ORIGINAL ARTICLE

SIMULTANEOUS DETERMINATION OF CEFAZOLIN OR CEFTIZOXIME IN PRESENCE OF ASCORBIC ACID FROM PHARMACEUTICAL FORMULATION AND HUMAN SERUM BY RP-HPLC

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ABSTRACT

A rapid, sensitive and specific RP-HPLC method involving HPLC-UV detection was developed and validated for simultaneous determination and quantification of cefazoline or ceftizoxime in presence of ascorbic acid. Chromatography was carried out on a pre-packed Kromasil 100, C_{18} (5µm 25 x 0.46 cm) column using acetonitrile: water (60:40; v/v) as mobile phase at a flow rate of 0.75 ml/minute and effluent were monitored at 265 nm for cefazoline and ascorbic acid while at 270 nm for ceftizoxime. The assay was linear over the concenteration range of 0.05-100µg/ml. The method is convenient for determination of cefazoline or ceftizoxime in presence of ascorbic acid with percent recovery ranging from 99.0-100.0% with an inter and intra day CV <3%. The method does not require more than 8 minutes for analysis with good peak resolution and low LOD 0.1µg /ml and LOQ 0.3µg/ml.

Keywords: Cefazoline, ceftizoxime, ascorbic acid, simultaneous determination, HPLC-UV.

INTRODUCTION

Cefazoline is 3-[(5-methyl-1-1,3,4-thiadazole-2-yl)-thio]methyl]-7-[2-(1H-tetrazol-1-yl) acetamidol]-3-cephem-4-carboxylic acid, while ceftizoxime is [6R-[6a, 7b (z)]]-7-[[2-amino-4-thiazol (methoximino) acetyl] amino]-8-oxo-5-thia-1-aza-bicyclo [4.2.0] oct-2-one-2-carboxylic (Gold and Mckee,1973). Cefazoline and ceftizoxime are first and third -generation injectable cephalosporins (Fillastre *et al*., 1973).

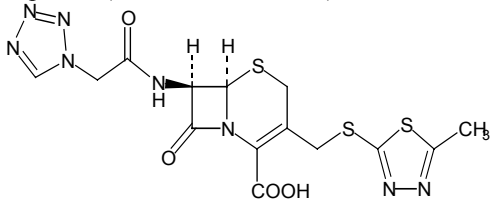

Fig. 1: Cefazoline

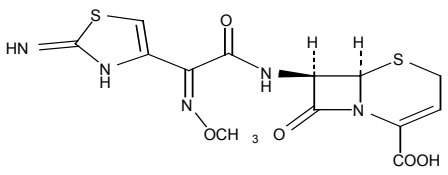

Fig. 2: Ceftizoxime

Cefazoline is more active against Gram-negative *bacilli* while less active against Gram-positive *cocci* than either cephalothin or cephaloridine (Dollery *et al.,* 1991; and Bryant, 1984). It provides higher serum levels, slower renal clearance, and a longer half-life than other first-generation cephalosporins. It has been satisfactory to treat infection of the respiratory tract, urinary tract, skin, soft tissues, joints, bones, and septicemias (Jaim and William, 2004).

A method for determination of cefazoline by HPLC using 0.02 M monobasic sodium phosphate (mobile phase) at pH 6.2 adjusted with 1N sodium bicarbonate, and detection at 254 nm with a flow rate 0.5ml/minute has been discussed by Klaus (Klaus, 1975). Ceftizoxime was determined using a reversed phase column with mobile phase NaOAc-MeOH-H-MeCN (Haneishi, *et al*., 1983).

Vitamins A, E and C play an important role in antioxidant activity and immunomodulation (Lykkesfeldt *et al.,* 1995). Ascorbic acid (AA) is the principal biologically active form but dehydroascorbic acid (DHA) also exhibits biological activity since it can be easily converted into AA in the human body (Ahmed *et al.,* 2004, Lee and Kader, 2000, Tudela, 2002, Grahm and Annette, 1992). Therefore, it is important to measure both AA and DHA when reporting total vitamin C levels. Vitamin C present in human milk plays several biochemical roles linked to the functioning of the immune system. It helps in the maintenance of a natural barrier against infection, stimulates leukocytes for their phagocytic and anti-

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microbial activity, augments antibody production and complement levels (Grahm and Annette, 1992) and also enhances synthesis of interferon (Thomas and Holth, 1978) for growth development and survival, infants need an optimum supply of ascorbic acid.

