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DETERMINATION AND VALIDATION OF SOME CEPHALOSPORINS BY USING N-BROMOSUCCINAMIDE IN HUMAN PLASMA AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Selective and highly sensitive spectrophotometric method was developed for the determination of three cephalosporins, namely cefadroxil, ceftriaxone and cefotaxime. Spectrophotometric method involves adding a measured excess of NBS to the drugs in acid medium followed by determination of residual NBS by reacting with a fixed amount of methyl orange and measuring the absorbance at 508nm. The measured absorbance is found to increase linearly with the concentration of cephalosporins serving as basis for quantitation. Under the described conditions, the proposed method is linear over the concentration range of 2.0-6.0 µg/ml, 1.5-4.5 µg/ml and 1.2-3.2 µg/ml for cefadroxil, ceftriaxone and cefotaxime respectively and the coefficients of variation was found to be in the range of 0.9992-0.9997. The recoveries of the title compounds in spiked plasma and in pharmaceutical dosage form ranged from 83.0 to 118.0% with a limit of detection (LOD) in the range of 0.0240- 0.088 µg/ml and limit of quantification in the range of 0.0720- 0.264 µg/ml (LOQ) of for all the three drugs.

Keywords: Cephalosporins, Oxidation, N-Bromosuccinamide (NBS), Methyl orange, Human plasma.

INTRODUCTION

Cefadroxil is chemically designated as 8-[2-amino-2-(4-hydroxyphenyl)-acetyl] amino-4-methyl-7-oxo-2-thia-6-azabicyclo [4.2.0] oct-4-ene-5-carboxylic acid. Cefadroxil is a first generation cephalosporin antibacterial drug that is the para-hydroxy derivative of cefalexin, and is used in the treatment of mild to moderate susceptible infections. It is a broad

spectrum antibiotic effective in Gram-positive, Gram-negative bacterial infections and is a bactericidal antibiotic. Cefadroxil is active against many bacteria, including *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus piogenes, Moraxella catarrhalis, Escherichia coli, Klebsiella and Proteus mirabilis* [1]. A wide variety of analytical methods have been reported for the determination of Cefadroxil in pure form, in pharmaceutical preparations and in biological fluids. These methods mainly involve spectrophotometry [2], atomic absorption spectrophotometry [3], fluorometry [4], chemiluminescence [5], polarography [6], high performance liquid chromatography [7], and capillary electrophoresis [8]. Cefadroxil and cefotaxime have been determined by flow injection spectrophotometric method [9]. Some simultaneous methods were developed for cefadroxil with different combination of drug in various dosage forms. Cefadroxil and cefotaxime in binary mixtures was estimated by derivative spectrophotometry [10]. Cefadroxil and cephalexin in combination have been determined simultaneously by coupling technique of synchronous fluorometry and H-point standard addition methods [11]. Similarly, cefadroxil and cephalexin were determined by HPLC method [12], cefadroxil and cefuroxime in biological sample like urine was measured at 260 nm [13].

Ceftriaxone is (6R,7R)-7-[[(2Z)-(2-amino-4-thiazolyl)(methoxyamino)-acetyl]amino]-8-oxo-3-[[1, 2, 5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)-thio]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid [14]. It is a cephalosporin beta-lactum antibiotics used in the treatment of bacterial infections caused by susceptible, usually gram positive organism. The bactericidal activities of ceftriaxone result from the inhibition of the cell wall synthesis and are mediated through ceftriaxone binding to penicillin binding proteins (PBPs). It inhibits the mucopeptide synthesis in the bacterial cell wall. The beta lactam moiety of ceftriaxone binds to caboxypeptidase, endopeptidase, transpeptidase, in the bacterial cytoplasmic membrane. These enzymes are involved in cell wall synthesis and cell division. By binding these, ceftriaxone results in the formation of defective cell walls and cell death.

Ceftriaxone is official in USP [15] and BP [16] describes HPLC method for estimation of drug in pharmaceutical formulation. Various methods were developed for ceftriaxone using HPTLC [17] and spectrophotometer [18], some simultaneous determination of ceftriaxone with other drug which includes high performance liquid chromatographic method for ceftriaxone and sulbactam in parentarals was developed [19].

Cefotaxime chemically (6R, 7R)-3-[(acetyloxy) methyl]-7-[[(2Z)-2-(2-aminothiazol-4-yl)-2-(methoxyimino) acetyl] amino]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylate is official in Indian Pharmacopoeia [20]. It is a

third generation cephalosporin, a broad antibacterial spectrum and is resistant to β-lactamases. Several analytical methods have been reported. High-performance liquid chromatography (HPLC) [21-23], thin-layer chromatography [24], and spectrophotometric [25–27] techniques were reported.

