Original Article

# LC–MS/MS assay for Acetazolamide, A Carbonic Anhydrase Inhibitor in Human Plasma and its Clinical Application

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## ABSTRACT

**Objective:** The objective of this research was to develop a novel liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of acetazolamide in human plasma. **Methods:** An analytical method based on LC-MS/MS (API-4000) has been developed and validated for the quantitative determination of acetazolamide in human plasma using acetazolamide d3 as Internal Standard (IS). After Solid phase extraction (SPE), analyte and the IS were chromatographed on a C18 columns using a isocratic mobile phase composed of 0.1% formic acid buffer and acetonitrile (30:70, v/v) pumped at a flow rate of 0.80 mL/min. **Results:** Precision and accuracy of the method was determined using five analytical batches in the concentration range of 50.3–12046 ng/mL. All the validation experiments were carried out as per the US FDA guidelines and results met the acceptance criteria. **Conclusion:** The proposed LC–MS/MS assay method is simple, rapid and sensitive for the determination of acetazolamide in human plasma. A chromatographic run time of 2.0 min, allow us to analyze more than 300 samples in a day.

**Key words:** Acetazolamide, Human plasma, LC–MS/MS, Method validation, Pharmacokinetics.

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## INTRODUCTION

Acetazolamide is a carbonic anhydrase inhibitor commonly used to treat glaucoma. The drug is also used for the treatment of epilepsy, mountain sickness, periodic paralysis, central sleep apnea and idiopathic intracranial hypertension.[1,2] Acetazolamide decreases the aqueous

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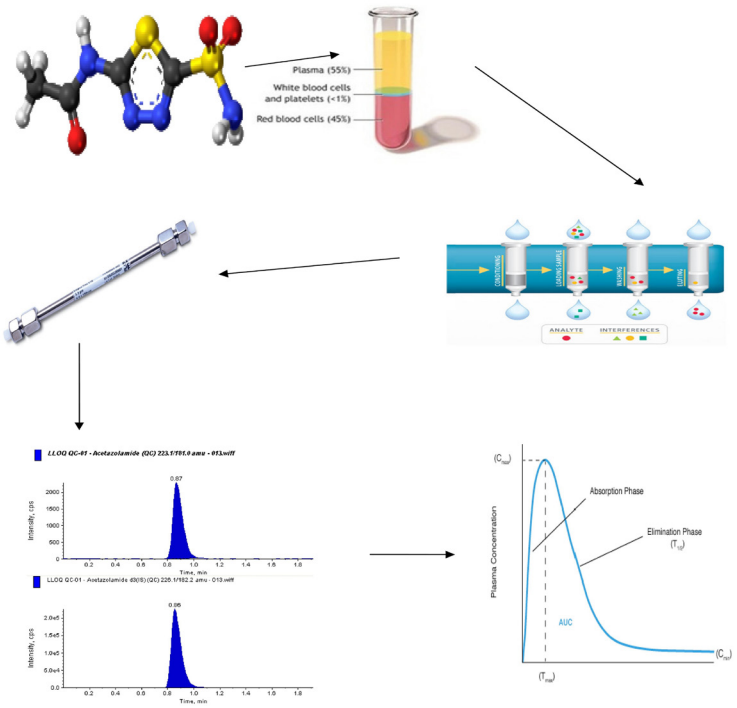


**Graphical Abstract**

humor formation, there by reduces the Intraocular pressure (IOP). However, the usefulness of the drug has been limited due to high incidence of side effects associated with its continued use. The etiology of many of the adverse effects is unclear, may be due to acidosis or carbon dioxide retention.[3] After oral administration, the peak blood levels were attained in 4–8 h with elimination half–life ($t_{1/2}$) about 10–15 h.[1,2]

Based on the literature, many HPLC based analytical methods have been reported for the determination of acetazolamide in human plasma,[4–6] rat plasma and tissues,[7] human urine[8] and in dosage forms.[9] The conventional HPLC methods must sacrifice time, resolution or sensitivity. Hence there is a necessity to develop fast or ultra–fast methods such as LC–MS/MS without any loss of separation efficiency and sensitivity. Recently, Li *et al*.[10] reported an LC–MS/MS method for the determination of acetazolamide in beagle dog plasma using Protein precipitation (PP) technique for the sample preparation. Till date, none of the method describes the development process, various validation experiments, stability studies and suitability for application to pharmacokinetic/ bioequivalence studies.

