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Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR QUANTIFICATION OF CEFOTAXIME IN PLASMA OF PATANWADI SHEEP

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ABSTRACT: Present research work was carried out to study the validation procedure of Cefotaxime using plasma of Patanwadi sheep. The samples were analyzed using RP- HPLC C18 column (250 X 4.6 mm) with UV detection (254 nm). The method was developed for extraction of Cefotaxime from plasma of sheep using acetonitrile and was validated. The mobile phase was a mixture of 0.05 M potassium dihydrogen phosphate buffer with pH 6 and acetonitrile (88: 12, v/v). The method was validated with respect to linearity, precision and accuracy. The intra day and inter day precision study of cefotaxime was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days (1st, 2nd and 3rd) for 6 different concentration of Cefotaxime (10, 5, 2.5, 0.625, 0.312 and 0.156 µg/ml) and the results were reported in terms of relative standard deviation (RSD). At all concentration studied the C.V. (Coefficient of Variance) was less than 7%. The recovery of Cefotaxime from plasma varied from 85-89%. The limit of detection (LOD) was found to be 0.156 ppm. Calibration graphs showed a linear correlation (r > 0.998) over the concentration ranges of 1.56 – 200 µg mL^{-1} for plasma. The results obtained indicated good precision of assay. The developed method was found to be simple, sensitive, accurate, precise and reproducible and can be used for analytical studies of Cefotaxime.

Key Words: RP-HPLC, Cefotaxime, Validation, Patanwadi sheep, Plasma.

INTRODUCTION

Cefotaxime is a semisynthetic third generation of Cephalosporin antibiotics, which is used effectively for the treatment of serious infections caused by a wide range of Gram-positive and Gram-negative susceptible microorganisms (Neu 1982). Cefotaxime acts by inhibiting bacterial cell wall synthesis by binding to one or more of the penicillin-binding proteins (PBPs) which in turn inhibit the final trans-peptidation step of peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis. Cefotaxime has greater activity than other available first and second-generation cephalosporins against Enterobacteriaceae. It is more active than older

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cephalosporins against *Pseudomonas aeruginosa* and *Acinetobacter spp.*, but most strains are still considered resistant. Cefotaxime is also active against some gram-negative bacilli that are resistant to aminoglycosides, including amikacin. It has activity comparable with other cephalosporins against gram-positive cocci, except staphylococci and enterococci, which are resistant (Dudley and Barriere 1982). Literature survey revealed that various spectrophotometric and HPLC methods are available for estimation of Cefotaxime sodium (Signs *et al.,* 1984). The pharmacokinetics of cefotaxime have been investigated after single injection in humans (Kampf *et al.,* 1984); rats (Hakim *et al.,* 1989); sheep (Guerrini *et al.,* 1983, 1985); dogs (Guerrini et al., 1986); cats (Mcelory *et al.,* 1986); goats (Atef *et al.,* 1990) and cattle (Sharma and Srivastava 1994; Sharma *et al.,* 1995). Literature for pharmacokinetics of Cefotaxime are available in most of the species but has not been studied in sheep of Indian breeds. So, the present study was designed to study the validation procedure of Cefotaxime by RP-HPLC using plasma of sheep.

MATERIALS AND METHODS

Experimental animals: The experiment was conducted on six healthy Patanwadi sheeps of about 2-3 years old, weighing 28-32 kg. Each sheep was housed in a separate pen and provided *ad libitum* water and standard ration. The sheep were kept under constant observation for two weeks before the commencement of the experiment and all necessary managerial procedures were adopted to keep the animals free from stress. The experiment was performed as per the guidelines of CPCSEA after approval from IAEC.

Experimental design: The study was carried out in cross-over design. All six animals were randomly allocated to receive a single intramuscular injection of Cefotaxime (2 mg/kg). Intramuscular injection was given into the deep gluteal muscles, using a 20 G × 25 mm needle. Blood samples (2 ml) were collected in sterile heparinized vials at 0 minute (before drug administration) 5, 10, 15, 30 and 45 minutes and at 1, 2, 4, 8, 12, 18, 24 and 36 hours after intramuscular administration. The sheep were monitored for any adverse reactions throughout the entire study period. Plasma was separated soon after collection by centrifugation at 3000 rpm for 10 minutes at 10 ^{o} C (Eppendorf 5804 R, Germany). Separated plasma samples were transferred to labeled cryovials and stored at -35 ^{o} C until assayed for Cefotaxime concentration.

Chemicals: Cefotaxime sodium injection IP (Taxim^{R} Alkem Laboratories Ltd., Mumbai, India) was procured from local market. Potassium dihydrogen phosphate, acetonitrile (HPLC grade) and water (HPLC grade) were purchased from S.D. Fine Chem Ltd., Merck India Ltd., and Sisco Research Laboratories Pvt. Limited, Mumbai, correspondingly.