The concentrations of the hydrophilic antioxidants ascorbic acid and dehydroascorbic acid in some biological samples have been considered possible biomarkers of oxidative stress (Buss *et al*., 2001). Therefore, AA and DHA could also be considered as biomarker of oxidation and affect storage time of human milk.

Several methods have been developed for the estimation of vitamin C levels (Arya *at al*., 2000, Wimalasiri and Wills, 1983, Ashoor *et al.,* 1984). High-performance liquid chromatography, using a UV detector is currently the most commonly used technique for the analysis of ascorbic acid in food (Lee and Kader, 2000, Wimalasiri and Wills, 1983, Ashoor *et al.,* 1984, Esteve *et al.,* 1995, Kall and Andersen, 1999, Furusawa, 2001, Perezvicente, 2002, Steffensen, 2002, and Sanchez-Moreno, 2003). Some HPLC methods require electrochemical (Bank *et al* 1985, Dhariwal, 1990 and Washko, 1989) or fluorimetric detection (Ancos *et al* 2000, Ostdal *et al*., 2000 and Nielsen, 2001) because of the low absorptivity of DHA in the ultraviolet range of the spectrum, but the necessary equipment is not always available in hospital laboratories. To solve this problem, some authors propose the previous reduction of DHA to AA using DL-dithiothreitol (Arya *et al.*, 2000, Bank *et al.,* 1985, Dhariwal, 1990, Washko *et al*., 1989, Ancos *et al.,* 2000, Ostdal *et al.,* 2000, Nielsen *et al*., 2001 and Esteve *et al.,* 1997). The quantification of the latter acid allows an indirect estimation of DHA levels. Enzymatic methods using commercial test kits are also used for determining ascorbic acid levels (Bensinger, 1978, Liu 1982, Lee, 1997 and Bandrakham, 2004). There are several problems associated with the determination of Vitamin C levels using enzymatic methods, for example, the reduction of dehydroascorbic acid to ascorbic acid cannot be measured, the minor specification of the sample and the low recovery of the ascorbic acid (Bandrakham, 2004).

The aim of this study was to develop and validate HPLC method for the simultaneous determination and quanification of cefazoline, ceftizoxime and ascorbic acid in raw materials, pharmaceutical formulations and in human serum.

EXPERIMENTAL

Materials, reagents and chemicals

Cefazolin, ceftizoxime and ascorbic acid reference standards were obtained from AGP (Pvt) Ltd. All reagents used were of analytical grade and obtained from Merck. HPLC grade acetonitrile (Tedia, USA) and deionized filtered water were used to prepare a mobile phase.

Chromatographic conditions

The liquid chromatographic system consisted of Shimadzu model LC-10AT *VP* pumps with a SPD-10AT *VP,* variable wavelength UV-Visible detector. Chromatographic system was integrated via Shimadzu model CBM-102 communication bus Module to a Pentium 4 PC for chromatographic determination and evaluation of data. Analysis was conducted on a Kromasil 100, C_{18} (5μm 25 x 0.46 cm) analytical reverse-phased column at ambient temperature. The samples were introduced through a Rheodyne injector valve with a 20-μL sample loop using acetonitrile: water (60:40; v/v) as mobile phase at a flow rate of 0.750 ml/minute. The mobile phase was filtered daily using 0.45 μm membrane filter (Millipore, Germany) degassed in an ultrasonic bath, and pumped at a flow rate of 0.750 ml/minute. The eluents were monitored at 265 and 270 nm.

Standard and working solutions

Standard solutions of cefazoline, ceftizoxime and ascorbic acid containing 100 μg/ml were prepared by dissolving them in freshly prepared deionised water. These were diluted to 50, 25, 12.5, 5, 1 0.9, 0.5, 0.09 and 0.05μg/ml working solutions and analyzed using the above technique.