This paper presents, for the first time, the complete development and validation of a simple, sensitive and selective LC–MS/MS method in multiple reaction monitoring (MRM) mode for the quantification of acetazolamide in human plasma using acetazolamide d3 as an Internal standard (IS) to avoid the possible matrix effect related problems and variability in recovery between analyte and the IS. This sensitive method requires only 100 µL human plasma for Solid–phase extraction (SPE) technique, minimum usage of organic solvents and demonstrates excellent performance in terms of ruggedness with a sample cut off time 2.0 min.

## MATERIALS AND METHODS

### Standards and chemicals

The reference sample of acetazolamide (99.46%) and acetazolamide d3 (99.20%) were obtained from Clearsynth Labs Limited (Mumbai, India). HPLC grade acetonitrile and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA), while analytical grade formic acid was from Merck Ltd (Mumbai, India). The blank $K_2$ human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India). Ultra pure water was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India).

### LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a Hypurity advance column (50×4.6 mm, 5 µm), a binary LC–20AD prominence pump, an auto sampler

(SIL–HTc) and a solvent degasser (DGU–20$A_3$) was used for the study. Aliquot of 10 µL of the processed samples were injected into the column, which was maintained at 35 ± 2°C temperature. An isocratic mobile phase composed of a mixture of 0.1% formic acid buffer and acetonitrile (30:70, v/v) was used to separate the analyte and the IS from the endogenous components. The mobile phase was pumped at a flow rate of 0.80 mL/min into the mass spectrometer electrospray ionization chamber. API–4000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turboionspray™ in positive ion mode was used for the quantification of analyte. The ion spray voltage was set at 5000 V with interface temperature 500°C. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 35, 40, 20, and 8 psi, respectively. The various potentials namely Declustering potential (DP), Collision energy (CE), Entrance potential (EP) and Collision cell exit potential (CXP) were set at 40, 22, 10, 10 V for acetazolamide and 42, 21, 10, 10 V for the IS. Detection of the ions was carried out in the Multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of $m/z$ 223.1 precursor ion to the $m/z$ 181.0 for acetazolamide and $m/z$ 226.1 precursor ion to the $m/z$ 182.2 product ion for the IS. Quadrupoles (Q1 and Q3) were set on unit resolution. Dwell time was set at 200 ms. The data acquisition was performed by Analyst software™ (version 1.4.2).

### Preparation of Spiked plasma samples

Stock solutions of acetazolamide and the IS were prepared in HPLC grade methanol (1 mg/mL). Working solutions were prepared using a mixture of methanol and water (50:50, v/v; diluent). Calibration samples were prepared at a concentration levels of 50.3, 101, 300, 601, 1202, 2403, 4806, 7227, 9637 and 12046 ng/mL in K2 EDTA human plasma. Similarly, Quality control (QC) samples were also prepared at concentrations of 50.8 (lower limit of quantitation quality control, LLOQ QC), 152 (low quality control, LQC), 1263 (medium quality control, MQC1), 6016 (MQC2) and 10026 ng/mL (high quality control, HQC).

### Extraction procedure

A 100 µL aliquot of human plasma sample was spiked with the IS dilution (20 µL of 50.0 µg/mL of acetazolamide d3). To this, 100 µL of formic acid buffer (5%) was added after vortex mixing for 10 s. The sample mixture was loaded onto a Orochem Celerity Deluxe SPE cartridge (30 mg/1 mL) that was pre–conditioned with 1.0 mL of methanol followed by 1.0 mL water. The cartridge was washed with 1.0 mL of water followed by 1.0 mL of 10% methanol. Analyte and the IS were eluted with 1.0 mL of methanol. Eluent was evaporated under gentle stream of nitrogen at 40°C and reconstituted with 500 µL of mobile phase. Aliquot of 10 µL of the sample was injected into the chromatographic system.