N- Bromosuccinamide (NBS) has been used as used as the oxidizing agent for the spectrophotometric determination pantoprazole and the residual amount of NBS is determined using methyl orange and indigo carmine as reagents. [28]. N-Bromosuccinamide/fluorescein system for spectrophotometric determination of H_2-receptor antagonists in their dosage forms [29]. However, the reaction between NBS with cefadroxil, ceftriaxone and cefotaxime has not been investigated so far. The present study describes the evaluation of NBS as a chromogenic reagent in the development of simple and rapid spectrophotometric method for the determination of cefadroxil, ceftriaxone and cefotaxime in its pharmaceutical dosage forms and also in spiked plasma samples[30-31].

Materials and Methods

Apparatus

A Shimadzu UV-visible spectrophotometer model 1800 with 1 cm matched quartz cell was used for the absorbance measurements. Systonics electronic balance was used for weighing the samples. A Remi Cooling centrifuge model 412LAG was used for the preparation of plasma samples.

Reagents

All employed chemicals were of analytical grade and high-purified water was used throughout the study. Cefadroxil, ceftriaxone and cefotaxime pure samples were obtained as a gift samples from Strides Arcolab Limited, Bangalore, India. Blood serum was kindly supplied by Blood blank, JSS Hospital, Mysore, Karnataka.

NBS (N- Bromosuccinamide) Solution:

9.0mg of NBS was accurately weighed transferred into a 100 ml calibrated flask, dissolved in 10ml distilled water, and make up the volume up to the mark with distilled water. The solution was freshly prepared and protected from light during the use.

1.0M Hydrochloric acid:

8.5ml of concentrated hydrochloric acid is measured accurately and transferred into a 100.0ml volumetric flask and made up to the mark with distilled water.

Methyl orange solution:

5.0mg of the methyl orange is weighed accurately and transferred into a standard volumetric flask, 30.0ml of water is added and sonicated for 5.0min then the solution is made up to 100.0ml.

10% (w/v) Trichloroacetic acid:

5.0 g of pure trichloroacetic acid is measured accurately and transferred into a 50.0ml volumetric flask and made up to the mark with distilled water.

Standard solutions

Cefadroxil, ceftriaxone and cefotaxime stock solutions (5.0mg) were prepared separately by dissolving in 100.0ml of distilled water. Working solutions of the drug were prepared by dilution of the stock solution. The dosage forms of cefadroxil, ceftriaxone and cefotaxime which are used in the determination was Droxil-500, Betazidim, and Nepecef® respectively with a labelled amount of 1 g and manufactured by Torrent pharmaceuticals ltd, Dist. Mehsana, India and Strides Arcolab Limited, Bangalore, India.

Procedure

Construction of calibration graph

Standard solutions cefadroxil, ceftriaxone and cefotaxime, having final concentrations in the range of 2.0-6.0 µg/ml, 1.5-4.5 µg/ml and 1.2-3.2 µg/ml were transferred into a series of 10.0 ml volumetric flasks. To each flask 1.5 ml of 1.0 M hydrochloric acid followed by 1.0 ml of NBS solutions (9.0 mg/ml) were added. The flasks were stoppered and let stand for 20 min with occasional shaking. Finally, 1.0 ml (50.0 µg/ml) of methyl orange solution was added to each flask, volume diluted to the mark with water, mixed well and absorbance measured at 508 nm against a water blank after 5 min and the calibration curve and absorption spectra are represented in the (Figure. 1 and 2) respectively.

