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AN IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR ACETAZOLAMIDE

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ABSTRACT

A high performance liquid chromatographic (HPLC) method for the quantitation of acetazolamide in a suspension dosage form is described. The method is simple, accurate, and precise. Acetazolamide was extracted from the suspension using a mobile phase which contained 65% phosphate buffer and 35% methanol and separated from other components by reversed-phase high performance liquid chromatography. The separation was carried out using a C18 column (250 x 4.5 mm i.d.). The photometric detector was set at 254 nm. The excipients present in the tablets from which the suspension was compounded did not interfere with the assay procedure. Sixteen replicate injections of 10.5 mg/mL samples resulted in a percent relative standard deviation of 2.02.

INTRODUCTION

Acetazolamide has been used for a variety of indications. Due to its carbonic anhydrase inhibitory effect, it reduces the rate of aqueous humor formation and decreases the intraocular pressure in patients with glaucoma. It is also very widely used as a diuretic. It is effective for adjunctive treatment of edema due to congestive heart failure or drug induced edema. Despite its extensive usage, very little information is available in the literature concerning the physicochemical and stability properties of acetazolamide. The USP-NF XXI method for the determination of acetazolamide in tablets was based on polarography. However, the USP-NF XXII edition has included a chromatographic procedure for analysis. The USP method for quantitation of acetazolamide in the injection form is based on UV spectrometry and it is not a stability indicating procedure. Three HPLC methods have been reported to quantify the drug in biological fluids.[1,2,3] In addition an enzymatic method[4] and an electron capture gas chromatographic procedure[5] have been reported. These HPLC methods require extensive and time consuming extraction and evaporation steps. Gupta et al.[6] has reported an assay for quantifying the drug in pharmaceutical dosage forms utilizing its sodium hydroxide conversion to the basic form. It has, however, been reported that acetazolamide appears to be very unstable in the presence of 0.1 *N* NaOH with a half life of about only 14 days.[6] Our assay procedure does not use sodium hydroxide and thus avoids this potential complicating factor. Moreover, the assay being proposed uses a slower flow rate with greater separation of peaks as compared to the assay reported. A simpler and more precise assay procedure was provided by this investigation.

EXPERIMENTAL METHOD

Chemical & Reagents: All reagents used were either USP, NF, HPLC or ACS grade and were used without further purification. Pure acetazolamide powder (Lot # 107F- 0807) and theophylline anhydrous crystals (Lot # 97F- 0733) were from Sigma Chemical Co., St. Louis, MO.63178.

Apparatus: The high performance liquid chromatographic (HPLC) system consisted of a Beckman 110B solvent delivery system; Beckman system organizer injector fitted with a 20 µL loop, Model 420 system controller programmer (Beckman Instruments Inc., San Ramon, CA 94583); a spectrophotometric UV detector (Number 731.7100000 from Knauer Corp., Berlin, W. Germany); and a Shimadzu C-R3A Chromatopac Integrator (Shimadzu Corp., Kyoto, Japan). The Column was 10µm, C18 Paratisil 10 0DS-3 (250 x 4.6 mm from Whatman Inc., Clifton, NJ 07014). The absorbance output was received by the integrator. The chart speed was 10 cm/minute.

Chromatographic Conditions: Phosphate buffer (0.020 mol/L) was prepared in deionized water using monobasic sodium phosphate and dibasic sodium phosphate. The buffer (pH = 5.0) was filtered through a 0.45 µm membrane filtration system (Millipore Corp., Milford,. MA 01757) to remove particulate matter. The mobile phase contained 65% phosphate buffer and 35% HPLC grade methanol. The mobile phase was degassed with helium or nitrogen before use.The flow rate was set at 1.0 mL/minute. The column pressure varied from 1500-2000 psi. The detector was operated at 254 nm and 0.04 AUFS. The analytical column was at ambient temperature approximately 22°C for all separations.

Standard Solution Preparation: Approximately 16.5 mg of pure acetazolamide powder was weighed, transferred into a 100 mL volumetric flask and diluted with methanol to give a concentration of 0.165 mg/mL. Also, approximately 100 mg of theophylline anhydrous crystals were transferred to a 100 mL volumetric flask and diluted with methanol to give a concentration of 1.0 mg/mL.These stock solutions were used to prepare 9 different dilutions. A volume of 1.0 mL of theophylline internal standard solution was used for each acetazolamide sample assay. Approximately 50 μL of the solution was injected into the chromatographic sample port fitted with a 20 μL loop. The areas under the curve for both the pure acetazolamide and the internal standard were recorded. The ratio was calculated by dividing the area under the curve for acetazolamide by the area under the curve for the internal theophylline standard. Each sample solution was injected three times, and the average readings for the ratio were recorded. The ratios of the areas under the curve for acetazolamide standard and the internal standard were linearly related to the acetazolamide concentration over the range of 0-50 μg/mL. Regression analysis of the calibration curve generated the equation used to calculate the acetazolamide concentration in the suspension sample. The calibration curve for acetazolamide standard concentrations is shown in Figure 1. A representation of chromatograms of the separation of acetazolamide from theophylline is shown in Figure 2.