### Method validation parameters

A thorough and complete method validation of acetazolamide in human plasma was carried out as per US FDA[11] guidelines. The parameters determined were carryover test, selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, long run evaluation and stability.

### Pharmacokinetic study design

A pharmacokinetic study was performed in healthy Indian male subjects ($n$=7) under fed condition. All the volunteers provided with written informed consent. Blood samples were collected after oral administration of 500 mg acetazolamide ER capsule at 0.5, 1, 1.5, 2, 2.5, 3, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 12, 16, 24, 36 and 48 h and collected in $K_2$ EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). A predose sample was also collected before administration of each drug formulation. The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 10°C. Plasma samples were spiked with the IS and processed as per the proposed extraction procedure. WinNonlin (Version 5.2) software package was use to calculate the pharmacokinetic parameters by non–compartmental model. An incurred sample reanalysis (ISR) was also performed as per FDA recommendations by selecting the 2 samples from each subject (14 samples in total) near $C_{max}$ and the elimination phase in the pharmacokinetic profile of the drug. The percent difference between the ISR value and the initial value should not be more than ±20%.[12]

## RESULTS

### Method development

The aim of the present work is to develop a simple, sensitive and rapid LC–MS/MS method for the quantification of acetazolamide in human plasma suitable for pharmacokinetic and bioequivalence studies. Initially, analyte and the IS were tuned in positive and negative ionization modes using ESI source. The high intense signals were obtained in positive ion mode than the negative mode.

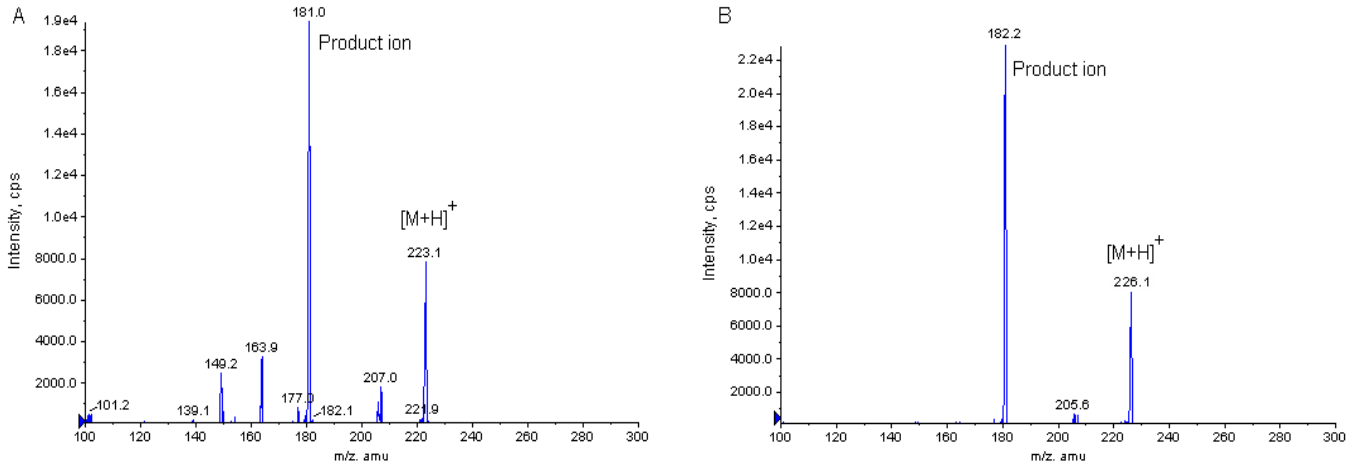


**Figure 1: Product ion mass spectra of [M+H]⁺ of (A) Acetazolamide and (B) Acetazolamide-d3 (IS)**

Data in the Multiple reaction monitoring (MRM) mode was considered, which showed better selectivity. The compound and source dependent parameters were suitably altered to get most intense signals and reproducible response. Data in the Multiple reaction monitoring (MRM) mode was considered, which showed better selectivity.[13] The positive ion spray mass spectrum revealed a protonated molecular by monitoring the transition pairs of *m/z* 223.1 precursor ion to the m/z 181.0 product ion for acetazolamide (Figure 1a) and *m/z* 226.1 precursor ion to the m/z 182.2 product ion for the IS (Figure 1b).

