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Short communication

A validated stability-indicating LC method for acetazolamide in the presence of degradation products and its process-related impurities

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ABSTRACT

The objective of the current study was to develop a validated, specific and stability-indicating reverse phase liquid chromatographic method for the quantitative determination of acetazolamide and its related substances. The determination was done for an active pharmaceutical ingredient, its pharmaceutical dosage form in the presence of degradation products, and its process-related impurities. The drug was subjected to stress conditions of hydrolysis (acid and base), oxidation, photolysis and thermal degradation as per International Conference on Harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. Significant degradation was observed during acid and base hydrolysis, and the major degradant was identified by LC–MS, FTIR and $^{1}H/^{13}C$ NMR spectral analysis. The chromatographic conditions were optimized using an impurity-spiked solution and the generated samples were used for forced degradation studies. In the developed HPLC method, the resolution between acetazolamide and, its process-related impurities (namely imp-1, imp-2, imp-3, imp-4 and its degradation products) was found to be greater than 2. The chromatographic separation was achieved on a C18, 250 mm × 4.6 mm, 5 μm column. The LC method employed a linear gradient elution, and the detection wavelength was set at 254 nm. The stress samples were assayed against a qualified reference standard and the mass balance was found to be close to 99.6%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

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1. Introduction

Acetazolamide, N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-acetamide, is a carbonic anhydrase inhibitor, mainly used to reduce intraocular pressure in the treatment or long-term management of glaucoma. However, the potential therapeutic value of acetazolamide as well as other carbonic anhydrase inhibitors is frequently limited due to a high incidence of side effects associated with their continued use [1]. Epstein and Grant reported that, 44 out of 92 patients undergoing carbonic anhydrase inhibitor therapy complained of systemic adverse reactions, such as malaise, fatigue, weight loss, depression, anorexia and loss of libido [2]. Acetazolamide is sometimes taken prophylatically. The drug promotes the excretion of bicarbonate, the conjugate base of carbonic acid, through the kidneys. By increasing the amount of bicarbonate excreted in the urine, the blood becomes more acidic, which stimulates ventilation and in turn increases the amount of oxygen in the blood. In addition, acetazolamide speeds up part of the acclimatization to acute mountain sickness process, helping to relieve symptoms.

A few chromatographic methods have appeared in the literature for the quantification of acetazolamide in human and rat plasma [3,4]. Several other methods have been published for the quantification of acetazolamide in human urine [5,6]. In addition, Vargas et al. reported the photosensitized degradation of acetazolamide [7] and del Pilar Corena et al. described the degradation and effect of methazolamide and acetazolamide in sheeps-head minnow [8]. To the best of our knowledge stability-indicating LC method for the quantitative estimation of acetazolamide in drug substance and in pharmaceutical dosage forms in the presence of degradation products and process-related impurities has been reported. The purpose of the present research work was to develop a suitable single stability-indicating LC method for the determination of acetazolamide and its related substances. The developed LC method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Forced degradation studies were performed on the drug substance and drug products to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines [9].

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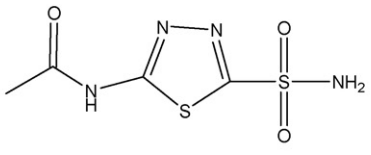
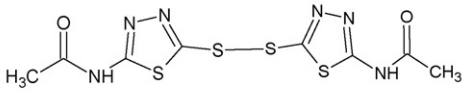


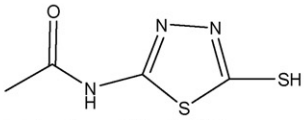
N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-acetamide

Imp-1: Molecular weight :222.25



N,*N*′-(dithiodi-1,3,4-thiadiazole-5,2-diyl)diacetamide

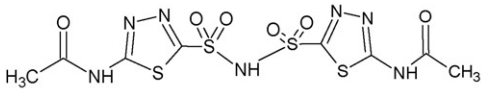
Imp-2: Molecular weight :348.45



Molecular weight :175.23

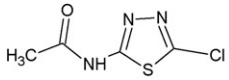
N-(5-mercapto-1,3,4-thiadiazol-2-yl)acetamide

Imp-3



N,*N*′-[iminobis(sulfonyl-1,3,4-thiadiazole-5,2-diyl)]diacetamide

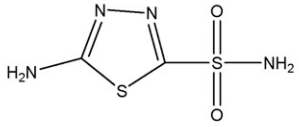
Imp-4 Molecular weight :427.46



N-(5-chloro-1,3,4-thiadiazole-2-yl)acetamide

Molecular weight: 177.61

Degradation imp (RRT 0.29)



N-(5-sulfamoyl-1, 3, 4-thiadiazol-2-yl)-amine

Molecular weight: 179.98

Fig. 1. Structures and names of acetazolamide and its impurities.

