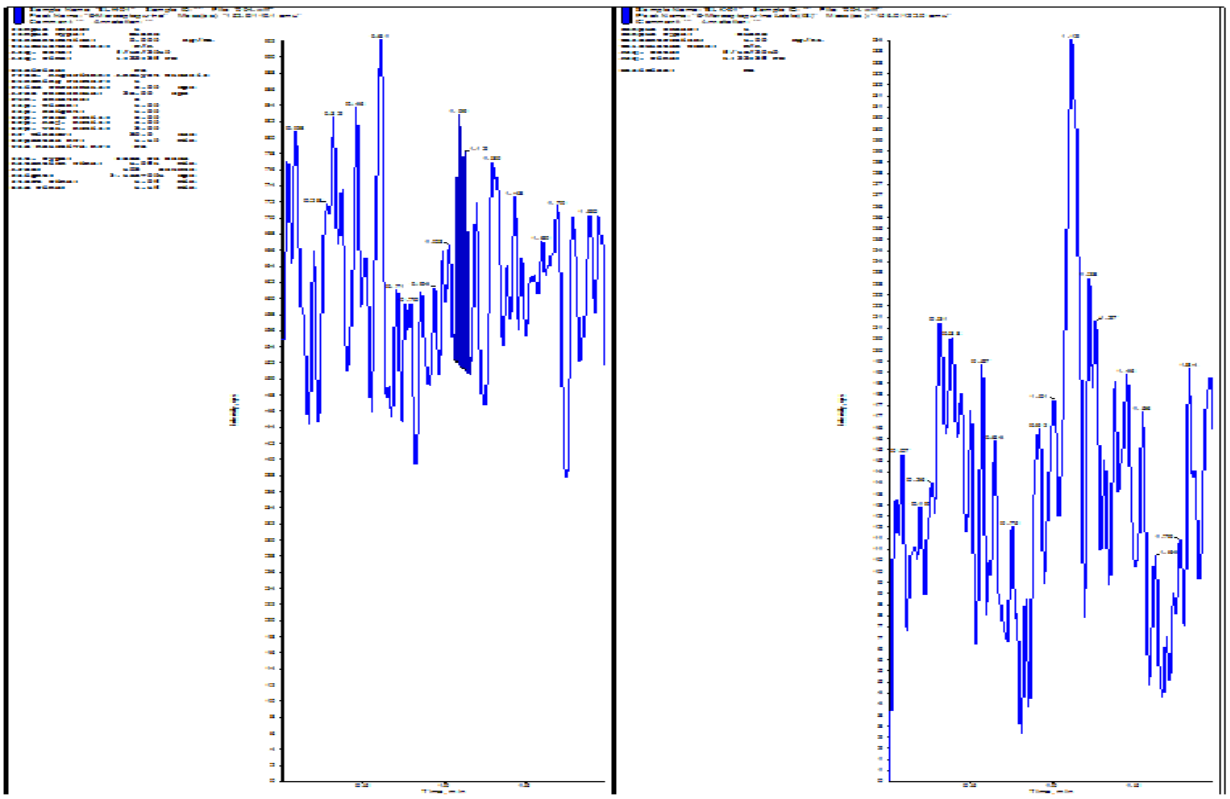

Figure 6: Blank of 6-Mercaptopurine extracted from plasma.