Assay Procedure for the Acetazolamide Suspension Sample: The acetazolamide suspension was prepared by levigating the powder with 70% sorbitol solution. The mixture was then incorporated into a suspension vehicle containing magnesium aluminum silicate and carboxymethylcellulose sodium. A 1.0 mL aliqout of the suspension was removed with a pipet and placed into a 50 mL volumetric flask. Individual samples were then diluted with the mobile phase. After mixing thoroughly for 5 minutes, a 2.0 mL portion was withdrawn and placed into a 25 mL volumetric flask. A 1.0 mL aliqout of the internal standard theophylline solution (1.0 mg/mL in methanol) was also added, and the mixture diluted with the mobile phase. A 25 - 50 μL sample of this prepared solution was injected into the column of the chromatograph.

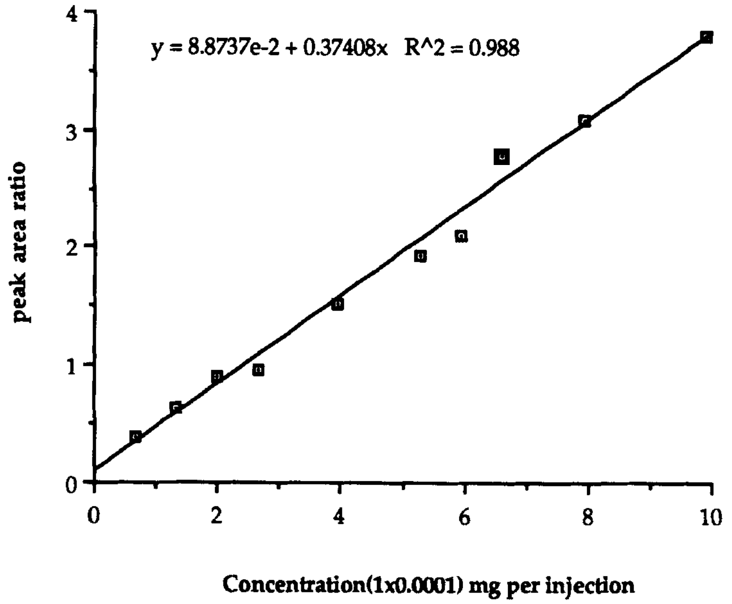


FIGURE : 1

Calibration Curve of Acetazolamide Standard Concentrations.

Calculations of Acetazolamide Concentration: The concentration of acetazolamide in the suspension was determined by dividing the area under the curve for the suspension sample by the area under the curve for the internal standard. The ratio obtained was substituted into the equation of the line obtained from the calibration curve. The concentration in the injection was determined and multiplying by the dilution factor determined the initial concentration of the drug in the suspension.

Stability Indicating Nature of Assay: Figures 3 and 4 show the chromatograms for the forced degradation of acetazolamide solution in 1.0*N* hydrochloric acid and 0.5 *N* sodium hydroxide respectively. Acetazolamide solutions were heated under acidic and alkaline conditions respectively for 2 hours, allowed to cool, internal standard added and