2. Experimental

2.1. Chemicals

Samples of acetazolamide and its related impurities were received from Versapharm Incorporated, Warminister, PA, USA (Fig. 1). All of the impurities and the acetazolamide standard were of >99% purity and are as follows: acetazolamide (99.1%), imp-1 (99.5%), imp-2 (99.4%), imp-3 (99.7%) and imp-4 (99.5%). Commercially available acetazolamide sustained-release capsules (Diamox Sequels) were purchased for this study. In addition, HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Analytical reagent grade sodium dihydrogen phosphate monohydrate, phosphoric acid and acetic acid were purchased from Merck. Highly pure water was prepared with the Millipore Milli-Q Plus water purification system.

2.2. Equipment

The LC system used for method development, forced degradation studies and method validation consisted of a Waters 2695 binary pump with an auto sampler and a 2996 photo diode array detector (PDA). The output signal was monitored and processed using Empower software on a Pentium computer (Digital equipment Co.). Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were carried out in a dry air oven (Lindberg-Blue, USA).

2.3. Chromatographic conditions

A Waters symmetry C18 250 mm × 4.6 mm, 5 μm column was used with a mobile phase containing a gradient of solvents A and B. The buffer was composed of 0.02 M sodium dihydrogen phosphate, with its pH adjusted to 3.0 with orthophosphoric acid. Buffer and acetonitrile in the ratio of 950:50 (v/v); was used as solvent A. Water and acetonitrile in the ratio of 150:850 (v/v); was used as solvent B. The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/5, 5/5, 25/50, 30/50, 30.1/5 and 35/5 (time (min)/%B). The column temperature was maintained at 27 °C and the detection wavelength was set at 254 nm. The injection volume was 20 μl. The diluent consisted of buffer and acetonitrile in a ratio of 50:50 (v/v).

2.4. LC–MS conditions

The LC–MS system (Agilent 1100 series liquid chromatography system coupled with a 6400 series triple quadrapole mass spectrometer) was used for the identification of unknown compounds formed during forced degradation. A Waters symmetry C18 250 mm × 4.6 mm, 5 μm column was used as the stationary phase. Water, acetonitrile and acetic acid in a ratio of 950:50:1 (v/v/v) was used as the mobile phase. A mixture of water and acetonitrile in a 50:50 (v/v); ratio was used as the diluent. The flow rate was 1.0 ml/min. The analysis was performed in positive and negative electrospray ionization modes. The capillary and cone voltages were 3.5 kV and 25 V, respectively. The source and dissolvation temperatures were 120 °C and 350 °C, respectively and the dissolvation gas flow was 500 l h^{-1}.

2.5. Preparation of standard solutions and sample solutions:

A stock solution of acetazolamide (5.0 mg/ml) was prepared by dissolving the appropriate amount of acetazolamide solid in the diluent. Working solutions of 1000 and 100 μg/ml were prepared from the stock solution for the determinations of related substances and assay, respectively. A stock solution of impurity (mixture of imp-1, imp-2, imp-3 and imp-4) at 1.0 mg/ml was also prepared in the diluent.

Five capsules were weighed and the powder was emptied from the capsules into a clean dry mortar and mixed well using a pestle. The powder equivalent to 100 mg of drug was transferred into a 100 ml volumetric flask, and 70 ml of diluent was added. The flask was attached to a rotary shaker and shaken for 10 min to disperse the powder completely. The mixture was sonicated for 10 min and then diluted to the appropriate volume with diluent to make a solution containing 1.0 mg/ml. This solution was centrifuged at 3000 rpm for 5 min. The solution was filtered through a 0.45 μm Nylon 66 membrane filter.

2.6. Stress studies/specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [10]. The specificity of the developed LC method for acetazolamide was determined in the presence of its impurities (namely imp-1, imp-2, imp-3 and imp-4) and degradation products. Forced degradation studies were also performed on acetazolamide to provide an indication of the stability-indicating property and specificity of the proposed method [11–15]. The stress conditions employed for the degradation study included light (carried out as per ICH Q1B), heat (60 °C), acid hydrolysis (1 M HCl), base hydrolysis (1 M NaOH) and oxidation (5% H_2O_2). For heat and light studies, the samples were exposed for 10 days, whereas the samples were treated for 24 h for acid, base hydrolysis and for oxidation. The peak purity of the acetazolamide stressed samples was checked by using a Waters 2996 photo diode array detector (PDA). The purity angle was within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the analyte peak.