Cefotaxime assay: Cefotaxime was assayed in plasma by slight modification of procedure described by Juan *et al.* (1999). 100 µl of plasma and 100 µl of acetonitrile were vortex-mixed for 30 s. After centrifuging for 5 min at 2500 rpm, the supernatant was added to 2 ml of dichloromethane. The mixture was vortex-mixed (30 s) and centrifuged (3000 rpm for 10 min), and a 20 µl aliquot of supernatant was injected into the HPLC.

Apparatus: The high performance liquid chromatography apparatus of Laballiance (USA) was used. It comprised of quaternary

Table 1. Partial validation of HPLC method used for Cefotaxime quantification in plasma of Patanwadi sheep.

Spiked concentration (µg ml^{-1})	No. of observations (n)	Observed concentration (µg ml^{-1}) Mean ± S.D.	C.V. (%)
Intra day			
10	3	1062.65±1.55	2.40
5	3	147.31±1.98	3.92
0.25	3	16.46±2.021	4.08
Inter day			
50	3	1922.85±1.065	1.13
10	3	983.90±1.123	1.25
5	3	112.48±0.034	0.11

gradient delivery pump (model AIS 2000) and UV detector (model 500). Chromatographic separation was performed by using reverse phase C18 column (Thermo, ODS; 250 X 4.6 mm ID) at room temperature. The data integration was performed using software Clarity (Version 2.4.0.190).

Chromatographic conditions: The mobile phase was a mixture of 0.05M potassium dihydrogen phosphate buffer with pH 6 and acetonitrile (88: 12, v/v). Mobile phase was filtered by 0.45 m filters and pumped into column at a flow rate of 1.0 ml min^{-1} at ambient temperature. The effluent was monitored at 254 nm wavelength. The sample was injected by using Hamilton syringe of 20 µl, and the total run time was 15 mins. Calibration curve was constructed by plotting area versus concentration and regression equation was computed for cefotaxime.

Cefotaxime standard solution: Stock solutions of cefotaxime was prepared by dissolving 500 mg of cefotaxime in 5 ml of distilled water. This stock solution was equivalent to 100 mg/ml.

Accuracy (Recovery test): The absolute recovery for plasma samples (10, 5 and 2.5 µg/ml) was determined by comparing peak areas obtained from freshly prepared sample extracts and those resulting from direct injection of an aqueous standard solution at the same concentration.

Precision: The intra day and inter day precision study of cefotaxime was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days (1^{st}, 2^{nd} and 3^{rd}) for 6 different concentration of Cefotaxime (10, 5, 2.5, 0.625, 0.312 and 0.156 µg/ml) and the results were reported in terms of relative standard deviation (RSD) 38.64 with correlation coefficient (R^2) was 0.998.

Statistical analysis: The data generated from the safety profile study were analyzed by Least Square Difference test using SPSS

Table 2. Recovery Percentage of Cefotaxime from Plasma of Patanwadi sheep by using HPLC method.

Spiked concentration (µg ml^{-1})	No. of observations (n)	Observed concentration (µg ml^{-1}) Mean ± S.D.	Recovery (%)
Intra day			
10	3	984.65±3.3	85.23
5	3	130.51±1.55	87.45
0.25	3	19.76±1.09	86.36
Inter day			
50	3	1853.23±0.98	85.99
10	3	1045.24±2.22	88.54
5	3	134.55±2.544	89.02

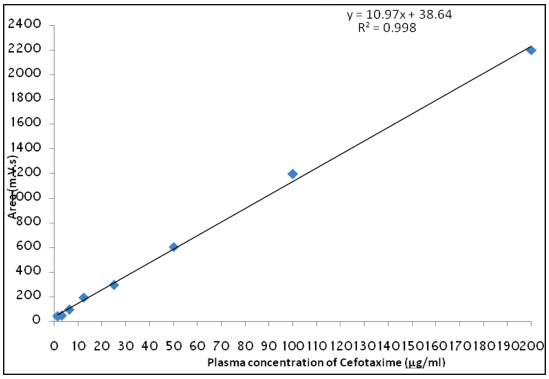

Fig. 1. Calibration curve showing linearity of area Vs concentration of Cefotaxime.

software (version 13.0.). All the data are presented as mean ± S.E.