Once the mass spectrometer conditions were set, the mobile phase composition was optimized with acetonitrile and methanol by varying its proportion with volatile buffers like ammonium formate, ammonium acetate, as well as acid additives like formic acid and acetic acid in varying strength. Finally an isocratic mobile phase composed of 0.1% formic acid buffer and acetonitrile (30:70, v/v) at a flow rate of 0.8 mL/min gave symmetric peak shape, best sensitivity and better separation for analyte and the IS. Among the various chromatographic columns tested for their suitability Hypurity advance (50×4.6, 5 µm) column gave good peak shape and response even at lowest concentration level for the analyte. The retention time of analyte and the IS (0.90 and 0.90 min, respectively) were low enough allowing a short run time of 2.0 min.

For LC–MS analysis, there should be a proper extraction procedure which can yields good recovery with no or minimal matrix effect. Hence, Solid phase extraction (SPE) was tested. SPE was tried with Oasis HLB, Starata X polymeric sorbent, Orochem celerity deluxe, Bond Elut Plexa and Orpheus $C_{18}$ extraction cartridges with/without acidic buffer addition. Of all the above, promising results were obtained with Orochem celerity deluxe SPE cartridge (30 mg/1 mL), which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte and the IS from the plasma. Stable labeled isotopes as internal standard increases the bioanalytical assay precision and accuracy. Hence, in the present work acetazolamide stable labeled isotope acetazolamide d3 was employed as an internal standard.

## Carryover test, selectivity and chromatography

No significant carryover was observed in blank sample after injection highest concentration of analyte (ULOQ; upper limit of quantitation). The degree of interference by endogenous plasma components with the analyte and the internal standard was assessed by inspection of chromatograms derived from processed blank plasma sample. As displayed in Figure 2a no significant interference in the blank plasma traces was observed from endogenous components in drug–free plasma at the retention time of analyte and the IS. Moreover, no significant interference was found from the IS to the MRM channel of the analyte (Figure 2b). Figure 2c shows a representative MRM chromatogram resulted from the analysis of LLOQ sample (50.3 ng/mL). A representative chromatograms resulting from the analysis of subject blank plasma sample and 7 h subject plasma sample after the administration of a 500 mg oral single dose of acetazolamide is shown in the Figure 3.

## Sensitivity

The Signal–to–noise ratio (S/N) was measured at a concentration of 50.3 ng/mL, which is set as a lowest limit of reliable quantification (LLOQ) for the analyte. The S/N ratio at this concentration was found to be ≥10. The precision and accuracy at LLOQ concentration were found to be 1.56% and 97.6%, respectively.