Assays were carried out for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) was calculated for all of the samples.

2.7. Method validation

The proposed method was validated per ICH guidelines [9].

2.7.1. Precision

The precision of the related substance method was investigated by injecting six individual preparations of (1000 μg/ml) acetazolamide spiked with 0.15% each of imp-1, imp-2, imp-3 and imp-4. The %RSD of the areas of each imp-1, imp-2, imp-3 and imp-4 was calculated.

The intermediate precision of the method was evaluated using a different analyst and instrument located within the same laboratory.

The precision of the assay method was evaluated by carrying out six independent assays of a test sample of acetazolamide against a qualified reference standard. The %RSD of six obtained assay values was calculated.

2.7.2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for imp-1, imp-2, imp-3 and imp-4 were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study was also carried at the LOQ level by injecting six individual preparations of imp-1, imp-2, imp-3 and imp-4 and calculating the %RSD of the areas.

2.7.3. Linearity

Linearity test solutions for the assay method were prepared from a stock solution at six concentration levels from 50% to 150% of the assay analyte concentration (50, 75, 100, 125 and 150 μg/ml). The peak area versus concentration data was analyzed with least-squares linear regression.

Linearity test solutions for the related substance method were prepared by diluting the impurity stock solution (2.5) to the required concentrations. The solutions were prepared at eight concentration levels from the LOQ to 200% of the specification level (LOQ, 0.02%, 0.05%, 0.10%, 0.15%, 0.20%, 0.25% and 0.3%). The slope and *y*-intercept of the calibration curve are reported.

2.7.4. Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150 μg/ml), and the percentage recoveries were calculated.

The drug substance did not show the presence of imp-2, but contained 0.06% of imp-1, 0.1% of imp-3 and 0.07% of imp-4. Standard addition and recovery experiments were conducted to determine the accuracy of the related substance method for the quantification of all four impurities (imp-1, imp-2, imp-3 and imp-4) in the drug substance as well as in the drug product. The study was carried out in triplicate at 0.075%, 0.15% and 0.225% of the analyte concentration (1000 μg/ml). The percentage of recoveries for imp-1, imp-2, imp-3 and imp-4 were calculated.

2.7.5. Robustness

To determine the robustness of the developed method, the experimental conditions were altered and the resolution between acetazolamide and imp-1, imp-2, imp-3 and imp-4 was evaluated. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.2 units (0.8 and 1.2 ml/min). The effect of pH on the resolution of the impurities was studied by varying the pH by ±0.1 units (buffer pH of 2.9 and 3.1). The effect of the column temperature on the resolution was studied at 22 °C and 32 °C instead of 27 °C. In all these varied conditions, the components of the mobile phase remained constant, as outlined in Section 2.3.

2.7.6. Solution stability and mobile phase stability

The solution stability of acetazolamide in the assay method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed for in 6 h intervals over the study period. The mobile phase stability was also examined by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h intervals up to 48 h. The prepared mobile phase remained constant during the study period. The %RSD of the acetazolamide assay was calculated for the mobile phase and solution stability experiments.

The solution stability of acetazolamide and its impurities in the related substance method was carried out by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h. The content of imp-1, imp-2, imp-3 and imp-4 was determined at 6 h intervals up to the study period.

The mobile phase stability was also investigated for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. The content of imp-1, imp-2, imp-3 and imp-4 was determined in the test solutions. The prepared mobile phase remained constant during the study period.

3. Results and discussion

3.1. Method development and optimization

The main objective of the chromatographic method was to separate imp-1, imp-2, imp-3, imp-4 and the generated degradation products from the analyte peak during stress studies. Impurities and degradation products were co-eluted by using different stationary phases, such as C8, cyano and phenyl with various mobile phases with buffers, such as phosphate, sulfate and acetate with different pH values (3–7), and organic modifiers, including acetonitrile and methanol, in the mobile phase. A sodium dihydrogen orthophosphate buffer with a pH value of 3.0 and acetonitrile (50:50, v/v) at a flow rate of 1.0 ml/min was chosen for the initial trail with a 250 mm × 4.6 mm ID column and 5 μm particle size C18 stationary phase. When an impurity-spiked solution was injected, the resolution between the impurities and analyte was poor. Imp-1 and imp-2 were almost co-eluted with the analyte (Fig. 2). To improve the resolution between the impurities and analyte, acetonitrile was replaced with methanol in the mobile phase