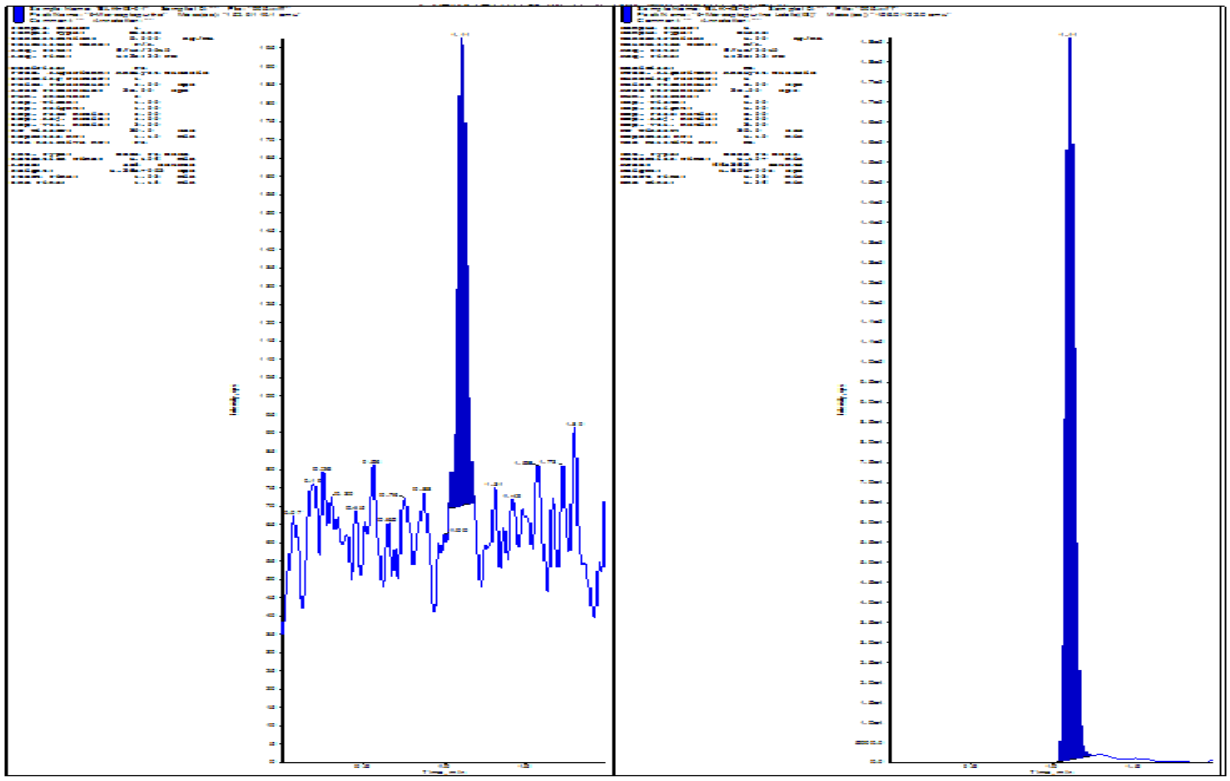

Figure 7: Chromatogram of 6-Mercaptopurine associated with its internal standard

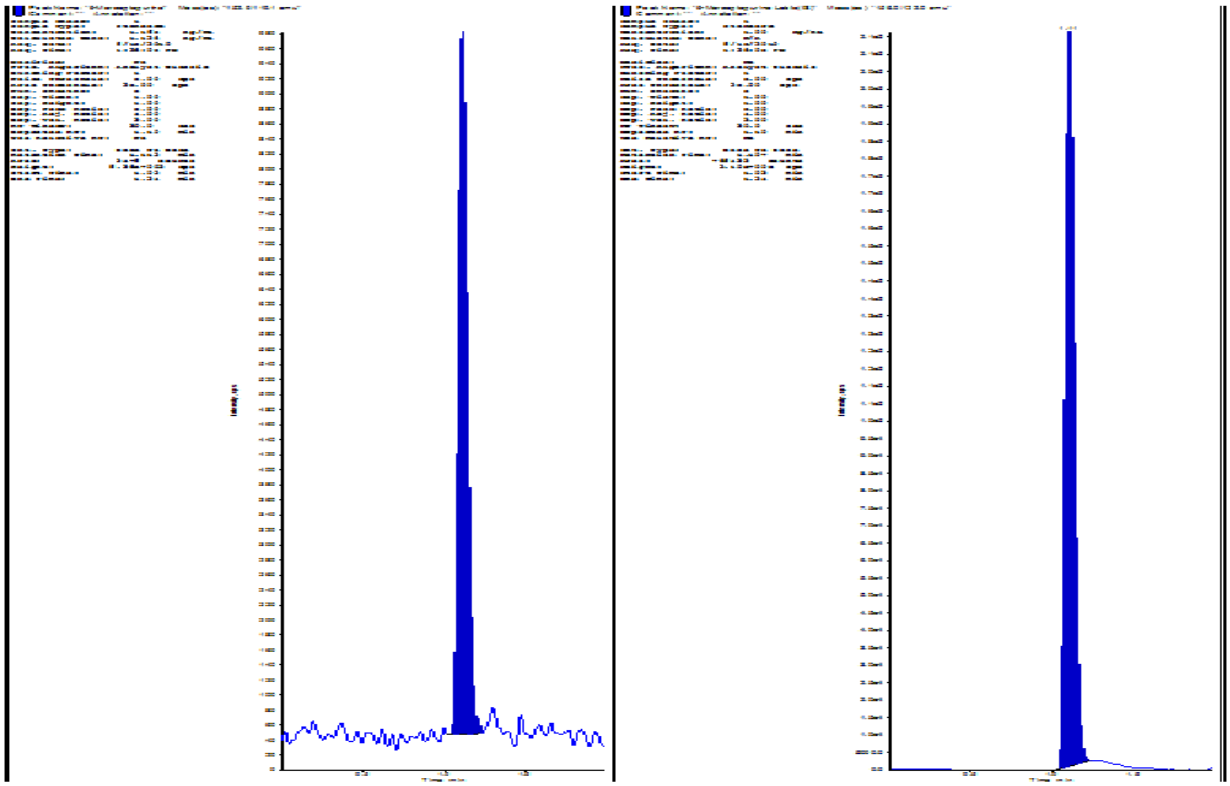

Figure 8: LLOQ chromatogram of 6-Mercaptopurine

standards of each nominal concentration meeting the above criteria, including the LOQ and the calibration standard at the highest concentration.

Accuracy and precision

The precision and accuracy were also assessed by analyzing method validation samples over three runs. The percentage accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. The intra-assay precision was assessed by analyzing six replicates at each QC level, while the inter-assay precision was determined over three runs conducted on two days by analyzing 30 samples. Data for both types of accuracy and precision (expressed as CV %) are presented in Tables 3, 4 and 5 azathioprine and 6-MP respectively. These results, as well as the respective values for the percentage accuracy were within the acceptance criteria.

Stability studies

The stability quality control plasma samples LQC-7.087 ng/mL and HQC-84.375 ng/mL for azathioprine and LQC-3.340 ng/mL and HQC-79.518 ng/mL for 6-MP (Table 6). To evaluate the freeze–thaw stability, a freeze-and-thaw cycle was defined as the storage of LQC and HQC samples at -80°C, followed by thawing at room temperature. Samples were analyzed after the fifth cycle, along with fresh reference samples of the same concentration. Back-calculated concentrations of fifth freeze–thaw cycles versus fresh were within the acceptance limit (<10% variation).

To evaluate the Bench top stability, six aliquots of LQC and HQC were maintained at room temperatures for 23 Hrs 23 Min without processing (which exceeds the time that samples normally remain at room temperature). After a stipulated period of storage over bench, stability samples were processed and analyzed against fresh CC and fresh QC.

To check long-term stability, aliquots of the two sample types were initially frozen at -70°C for 30 days, and then thawed and analyzed. The 30 day period is more than adequate for the analysis of samples from a bioequivalence study, since the current method allows the measurement of more number of samples per day. All of the molecules were stable (<10% variation) under these conditions. Stock solution stability was estimated by comparing fresh and old dilutions in mobile phase of these solutions (stored at 4°C). The measurements proved that the concentrations of azathioprine and 6-MP in stock solution remained intact (variation <5%).