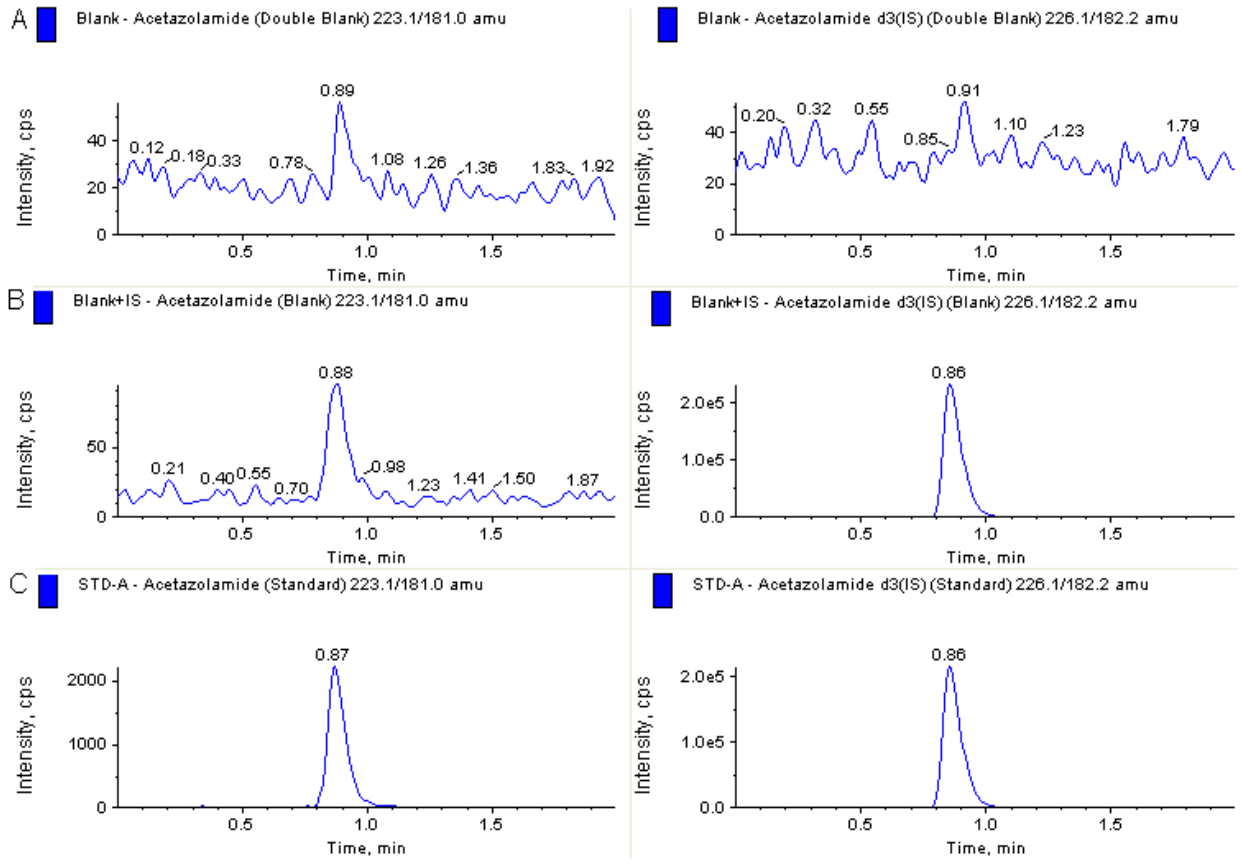


**Figure 2: Typical MRM chromatograms of Acetazolamide (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C)**

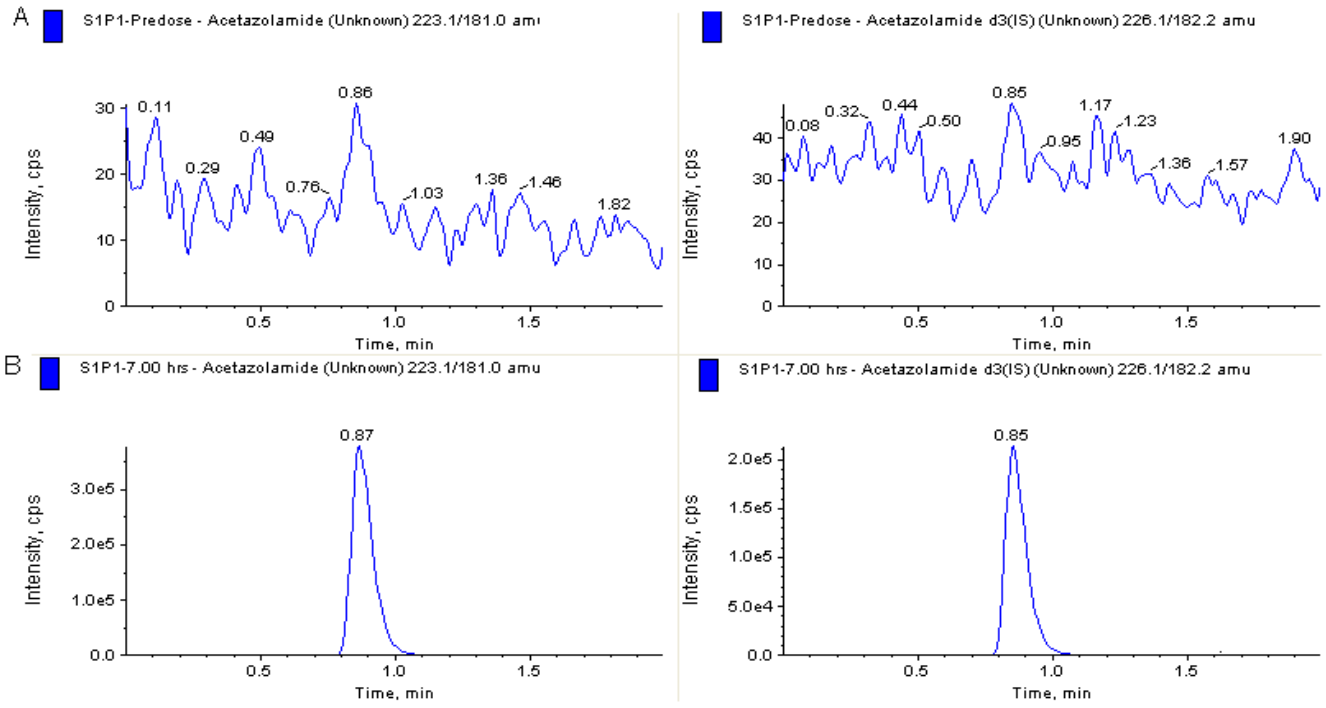


**Figure 3: MRM chromatograms resulting from the analysis of subject blank plasma sample (A) and 7 h subject plasma sample (B), after the administration of a 500 mg oral single dose of acetazolamide ER capsule. The sample concentration was determined to be 9666 ng/mL**

**Table 1: Precision and accuracy data for Acetazolamide**

| Quality control | Run | Concentration found Mean ± SD (ng/mL) | Precision (%) | Accuracy (%) |
|---|---|---|---|---|
| **Intra–day variations (n=12 at each concentration)** | | | | |
| **LLOQ** | | 50.5±4.56 | 9.04 | 99.4 |
| **LQC** | | 148±15.3 | 10.3 | 97.6 |
| **MQC1** | | 1242±11.2 | 0.91 | 98.3 |
| **MQC2** | | 5668±85.8 | 1.51 | 94.2 |
| **HQC** | | 9735±145 | 1.49 | 97.1 |
| **Inter–day variations (n=30 at each concentration)** | | | | |
| **LLOQ** | | 51.3±3.27 | 6.37 | 101 |
| **LQC** | | 150±12.7 | 8.43 | 99.0 |
| **MQC1** | | 1251±25.9 | 2.07 | 99.0 |
| **MQC2** | | 5873±201 | 3.42 | 97.6 |
| **HQC** | | 9925±265 | 2.67 | 99.0 |

Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 50.8, 152, 1263, 6016 and 10026 ng/mL, respectively.