Figure 1: Calibration graphs of cefadroxil, ceftriaxone and cefotaxime

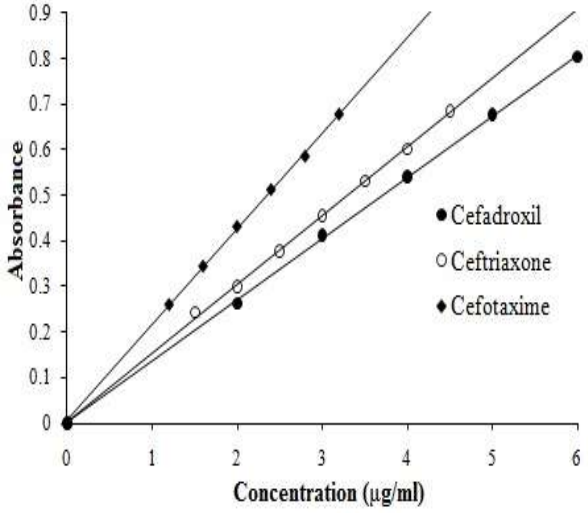
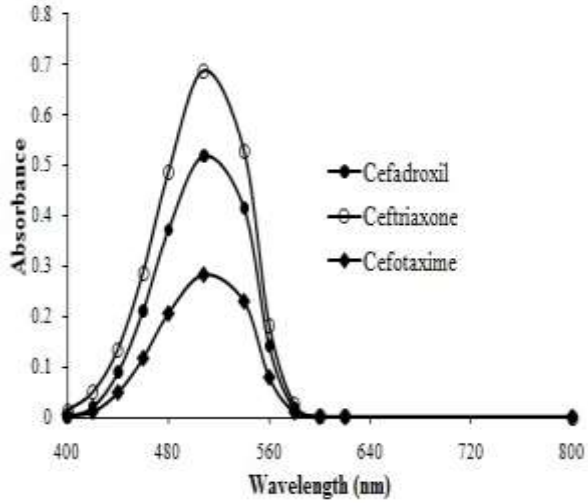


Figure 2. Absorption spectra of NBS with cefadroxil, ceftriaxone and cefotaxime against the reagent blank



Assay procedure for dosage forms

In injection dosage forms:

An accurately weighed amount of the powder equivalent to 5.0 mg of each drug (ceftriaxone and cefotaxime) was transferred into a 100-ml

calibrated flask. The volume was brought to the mark with deionised water then sonicated for 5 min and filtered if necessary. The procedure was completed as described under calibration graph. The nominal content of the drugs in each solution was calculated either from linear regression equation (Table 1) or from the previously plotted calibration graphs.

Table 1. Optical characteristics

S. No	Parameter	Values		
		Cefadroxil	Ceftriaxone	Cefotaxime
1.	λ_{max}/ nm	508	508	508
2.	Beers law limits (µg/ml)	2.0-6.0	1.5-4.5	1.2-3.2
3.	Molar absorptivity (1 /mol/cm)	4.77×10^4	8.98×10^4	9.90×10^4
4.	Correlation coefficient (R)	0.9997	0.9992	0.9995
5.	Sandell's sensitivity(ng cm^{-2})	0.0076	0.0061	0.0045
6.	Regression equation (y)	y = 0.1349x + 0.0002	y = 0.1506x + 0.0039	y = 0.2105x + 0.0052
7.	Slope, b	0.1349	0.1506	0.2105
8.	Intercept, c	0.0002	0.0039	0.0052
9.	Relative standard deviation%	0.96	1.66	0.35
10.	Limit of detection (µg/ml)	0.0628	0.088	0.024
11.	Limit of quantification(µg/ml)	0.188	0.264	0.072

In tablet dosage form:

The content of five tablets was crushed using the mortar and pestle and the powder equivalent to 5.0 mg of active ingredient (cefadroxil) was taken. Suitable concentration was prepared after proper dilution of the stock solution. And the solution was analyzed as per the procedure.

Assay of drugs in spiked human plasma

To 5.0 ml of human serum one of concentration of studied compounds within the concentration range was selected for all the three drugs and added to the serum sample in three separate centrifugation tubes. Then 5.0 ml of 10% (v/v) trichloroacetic acid was added for deproteination to each tube. The mixture was blended in a cooling centrifuge at 2500 rpm for 15 min. Then the protein-free supernatant was transferred into three separate 10.0 ml calibrated flask and the procedure was completed as described under calibration graph and volume is made up with deionised water.

Effect of Reagent Concentration

The effect of varying the concentration of NBS was carried out using reagent concentrations of 0.1, 0.2, 0.4, and 0.6, 0.8, 1.0, 1.2 and 1.4ml in 1.0M HCl. After mixing 1.0ml of each reagent concentration with the drug solutions of cefadroxil, ceftriaxone and cefotaxime and left for 20min with intermediate shaking for the reaction to complete and the solutions are added with 1.0ml of methyl orange and the solutions are made up to 10 ml with water, the absorbance readings of the complex formed were made at 508nm on the UV-visible spectrophotometer (Figure 3). The residual concentration of NBS is giving colour with methyl orange so the volume of NBS is optimised at found that 1.0ml of NBS gave good

results and so further studies are carried out using 1.0ml NBS.

Effect of the concentration of HCl

The influence of the volume of 1M HCl on the reaction has been studied and the results are shown in Figue 4. The highest absorbance was obtained by using 0.8 ml of 1 M HCl; above this volume the absorbance remained constant upto 1.2 ml. So further studies are carried out using 1.0 ml of 1M HCl.