Drug-plasma solutions

Multiple blood samples (10 ml) of ten healthy non-smoker volunteers (age ranging from 22-25 years) not involved in any strenuous activity and not taking any medicaments were collected in evacuated glass tubes. The blood was then centrifuged at 3000 rpm for 10 minutes and the plasma separated and deprotinated by acetonitrile. The supernatant obtained was filtered through a 0.45 μM filter. Serum thus obtained was mixed in ratio of 1:1 with drug solutions and stored at –20°C pending drugs analysis. The recovery of cefazolin, ceftizoxime and ascorbic acid from serum was determined under the stated chromatographic conditions.

RESULTS AND DISCUSSION

The proposed HPLC method required fewer reagents and material, and it is simpler and less time-consuming. Further, it is possible to analyze more samples concurrently than the enzymatic method which enzymatic method does not determine the total vitamin C because of the reduction of the dehydroascorbic acid to ascorbic acid. The near-absence of sample preparation and the easy use of HPLC technique make it an ideal quality control tool for food and pharmaceutical industries.

Methods development and optimization

In reported methods the mobile phase consisted of complicated buffers causing column corrosion. In our method, the mobile phase simply consisted of acetonitrile and water (40:60). Other ratios (10:90, 30:70, 20:80 v/v)

Table 1: Calibration data, recovery, within-day and between day precision and accuracy for ascorbic acid.

Drug injected µg/ml	————>Intra day<————			————>Inter day<————			——>Human serum ——>		
	Found µg/ml	%,RSD	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)
0.05	0.05	0.0100	100.0	0.05	0.0100	100.0	0.05	0.0112	100.99
0.09	0.09	0.0382	100.0	0.09	0.0482	100.0	0.09	0.0000	100.40
0.5	0.49	0.0665	98.00	0.48	0.0675	98.00	0.50	0.0001	99.96
0.9	0.90	0.0025	100.0	0.90	0.0035	100.0	0.89	0.0002	100.0
1	0.98	0.0477	98.00	0.99	0.0477	98.00	0.99	0.0000	99.60
5	4.98	0.0004	99.60	4.98	0.0005	99.60	4.98	0.0000	99.00
12.5	12.49	0.0002	99.92	12.48	0.0003	99.92	12.50	0.0003	98.89
25	25.00	0.0004	100.0	25.00	0.0014	100.0	24.99	0.0016	99.80
50	50.39	0.0045	100.7	50.38	0.0055	100.7	50.20	0.0000	100.0
100	100.0	0.0001	100.0	100.0	0.0001	100.0	99.90	0.0002	99.00

Table 2: Calibration data, recovery, within-day and between day precision and accuracy for cefazolin.

Drug injected µg/ml	————>Intra day<———			———>Inter day<———			———>Human serum —>		
	Found µg/ml	RSD, %	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)
0.05	0.05	0.0060	100.00	0.05	0.0060	100.00	0.05	0.0111	100.9
0.09	0.09	0.0062	100.00	0.09	0.0062	100.00	0.09	0.0010	100.30
0.5	0.49	0.0284	99.99	0.49	0.0284	99.99	0.50	0.0011	99.90
0.9	0.90	0.0237	99.99	0.90	0.0237	99.99	0.89	0.0002	100.0
1	1.00	0.0176	100.01	1.00	0.0176	100.01	0.99	0.0159	98.67
5	5.01	0.0005	100.20	4.90	0.0005	100.20	4.98	0.0002	99.73
12.5	12.50	0.0002	100.01	12.50	0.0002	100.01	12.50	0.0003	99.95
25	25.00	0.0001	100.01	24.91	0.0001	100.01	24.99	0.0015	100.01
50	50.00	0.0000	100.01	49.99	0.0000	100.01	50.20	0.0018	100.59
100	100.00	0.0012	100.00	99.99	0.0012	100.00	99.90	0.0001	100.33

were also tested for system suitability study and optimization. The variation in the mobile phase leads to considerable changes in the chromatographic parameters, like peak symmetry and retention time. However, the ratio of 40:60 (v/v) yielded best results.