**Table 2: Stability data for Acetazolamide in plasma (n=6)**

| Stability test | QC (spiked concentration, ng/mL) | Mean ± SD (ng/mL) | Precision (%) | Accuracy/ Stability (%) |
|---|---|---|---|---|
| **Aautosampler stability (at 10°C for 65 h)** | 152<br>10026 | 136±1.85<br>9220±92.5 | 1.36<br>1.00 | 89.7<br>92.0 |
| **Wet extract stability (at 2–8°C for 61 h)** | 152<br>10026 | 136±0.87<br>9167±106 | 0.64<br>1.15 | 89.8<br>91.4 |
| **Bench top stability (12 h at room temperature)** | 152<br>10026 | 141±1.72<br>9403±125 | 1.22<br>1.33 | 92.7<br>93.8 |
| **Freeze–thaw stability (4 cycles)** | 152<br>10026 | 136±2.74<br>9232±94.3 | 2.02<br>1.02 | 89.8<br>92.1 |
| **Reinjection stability (25 h)** | 152<br>10026 | 144±2.92<br>10316±103 | 2.03<br>1.00 | 94.7<br>103 |
| **Long–term Stability (at–70°C for 101 days)** | 152<br>10026 | 158±1.86<br>10258±201 | 1.18<br>1.96 | 104<br>102 |
| **Short–term Stability (at –20°C for 15 days)** | 152 10026 | 139±3.52<br>9283±56.3 | 2.54<br>0.61 | 91.5<br>92.6 |

## Linearity and precision and accuracy

Linearity of the proposed method was established over the concentration range of 50.3–12046 ng/mL using five calibration curves. The mean linear equation obtained for acetazolamide was $y=(0.000180 \pm 0.000022)x + (0.000357 \pm 0.000398)$, where $y$ is the peak area ratio of the analyte/IS and $x$ the concentration of the analyte. The mean correlation coefficient values were in the range of 0.9984–0.9992 during entire course of validation.

Intra–day and Inter–day precision and accuracy results of acetazolamide for five analytical runs in spiked quality control samples are summarized in Table 1.

## Recovery and dilution integrity

With the proposed SPE method, the mean overall recovery obtained for acetazolamide was 79.4 ± 3.04% with the precision (%CV) range of 1.29%–6.69% and the recovery of the IS was 77.1% (measured at a concentration of 50.00 µg/mL).

The upper concentration limit of acetazolamide can be extended to 20052 ng/mL by using half (1:2) or quarter (1:4) dilution with screened human blank plasma. The precision (%CV) for dilution integrity of half and quarter dilution was found to be 1.40% and 1.02% and the accuracy results were found to be 97.2% and 95.0%, respectively.

## Long run evaluation

A total of 196 samples were analyzed in a single run. 40 sets of each of LQC, MQC1, MQC2 and HQC samples (stored at –70°C) and 6 sets each of freshly spiked quality control samples were processed and analyzed along with freshly spiked calibration curve standards. 160 QC's out of 160 QC's of long run evaluation and 24 QC's out of 24 QC's of freshly spiked were within 15% of their respective nominal values.

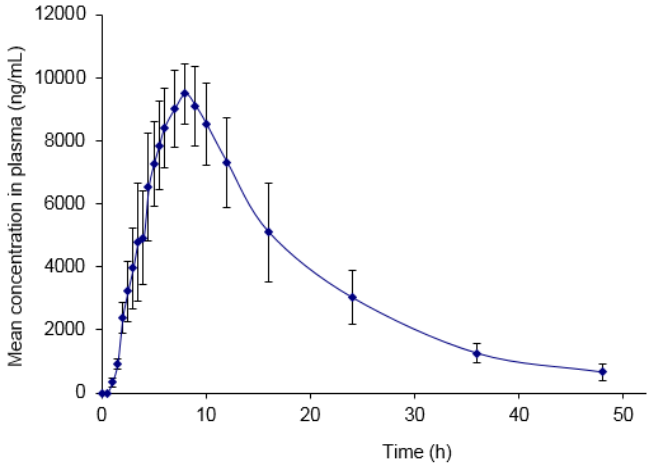


**Figure 4: Mean plasma concentration–time profile of Acetazolamide in human plasma following oral dosing of acetazolamide (500 mg ER capsule) to healthy volunteers (n=7)**