RESULTS AND DISCUSSION

Cefotaxime from plasma was estimated by high performance liquid chromatography (HPLC) at 254 nm wavelength. Extraction of Cefotaxime from plasma by adding acetonitrile is advantageous over other agents as it deproteinizes without causing a drastic change in pH, so does not affect the stability of drug present in the sample. The chromatogram showed peak of Cefotaxime and the retention time (RT) here was found to be 9.03 min (Fig. 2). The recovery of Cefotaxime from plasma varied from 85-89% (Table 2 and Fig. 3). Since, the value was above 80% the recovery was found to be satisfactory. Therefore, the method was considered to be the best. The limit of detection (LOD) was found to 0.156 ppm. Precision of an analytical method describes the closeness of individual measures of Cefotaxime when the procedure is applied repeatedly. Accuracy describes the closeness of mean test results obtained by the method to the true concentration. Precision and accuracy of the assay were assessed in conjunction with the linearity study, including mean and coefficient of variance (C.V.) for each standard concentration (Table 1). At all concentration studied the C.V. was less than 7%. These results

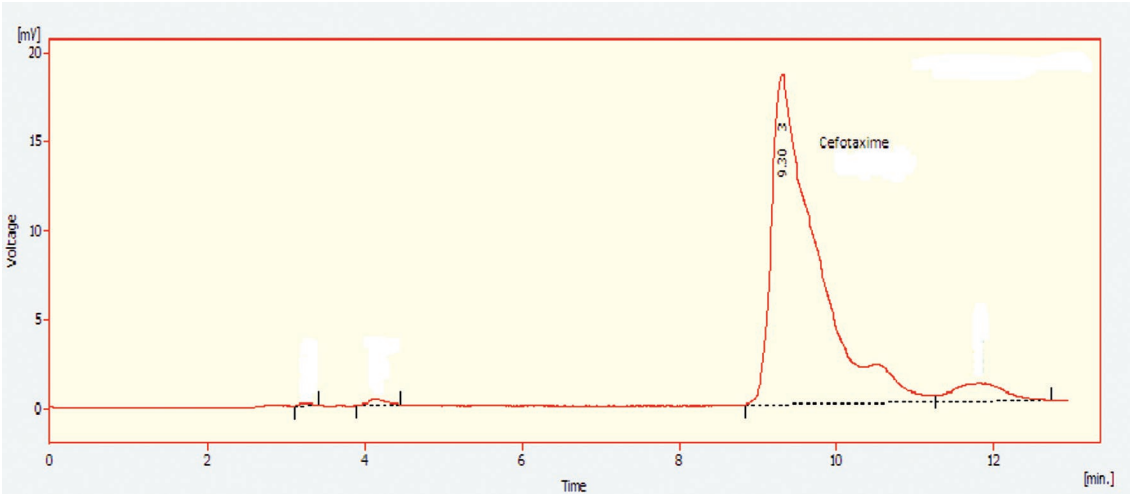

Fig. 2. Chromatograms of Cefotaxime (10 µg ml^{-1}) standard in mobile phase.

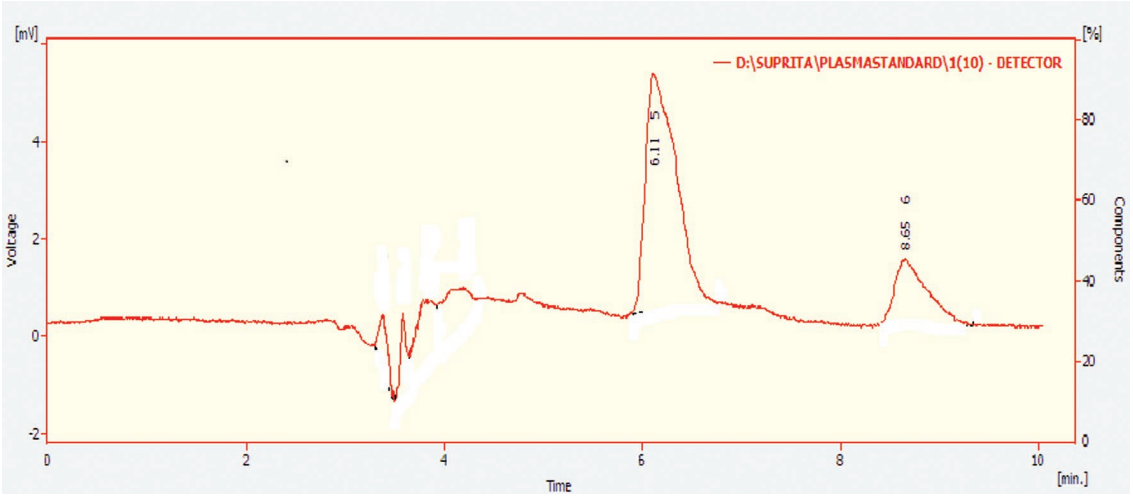

Fig. 3. Chromatograms of Cefotaxime (10 µg ml^{-1}) in plasma of Patanwadi sheep.

indicate good precision of assay.

Linearity: Cefotaxime exhibits maximum absorption at 254 nm and in the range of 10 µg/ml. Linear regression of Area Vs Concentration yielded equation y = 10.97x + 38.64 with correlation coefficient (R^2) was 0.998 (Fig.1).

CONCLUSION

The developed method was found to be simple, sensitive, accurate, precise and reproducible and can be used for analytical studies of Cefotaxime.

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