and injected the impurity-spiked solution. The resolution between the impurities and analyte was slightly improved but one of the impurities, which was eluted at ~RRT 2.5 (RT ~6.6) in the initial trail (buffer:acetonitrile: 50:50, v/v), was not eluted even after 60 min of run time. To optimize the resolution between the impurities and the retention time of the process impurity, trails were carried out with different mobile phase ratios using buffer and acetonitrile (buffer:acetonitrile: 90:10, 80:20, 70:30, v/v). Isocratic trails were not successful in achieving a favorable resolution between the impurities and analyte peak and the elution of the process impurity. Therefore, a gradient method was selected using buffer and acetonitrile in a ratio of 950:50 (v/v) as mobile phase A and water and acetonitrile in a ratio of 150:850 (v/v) as mobile phase B. Different gradient programs were investigated and satisfactory results

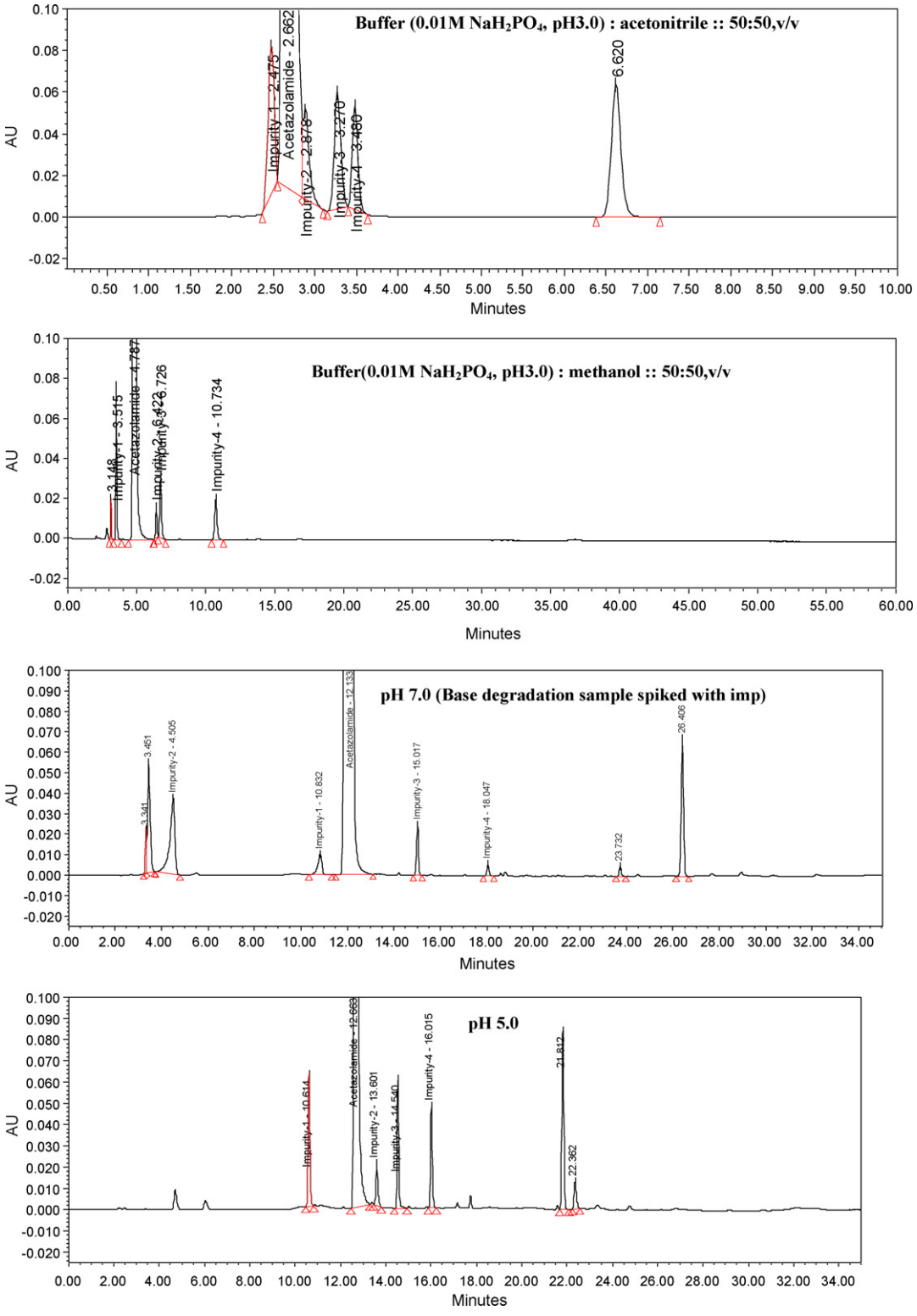


Fig. 2. Typical chromatogram from the method development trials and stressed acetazolamide samples.