The chromatogram of cefazoline and ascorbic acid is shown in fig. 3 while fig. 4 shows the resolution of ceftizoxime and ascorbic acid. There was clear resolution of each compound with retention times 4.1 (cefazoline) and 2.9 minutes (ascorbic acid), 4.3 (ceftizoxime) and 2.9 minutes (ascorbic acid) respectively.

VALIDATION OF THE METHODS

Linearity and sensitivity

The response for the detector was determined to be linear over the range of 100–0.05 µg/, using standard calibration curve ($n = 6$). Cefazoline, ceftizoxime and ascorbic acid solutions were analyzed at intervals of four hours on day one; this process was repeated for four days. A calibration curve was constructed at working concentrations (100, 50.0, 25, 12.5, 5, 1, 0.9, 0.5, 0.09 and 0.05 µg/ml) of cefazoline, ceftizoxime and ascorbic acid, the proposed method was evaluated by its correlation coefficient and intercept value, calculated in the corresponding statistic study. Slopes and intercepts were obtained by using regression equation ($y = a + bx$) and least square treatment of the results used to confirm linearity of the method developed. Correlation coefficients for cefazoline, ceftizoxime and ascorbic acid is 0.999.

Precision and accuracy

The accuracy of the assay was measured by analyzing independently prepared solutions of cefazoline, ceftizoxime and ascorbic acid (100, 50.0, 25, 12.5, 5, 1, 0.9, 0.5, 0.09 and 0.05 µg/ml). Precision must be considered at two levels, repeatability and intermediate precision. On that account, six-sample replicates were consecutively tested with the same equipment and different equipments at a concentration of (100, 50.0, 25, 12.5, 5, 1, 0.9, 0.5, 0.09 and 0.05 µg/ml) of the regular analytical working value. Cefazoline, ceftizoxime and ascorbic acid precision was assessed within-laboratories variations, within-day precision and between-day precision by using different equipments, analysts and days to analyze samples six times.

The results in tables 1-4 indicated that the percentage CV for the within-day ($n = 6$) and between-day ($n = 6$) are numerically all below 3%. The obtained results indicated a good method precision with relative standard deviation (<3%).

Table 3: Calibration data, recovery, within-day and between day precision and accuracy for ceftizoxime.

Drug injected µg/ml	————>Intra day<————			————>Inter day<————			——>Human serum ——>		
	Found µg/ml	RSD, %	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)	Found µg/ml	RSD, %	Accuracy (%)
0.05	0.05	0.0060	100.00	0.05	0.0060	100.00	0.05	0.0100	100.0
0.09	0.09	0.0062	100.00	0.09	0.0062	100.00	0.09	0.0020	100.10
0.5	0.49	0.0284	99.99	0.48	0.0270	99.99	0.50	0.0111	99.80
0.9	0.90	0.0237	99.99	0.89	0.0200	99.99	0.89	0.0012	100.0
1	1.00	0.0176	100.01	1.00	0.0176	100.01	0.90	0.0159	98.67
5	5.01	0.0005	100.20	5.01	0.0005	100.20	4.99	0.0002	99.73
12.5	12.50	0.0002	100.01	12.49	0.0001	98.98	12.49	0.0003	99.95
25	25.00	0.0001	100.01	24.99	0.0001	99.90	24.95	0.0015	100.01
50	50.00	0.0000	100.01	49.99	0.0002	99.80	50.27	0.0018	100.59
100	100.00	0.0012	100.00	100.0	0.0012	100.00	100.25	0.0001	100.33

Table 4: Statistical regression characteristics of method for ascorbic acid, cefazolin and ceftizoxime.