**Table 3: Incurred samples re–analysis data of Acetazolamide**

| Sample | Initial conc. (ng/mL) | Re–assay conc. (ng/mL) | Difference [a] (%) |
|---|---|---|---|
| 1 | 9666 | 9126 | 5.75 |
| 2 | 530 | 498 | 6.27 |
| 3 | 8959 | 9416 | -4.97 |
| 4 | 585 | 611 | -4.34 |
| 5 | 9665 | 10235 | -5.73 |
| 6 | 1248 | 1199 | 3.99 |
| 7 | 9749 | 10197 | -4.49 |
| 8 | 775 | 839 | -7.87 |
| 9 | 10828 | 9676 | 11.2 |
| 10 | 405 | 385 | 4.97 |
| 11 | 9442 | 8930 | 5.57 |
| 12 | 564 | 520 | 8.20 |
| 13 | 10016 | 9814 | 2.04 |
| 14 | 520 | 602 | -14.5 |

[a]Expressed as [(initial conc.–re–assay conc.)/average]×100%.

### Stability studies

The mean % nominal values were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels and the precision (%CV) values were within 15% (Table 2) for all the stability tests carried out during the entire course of method validation. All the above stability results were found to be within the acceptable limits during the entire validation.

### Clinical application and incurred samples reanalysis

The proposed method was successfully used to estimate the real time plasma samples obtained from the pharmacokinetic study ($n$=7). The mean concentration ($C_{max}$) in plasma (10090 ± 687 ng/mL) for acetazolamide was attained at 7.71 ± 1.38 h ($t_{max}$) with a half–life ($t_{½}$) of 11.2 ± 3.53 h. The area under the plasma concentration–time curve from time zero to last measurable time point ($AUC_{0–t}$) and area under the plasma concentration time curve from time zero to infinity time point ($AUC_{0–inf}$) for acetazolamide were 169562 ± 23937 and 181260 ± 24439 ng*h/mL, respectively. Figure 4 depicts the mean plasma concentration *vs* time profile of acetazolamide in healthy subjects. The obtained study data is validated through ISR. The concentration difference between the initial and the ISR values for all the tested samples are presented in the Table 3 and the results indicating good reproducibility of the proposed method.

## CONCLUSION

The LC–MS/MS assay presented in this paper is simple, rapid and sensitive for the determination of acetazolamide in human plasma. To the best of knowledge, this is the first LC–MS/MS report describes the complete method development and validation process for the determination of acetazolamide in human plasma suitable for pharmacokinetic or bioavailability/bioequivalence application. With the reported LLOQ (50.3 ng/mL) we could quantify acetazolamide up to 48 h post–dosing in human volunteers. The simple solid phase extraction method gave high and reproducible recoveries for the analyte and the IS from plasma. A sample turnover rate of less than 2.0 min makes it an attractive procedure in high–throughput bioanalysis of acetazolamide.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATION

| | |
|---|---|
| LC-MS/MS: | Liquid Chromatography/ Tandem Mass Spectrometry |
| MRM: | Multiple Reaction–Monitoring Mode |
| US FDA: | United States Food and Drug Administration |
| IOP: | Intraocular Pressure |
| HPLC: | High Performance Liquid |

| | | | |
|---|---|---|---|
| | Chromatography | | Quality Control |
| PP: | Protein Precipitation | LQC: | Low Quality Control |
| IS: | Internal Standard | MQC: | Medium Quality Control |
| SPE: | Solid–Phase Extraction | HQC: | High Quality Control |
| DP: | Declustering Potential | EDTA: | Ethylenediaminetetraacetic acid |
| CE: | Collision Energy | ER: | Extended Release |
| EP: | Entrance Potential | ISR: | Incurred Sample Reanalysis |
| CXP: | Collision Cell Exit Potential | ESI: | Electro Spray Ionisation |
| LLOQ QC: | Lower Limit of Quantitation | ULOQ: | Upper Limit of Quantitation |

## Highlights of Paper

- First LC-MS/MS report for the determination of acetazolamide in human plasma.
- The method employs only 100 µL of plasma volume and achieved good sensitivity.
- Isotope labeled compound used as an internal standards to get better precision and accuracy.
- The total analysis time is shortest compared to all existing methods.
- Method reproducibility was demonstrated by incurred sample reanalysis.

## Author Profile

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