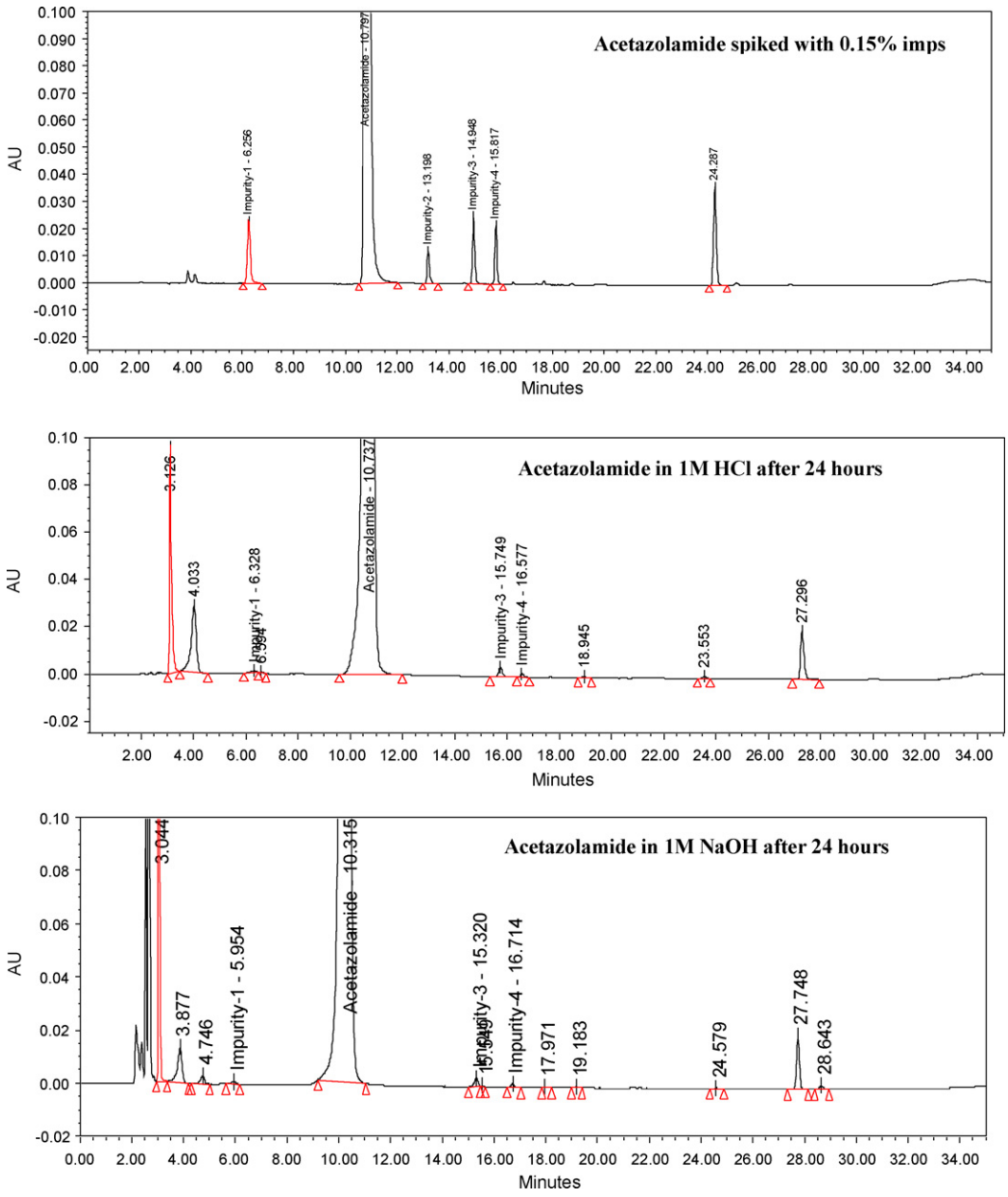


Fig. 2. (*Continued*).

were obtained when a gradient program of (time (min)/%B): 0/5, 5/5, 25/50, 30/50, 30.1/5 and 35/5 was used.