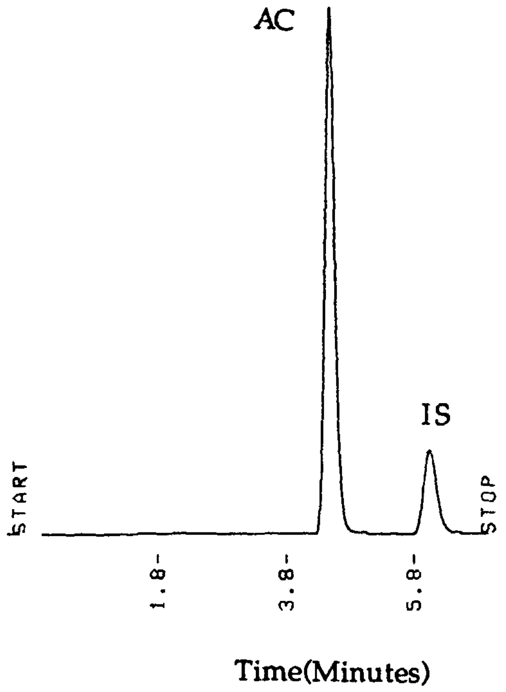
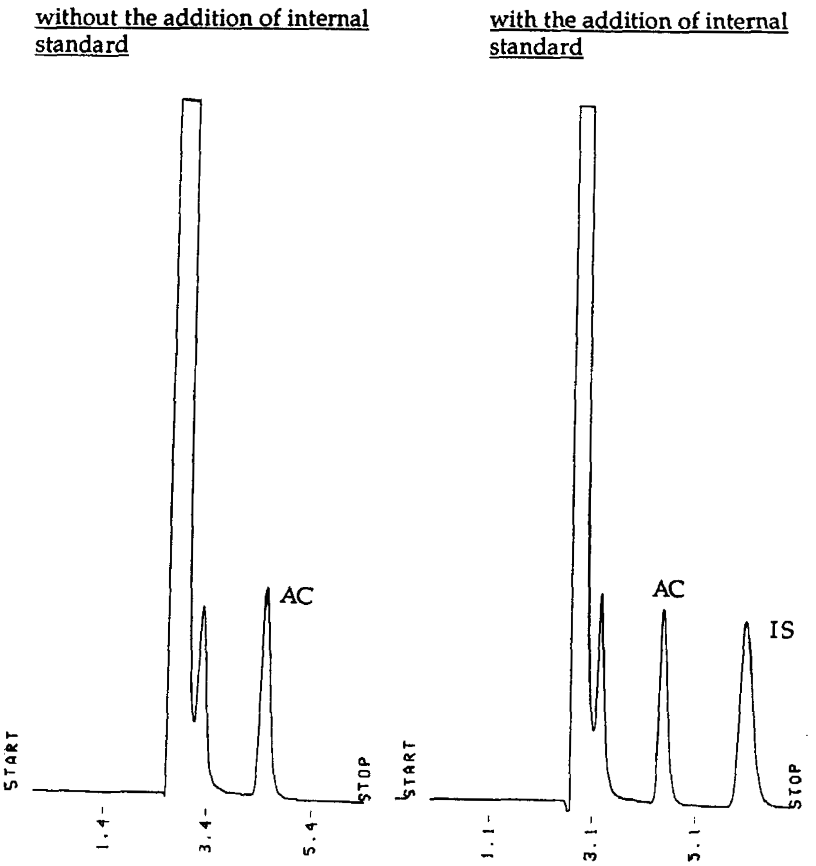


FIGURE : 2

Sample Chromatogram Showing the Separation of Pure Acetazolamide (AC) from Theophylline as Internal Standard (IS).

subjected to the same assay procedure. Under acidic condition the acetazolamide peak decreased and a new peak appeared with a retention time of 2.7 minutes. With the heated alkaline condition a small peak appeared at 3.3 minutes. Peaks for intact acetazolamide and the internal standard still appeared at the same retention time in both cases. None of the degradation products of the drug interfered with either of the two peaks and were also quite well separated from them. This ensures that the high pressure liquid chromatographic procedure was stability indicating.