Figure 3: Effect of NBS concentration

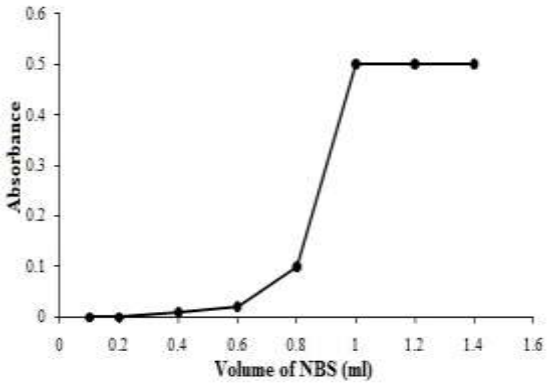
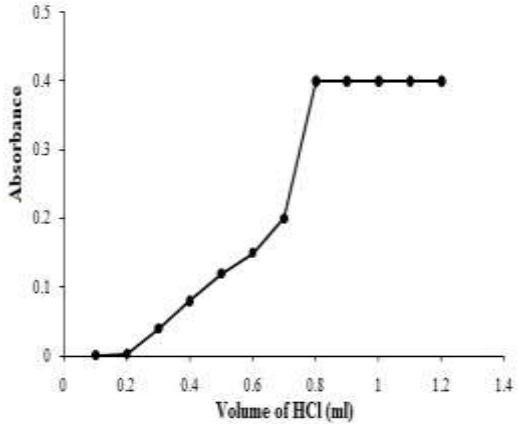


Figure 4: Effect of HCl concentration

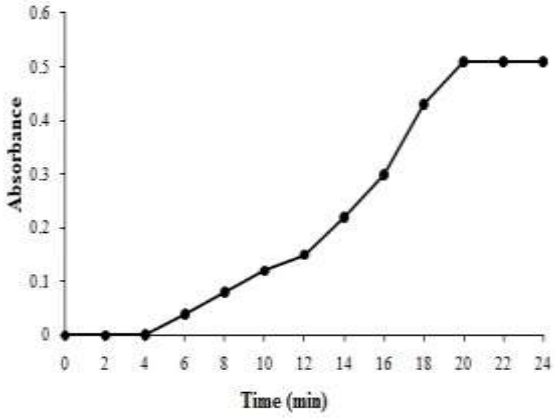


Effect of time on the reaction process

The time of reaction should be governed carefully so the optimization of the time is done. The highest absorbance is seen after 20min the NBS solution is added to the drug solutions.

The reaction time is studied from 2min - 24min after the NBS is added to the drug sample. A rapid increase in the absorbance is seen from 20min and it is constant till 24min so the further experiment is done after 20min the NBS solution is added. The results are shown in Figure 5.

Figure 5: Effect of time on reaction



Method validation

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The linear response of cefadroxil, ceftriaxone and cefotaxime were determined by analysing three independent levels of the calibration curve in the range of 2.0- 6.0, 1.5- 4.5 and 1.2- 3.2 µg/ml respectively for cefadroxil, ceftriaxone and cefotaxime in triplicate.

Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method. It provides an indication of random error. The precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variance of a series of measurements.

Repeatability

It is a precision under a same condition (same analyst, same apparatus, short interval of time and identical reagents) using same sample. Method precision of experiment was performed by preparing the standard solution of cefadroxil, ceftriaxone and cefotaxime with the concentration of 2.0µg/ml for six times and analysed as per the proposed method (Table 2). Percentage relative standard deviation (%RSD)

Table 2: Evaluation of precision

Drug	S.No	Label Claim (g)	Amount found* (g)		% Purity*		%RSD	
			Dosage form	Plasma	Dosage form	Plasma	Dosage form	Plasma
Cefadroxil	1		0.4976	0.456	99.52	91.2	0.77	2.0
	2	0.5	0.480	0.461	98.05	92.2		
	3		0.485	0.474	98.453	94.8		
Ceftriaxone	1		0.976	0.923	97.6	92.3	0.46	1.74
	2	1.0	0.980	0.901	98.0	90.1		
	3		0.985	0.932	98.5	93.2		
Cefotaxime	1		0.918	0.908	91.8	90.8	1.08	3.18
	2	1.0	0.899	0.879	89.9	87.9		
	3		0.913	0.852	91.3	85.2		

Accuracy (% Recovery)