The effect of the buffer pH was also studied under the above conditions. At pH 7.0, the resolution between imp-2 and one of the degradants in base degradation was poor and tailing of the acetazolamide peak was 1.4. At pH 5.0, the resolution between acetazolamide and imp-2 was poor and tailing of acetazolamide peak was 1.3. At pH 3.0, the resolution between all of the impurities, degradants and acetazolamide was >2 and tailing of acetazolamide was 1.1. Therefore, a pH value of 3.0 was selected for further method development.

Under the above described conditions, impurity-spiked solution and degradation samples were injected on to cyano, phenyl and C8 columns. On the cyano and phenyl columns, imp-3 and imp-4 were co-eluted however, on C8 columns, the peak shapes were not good and the resolution between acetazolamide and imp-2 was poor. In contrast, when a Waters symmetry C18 250 mm × 4.6 mm, 5 μm column was used, satisfactory results were obtained. Based on these experiments, the conditions were further optimized as described below.

A Waters symmetry C18 250 mm × 4.6 mm, 5 μm column was used as the stationary phase. The buffer consisted of 0.02 M sodium dihydrogen phosphate monohydrate, and its pH was adjusted to 3.0 using phosphoric acid. Mobile phase A contained a mixture of buffer and acetonitrile in a ratio of 950:50 (v/v). The mobile phase B contained a mixture of water and acetonitrile in the ratio of 150:850 (v/v). The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/5, 5/5, 25/50, 30/50, 30.1/5 and 35/5 (time (min)/%B). The column temperature was maintained at 27 °C and the detection was monitored at a wavelength of 254 nm. The injection volume was 20 μl.

Using the optimized conditions, acetazolamide, imp-1, imp-2, imp-3 and imp-4 were well separated with a resolution of greater than 2 and typical retention times for imp-1, acetazolamide, imp-2, imp-3 and imp-4 of about 6.2, 10.8, 13.2, 14.9 and 15.8 min, respectively. The system suitability results are given in Table 1 and the

Table 1
System suitability report.

Compound	USP resolution (R_S)	USP tailing factor	No. of theoretical plates (USP tangent method)
Imp-1	–	1.2	14,772
Acetazolamide	20.9	1.2	39,759
Imp-2	12.7	1.4	110,972
Imp-3	11.0	1.2	140,190
Imp-4	5.5	1.1	171,110

Table 2
Summary of forced degradation results.

Stress condition	Time	%Assay of active substance	Mass balance (% assay + % impurities + % degradation products)	Remarks
Acid hydrolysis (1M HCl)	24 h	94.2	99.7	One major degradation product was formed
Base hydrolysis (IM NaOH)	24 h	92.5	99.5	One major degradation product was formed
Oxidation (5% H_2O_2)	24 h	98.5	99.7	Mild degradation observed
Thermal (60 °C)	10 days	98.5	99.8	No degradation products formed
Light (photolytic degradation)	10 days	98.6	99.8	No degradation products formed

Table 3
Regression and precision data.

Parameter	Acetazolamide	Imp-1	Imp-2	Imp-3	Imp-4
LOD (μg/ml)	0.04	0.04	0.06	0.03	0.03
LOQ (μg/ml)	0.16	0.16	0.25	0.12	0.09
Regression equation					
Slope (m)	1,052,219.7	232,207.1	66,689.4	332,333.4	454,828.3
Intercept (C)	1,060,977.1	−707.7	−242.2	−86.5	246.9
Correlation coefficient	0.9997	0.9951	0.9965	0.9992	0.9996
Precision (%RSD)[a]	0.42	0.84	1.25	1.42	0.68
Intermediate precision (%RSD)[a]	0.33	0.98	1.33	1.84	0.94
Accuracy at 100% for drug substance	99.2 ± 0.6	100.2 ± 0.8	100.6 ± 0.5	99.6 ± 1.0	99.0 ± 0.8
Accuracy at 100% for drug product	99.6 ± 0.8	100.6 ± 0.4	101.4 ± 0.2	99.9 ± 0.5	99.6 ± 0.6

Linearity range was LOQ to 150% with respect to 1.0 mg/ml acetazolamide for impurities; linearity range was 50–150% with respect to 0.1 mg/ml of acetazolamide for the assay.

[a] Six determinations using LOQ solutions for impurities and 0.1 mg/ml for acetazolamide.

developed LC method was determined to be specific for acetazolamide and the four impurities, imp-1, imp-2, imp-3 and imp-4 (Table 2).