Table 7: Summary data of Recovery studies.

	AZA (%)	6-MP (%)
LQC	99.32	100.63
MQC1	99.67	99.48
MQC	100.23	100.36
HQC	98.22	101.32
Avg	99.36	100.44

Autosampler stability was another part of the method validation that was tested. This was assessed by comparing QC samples included at the beginning, at half-way and upon completion of each of the three analytical runs. The stabilities of all molecules in the autosampler (5°C) were acceptable.

Recovery Studies

The recovery of the proposed method, were spiked with different concentrations of azathioprine and 6-MP. The recovery was obtained by performing the mean response of each concentration and dividing with the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the actual concentration (Table 7). The difference between the unextracted samples, spiked plasma residues and the extracted sample was done in order to minimize the matrix effects, giving a true recovery. The recoveries observed for azathioprine and mercaptopurine was 99.36% and 6-MP was 100.44%.

CONCLUSION

A SPE–Evaporation protocol described above was developed for the simultaneous quantification of Azathioprine and 6-Mercaptopurine in human plasma. Azathioprine is a prodrug, which convert into active metabolite 6-Mercaptopurine in the blood after it has been administered. The current method includes, for the first time, a single sample preparation procedure and a rapid LC–MS/MS protocol. Therefore, more number of samples can be analyzed daily, while only small quantities of plasma and solvent are consumed. The method developed here was validated over the concentration 2.455 ng/mL to 106.568 ng/mL for azathioprine

and 1.165n g/mL to 101.143 ng/mL for 6-MP. These ranges are suitable for measuring these drugs in plasma samples obtained for a pharmacokinetic or bioequivalence study. The method possessed excellent precision and accuracy and proved to be reliable. It is expected that this approach can be applied to the extraction and analysis of other pharmaceutical compounds with different physico-chemical properties from biological samples.

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