Accuracy of an analysis is determined by systemic error involved. It is defined as closeness of agreement between the actual (true) value and analytical value and obtained by applying test method for a number of times. Accuracy may often be expressed as % recovery by the assay of known, added amount of analyte (Table 3). It is measure of the exactness of the analytical method. The recovery experiments were carried out in triplicate by spiking preciously analysed drug samples (cefadroxil 1.0 µg/ml, ceftriaxone 0.5 µg/ml and cefotaxime each of 0.4 µg/ml) with three different concentrations of standards (cefadroxil 2.0, 3.0, 4.0 µg/ml, ceftriaxone 1.5, 2.0, 2.5 µg/ml and 1.2 1.6, 2.0 µg/ml cefotaxime)

The recovery of the drug is also tested by spiking the drug in the plasma sample (Table 3) by the standard addition method and the remaining procedure is done as given under the

construction of calibration graph and the amount of drug recovered is found out.

Table 3: Results of recovery study by standard addition method for cefadroxil

Drug	Standard Concentration (µg/ml)	Sample Concentration (µg/ml)	Absorbance		Amount obtained		% Recovery	
			Dosage form	Plasma	Dosage form	Plasma	Dosage form	Plasma
	2	1	0.240	0.20	1.9	1.8	95	90
Cefadroxil	3	1	0.390	0.350	2.8	2.5	93.3	83.3
	4	1	0.530	0.480	3.9	3.5	97.5	87.5
	1.5	0.5	0.12	0.98	1.4	1.3	93.3	86.6
Ceftriaxone	2.0	0.5	0.280	0.230	1.99	1.8	99.5	90.0
	2.5	0.5	0.358	0.360	2.40	2.24	96.0	89.6
	1.2	0.4	0.28	0.240	1.3	1.0	108	83
Cefotaxime	1.6	0.4	0.42	0.399	0.399	1.8	112.5	118
	2.0	0.4	0.480	0.478	0.478	2.1	109	105

Limit of Detection

It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. Limits of detection can be calculated using following equation as per ICH guidelines.

LOD = 3.3 x N/S

Where, N is the standard deviation of the peak areas of the drug and S is the slope of the correspoding calibration curve.

Limit of Quantification

It is the lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions. Limit of quantification can be calculated using following equation as per ICH guidelines.

LOQ = 10 x N/S

Where, N is the standard deviation of the peak areas of the drug and S is the slope of the corresponding calibration curve.

Results and Discussion

N-Bromosuccinimide acts as an oxidizing agent. The oxidizing action of N-bromosuccinimide has been widely used to bring about the selective oxidation of the beta lactum ring containing compounds. In the spectrophotometric methods, cefadroxil, ceftriaxone and cefotaxime was added to a fixed and known amount of NBS, and after the reaction was judged to be complete, residual NBS was determined by reacting with a fixed amount of methyl orange. When the drugs (cefadroxil, ceftriaxone and cefotaxime) are added in increasing amounts to a fixed amount of NBS, consumed the latter, and the fall in NBS

concentration occurred. When a fixed amount of methyl orange dye was reacted with decreasing amounts of NBS, a concomitant increase in the dye concentration occurred. This was observed as a proportional increase in the absorbance at the respective λmax with increasing concentration of cefadroxil, ceftriaxone and cefotaxime, as shown by the correlation coefficients of 0.9993, 0.995 and 0.9988 respectively for cefadroxil, ceftriaxone and cefotaxime. Preliminary experiments were conducted to determine the maximum concentration of methyl orange spectrophotometrically by measuring the absorbance of their acidic solutions at their respective λmax, and the upper limit was found to be 5mg/ml and NBS concentration of 9.0 mg/ml was found to give good results. NBS was sufficient to destroy the orange colour of 50.0 µg/ml methyl orange. Hence, different amounts of cefadroxil, ceftriaxone and cefotaxime reacted with 9.0 mg/ml of NBS before determining the residual NBS as described under the respective procedure. Hydrochloric acid was found to be a convenient medium for the two steps involved for all the three drugs (cefadroxil, ceftriaxone and cefotaxime). For a quantitative reaction between cefadroxil, ceftriaxone and cefotaxime and NBS, a contact time of 20 min was found sufficient in all the three drugs. Constant absorbance readings were obtained when the reaction times were extended up to 24 min for cefadroxil, ceftriaxone and cefotaxime was necessary for the bleaching of dye color by the residual NBS. The measured color was stable for several hours even in the presence of the reaction product.

Conclusion

All these factors lead to the conclusion that the proposed method is accurate, precise, simple, sensitive, and rapid and can be applied successfully for the spectrophotometric estimation of cefadroxil, ceftriaxone and cefotaxime in human plasma and pharmaceutical formulation without any interference.

Acknowledgement

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