3.2. Method validation

3.2.1. Precision

The %RSD of acetazolamide during the assay method precision study was within 0.5% and the %RSD values of the area of imp-1, imp-2, imp-3 and imp-4 in the related substance method precision study were within 1.5%. The %RSD of the results obtained in the intermediate precision study was within 0.4% and the %RSD of the areas of imp-1, imp-2, imp-3 and imp-4 were well within 2%, revealing the high precision of the method (Table 3).

3.2.2. Limit of detection and limit of quantification

The limits of detection and quantification of acetazolamide, imp-1, imp-2, imp-3 and imp-4 (analyte concentration of 1000 μg/ml) for a 20 μl injection volume are given in Table 3. The precision at the LOQ concentration for imp-1, imp-2, imp-3 and imp-4 was below 2%.

3.2.3. Linearity

The linear calibration plot for the assay method was obtained over the tested calibration range (50–150 μg/ml) and the obtained correlation coefficient was greater than 0.999. The results revealed an excellent correlation between the peak area and analyte concentration. The slope and *y*-intercept of the calibration curve were 1,052,220 and 1,060,977, respectively.

The linear calibration plot for the related substance method was determined over the calibration ranges (LOQ to 0.3%) for imp-1, imp-2, imp-3 and imp-4, a correlation coefficient of greater than 0.99 was obtained. The linearity was checked for the related substance method over the same concentration range for 3 consecutive days. The %RSD values of the slope and *y*-intercept of the calibration curves were 3.5 and 6, respectively. These results showed an excellent correlation between the peak areas and concentrations of imp-1, imp-2, imp-3 and imp-4 (Table 3).

3.2.4. Accuracy

The percentage recovery of acetazolamide in the drug substance and product ranged from 98.9 to 100.6 and from 98.2 to 102, respectively. The percentage recoveries of imp-1, imp-2, imp-3 and imp-4 in the drug substance and product ranged from 98.1 to 101.5 and from 98.9 to 102.1, respectively. The HPLC chromatograms of spiked samples at the 0.15% level of all four impurities in the acetazolamide drug substance sample are shown in Fig. 2.

3.2.5. Robustness

In all of the deliberately varied chromatographic conditions carried out as described in Section 2.3 (flow rate, pH and column temperature), the resolution between the closely eluting impurities, namely imp-3 and imp-4 was greater than 5.0, illustrating the robustness of the method. The assay variability of acetazolamide and the impurities was within ±1% and within ±2%, respectively.

3.2.6. Solution stability and mobile phase stability:

The %RSD of assaying acetazolamide during the solution stability and mobile phase stability experiments was within 1%. No

Table 4
Spectral data for the isolated compound at RRT 0.29 in acid and base hydrolysis.

Spectral technique	Isolated compound (0.29 RRT imp)
m/*z* (ESI +ve)	181.0 (M+H)
IR (cm^{-1})	3431.36, 3325.28 (primary amine) 1640 (NH bending), 1320.5 (–C–N stretching)
^{1}H NMR CD3OD, 300 MHz; δ (ppm)	4.0 (S) (C–NH), 2.0 (S) (amine)
^{13}C NMR CD3OD, 60 MHz; δ (ppm)	161.6

significant changes were observed in the content of imp-1, imp-2, imp-3 and imp-4 during the solution stability and mobile phase stability experiments when performed using the related substances method. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays and related substance determinations were stable up to 48 h.

4. Results of forced degradation studies

Degradation was not observed in acetazolamide stressed samples subjected to light and heat. Significant degradation of the drug substance and product was detected under acid and base hydrolysis, leading to the formation of one major unknown degradation product at 0.29 RRT (Fig. 2). Peak purity test results derived from the PDA detector, confirmed that the acetazolamide peak and the degraded peaks were homogeneous and pure in all of the analyzed stress samples. Assay studies were carried out for the stress samples (at 100 μg/ml) against a qualified reference standard of acetazolamide.

The mass balance of the stressed samples was close to 99.6%. The assay of acetazolamide was unaffected in the presence of imp-1, imp-2, imp-3, imp-4 and its degradation products, confirming the stability-indicating power of the developed method.

4.1. Identification of major degradation product (RRT 0.29) formed in acid and base hydrolysis

A LC–MS study was carried to determine the *m*/*z* value of the major degradation product formed under acid and base hydrolysis using an Agilent 1100 series liquid chromatography system coupled with a 6400 series triple quadrapole mass spectrometer. The volatile mobile phase contained water, acetonitrile and acetic acid in a ratio of 950:50:1 (v/v/v) and the conditions were described in Section 2.4. The *m*/*z* value obtained for the degradation product resolving at 0.29 RRT in ESI positive mode was 181 (M+H) and corresponds to a molecular weight of 180. The impurity was isolated using preparative LC–MS, and its structure was confirmed by characterization through FTIR, and $^{1}H/^{13}C$ NMR spectral analysis (Table 4).