Statistical regression characteristics	Ascorbic acid			Cefazolin			Ceftizoxime		
	Intra day	Inter day	Serum	Intra day	Inter day	Serum	Intra day	Inter day	Serum
Correlation coefficient	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999
Standard error of estimate	0.124798	0.045663	0.079273	0.079273	0.041169	0.922810	0.00499	0.00900	0.0922
Standard error	0.046703	0.045663	0.029667	0.029667	0.015400	0.051653	0.00187	0.00336	0.0516
Intercept	-0.0014	0.0005	0.0006	-0.0113	0.0011	-0.0031	0.0023	0.0023	0.0020
Slope	1	1	1	1	1	1	1	1	1

Specificity of the method

The specificity of the chromatographic method was determined to ensure separation of cefazoline, ceftizoxime and ascorbic acid as shown in figs. 1-2. This is evidenced by the lack of interfering peaks in the chromatograms containing both drugs respectively. The method demonstrated good resolution between cefazoline, ceftizoxime and ascorbic acid and was found to be free of interferences.

Quantification Limit

The limit of detection (LOD), and limit of quantification (LOQ), of the methods was (0.1µg/ml) and (0.3µg/ml) respectively**.** The detection limit of the method was calculated to compare sample solution with blank sample, signal to noise ratio was found > 3.

Ruggedness

Ruggedness of this method was evaluated in two different labs with two different instruments. Lab 1 was in the Department of Chemistry, University of Karachi while another Lab was at the Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, University of Karachi. The method did not show any notable deviation in results from acceptable limits.

Robustness

Stability of standard and sample solutions was determined by assay after 24 and 48 hours stored at –20°C against fresh standard solutions. The results showed that all the cephalosporins were stable and did not show significant much variation in the time span of 48 hours.

System suitability

System suitability of the method was evaluated by analyzing the peak symmetry of cefazolin, ceftizoxime and ascorbic acid peaks (symmetry factor), theoretical plates of the column, resolution between the peaks of cefazolin, ceftizoxime and ascorbic acid, mass distribution ratio (capacity factor) and relative retention.

CONCLUSION

This LC-method is specific, sensitive, rapid and easy to perform. The low limit of quantification, small sample volume and short chromatographic time of this method makes it suitable for use in routine assay. The method enables simultaneous determination of cefazolin, ceftizoxime and ascorbic acid because of good separation and resolution of the chromatographic peaks. The obtained results are in a good agreement with the declared

contents of dosage formulations. The accuracy and precision of the results are confirmed by the statistical parameters. Reliability, simplicity, economical nature and good recovery of this method make it advantageous for simultaneous determination of several cephalosporins along with ascorbic acid in a single chromatographic run. It is however, necessary to ensure that accurate results are obtainable. Therefore, there has to be a compromise between the number of cephalosporins that can be simultaneously determined and the validity of the results.

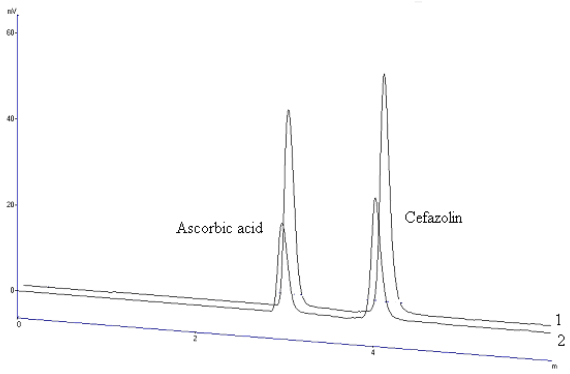

Fig. 3: (1) In dosage form (2) In human serum

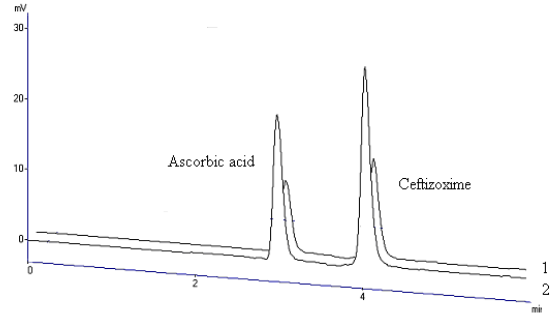

Fig. 4: (1) In dosage form (2) In human serum

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