without the addition of internal standard

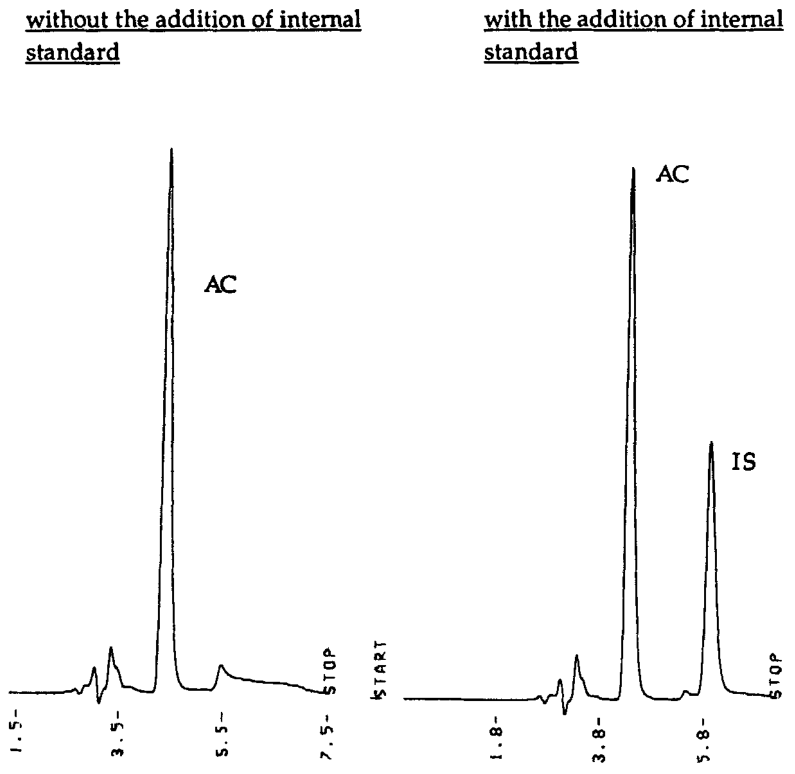
with the addition of internal standard

FIGURE: 3

Chromatograms of Forced Degradation of Acetazolamide Solution with 1.0 *N* Hydrochloric Acid. (AC):Acetazolamide, (IS):Theophylline as Internal Standard.

RESULTS AND DISCUSSION

Figure 1 shows the linear relationship between the ratio's of area under the curve and the acetazolamide concentration in methanol. The stock solution from which all the dilutions were made had a concentration of 0.16 mg/mL. The concentration of acetazolamide was calculated from the equation of the calibration curve which was obtained by the regression



without the addition of internal standard

with the addition of internal standard

FIGURE : 4

Chromatograms of Forced Degradation of Acetazolamide Solution with 0.5 *N* Sodium Hydroxide. (AC):Acetazolamide, (IS): Theophylline as Internal Standard.

analysis of the curve. Typical chromatograms for the samples containing pure acetazolamide and theophylline as the internal standard are shown in Figure 2. The retention times of acetazolamide and theophylline were 4.4 minutes and 5.8 minutes repsectively. The extraction procedure for acetazolamide from the suspension sample consisted of using the mobile phase which contained 65% phosphate buffer and 35% methanol. The recovery of the drug from the sample is shown in Table I.

TABLE :I

Recovery Data for Acetazolamide Samples

Concentration (μg/mL)	Peak area ratio[a] (expressed as % RSD)	AmountRecovered %
3.30	0.3900(3.34)	121.81
6.60	0.6384(3.96)	111.51
19.80	1.5328(2.33)	97.58
26.40	1.9363(0.52)	94.54
33.00	2.7861(4.37)	109.09
39.60	3.0874(2.45)	100.97
49.50	3.8084(3.61)	100.59

[a] Peak area ratio values are averaged based on triplicate injections

The reproducibility of the assay results depended to some extent on the difficulty experienced with measuring samples of suspension for analysis, instrumental error and the distribution of particulate matter within the preparations when shaken prior to sampling. Different compositions for the mobile phase (buffer/methanol) were also examined but produced less acceptable results. Isopropanol was also used as a potential solvent instead of methanol. There were several differences observed with this solvent change. The retention times of acetozolamide and the internal standard were shortened considerably and the two peaks were mixed one with the other when isopropanol was used so no separation was observed. The use of isopropanol as a solvent for the separation was therefore ruled unacceptable. Sonification was also examined to see if this procedure had any effect on the extraction of the drug from the suspension. The result showed that samples which did not undergo sonification had greater recovery than the ones which did undergo sonification. Thus, it was concluded that sonification would not have a positive effect on the extraction of the drug. Attempts to develop a more efficient extraction procedure and the best solvent system were made, but the results were not as significant as compared to the mobile phase conditions initially specified.

An attempt was made to identify any single peak which was not attributable to the acetozolamide in the suspension sample. These peaks could only be derived from the suspending agents or any of the sweeteners or preservatives used in the suspension formulation. An identical suspension was made without the drug and a sample was injected to confirm that any of the peaks seen were from the suspension components and not from the drug itself. A number of researchers have reported on the stability of suspensions and have faced this same problem with extraneous peaks. However, no one has identified the peaks. The extraneous peak obtained in the system under investigation was derived from the methyl/propyl parabens which were used as the preservative for the suspension formulation. It appeared, fortunately at a much greater retention time. A system containing an identical concentration of the parabens to that of the suspension was prepared using a stock solution of the parabens in propylene glycol. A sample was injected. The retention times and the areas under the curves were almost identical for the paraben stock solutions and the suspension sample. This confirmed that the extraneous peak obtained was from the parabens.

CONCLUSION

In the analysis of the acetazolamide suspension, the high performance liquid chromatographic procedure offered the desired accuracy, sensitivity, and specificity. By the appropriate choice of column, mobile phase, and operation of the UV detector at 254 nm the suspension excipients were found not to interfere with any of the assay procedures. The assay procedure is simple, precise and accurate with a percent relative standard deviation of 2.02 based on sixteen injections of a sample of approximately 10.5 mg/mL.

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