The mass data was generated for the process-related impurity that eluted at RRT ~2.6 in positive electron spray ionization (ESI) mode. The *m*/*z* value obtained for the degradation product resolving at ~2.6 RRT was 634.1. However, FTIR, and $^{1}H/^{13}C$ NMR spectral data for this impurity is not available as this impurity could not be isolated.

5. Conclusions

In this paper, a sensitive, specific, accurate, validated and well-defined stability-indicating LC method for the determination of acetazolamide in the presence of degradation products and its process-related impurities was described. The behavior of acetazolamide under various stress conditions was studied, and the hydrolysis (acid and base) degradant was identified by LC–MS and presented. All of the degradation products and process impurities were well separated from the drug substance and drug product demonstrates the stability-indicating power of the method. The information presented in this study could be very useful for quality monitoring of active pharmaceutical ingredients in their, dosage forms and be used to check drug quality during stability studies.

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References

[1] P.J. Wistrand, The use of carbonic anhydrase inhibitors in ophthalmology and medicine, Ann. N. Y. Acad. Sci. 429 (1984) 609–619.
[2] D.L. Epstein, W.M. Grant, Carbonic anhydrase inhibitor side effects: serum chemical analysis, Arch. Opthalmol. 95 (1997) 1378–1382.
[3] N. Ichikawa, K. Naora, H. Hirano, K. Iwamoto, Quantitation of acetazolamide in rat plasma, brain tissue and cerebrospinal fluid by high-performance liquid chromatography, J. Pharm. Biomed. Anal. 17 (1998) 1415–1421.
[4] A. Zarghi, A. Shafaati, Rapid determination of acetazolamide in human plasma, J. Pharm. Biomed. Anal. 28 (2002) 169–172.
[5] R. Herráez-Hernández, P. Campíns-Falcó, A. Sevillano-Cabeza, Determination of acetazolamide in human urine samples by reversed-phase high-performance liquid chromatography in the presence of xanthines, J. Chromatogr. Biomed. Appl. 582 (1992) 181–187.
[6] P. Campíns-Falcó, R. Herráez-Hernández, A. Sevillano-Cabeza, Application of column-switching techniques to the determination of medium polarity drugs: determination of acetazolamide in urine, J. Chromatogr. B: Biomed. Sci. Appl. 654 (1994) 85–90.
[7] F. Vargas, M.V. Hisbeth, J.K. Rojas, Photolysis and photosensitized degradation of the diuretic drug acetazolamide, J. Photochem. Photobiol. A 118 (1998) 19–23.
[8] M. del Pilar Corena, P. van den Hurk, H. Zhong, C. Brock, R. Mowery, J.V. Johnson, P.J. Linser, Degradation and effects of the potential mosquito larvicides methazolamide and acetazolamide in sheepshead minnow (*Cyprinodon variegatus*), Ecotoxicol. Environ. Saf. 64 (2006) 369–376.
[9] ICH Q2 (R1), Validation of Analytical Procedures: Text and Methodology, 2005.
[10] ICH Q1 (R2), Stability Testing of New Drug Substances and Products, 2000.
[11] M. Bakshi, S. Singh, Development of validated stability indicating assay methods—critical review, J. Pharm. Biomed. Anal. 28 (2002) 1011–1040.
[12] D.V. Subbarao, P. Radhakrishnanand, M.V. Suryanarayana, V. Himabindu, A stability indicating LC method for Candesartan cilexetil, J. Chromatogr. 66 (2007) 499–507.
[13] D.V. Subbarao, P. Radhakrishnanand, V. Himabindu, Stress degradation studies on Tadalafil and development of a validated stability indicating LC assay for bulk drug and pharmaceutical dosage form, J. Chromatogr. 67 (2008) 183–188.
[14] D.V. Subbarao, P. Radhakrishnanand, Stress degradation studies on Dutasteride and development of a stability indicating HPLC assay for bulk drug and pharmaceutical dosage form, J. Chromatogr. 67 (2008) 841–845.
[15] J.T. Carstensen, C.T. Rhodes, Drug Stability Principles and Practices, 3rd ed., Marcel Dekker, New York, 2000.