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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING GREEN HPLC-UV METHOD FOR DETERMINATION OF CEPHALEXIN IN PHARMACEUTICAL DOSAGE FORMS AND HUMAN URINE USING MICELLAR MOBILE PHASE

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ABSTRACT

Objective: Development and validation of simple, stability indicating and green high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for determination of cephalexin in pure form, pharmaceutical dosage forms and human urine samples.

Methods: The method is based on using of a micellar mobile phase for separation of cephalexin and its degradation products. The analyte was chromatographed on a Kinetex C_{18} 75×4.6 mm, 2.6 µm column. Micellar mobile phase composed of 0.1M sodium dodecyl sulphate (SDS) and 10 % isopropanol (IPA), pH was adjusted to 3±0.05 with phosphoric acid, the flow-rate was 1.0 mL/min, the UV detector was set at 254 nm and the injection volume was 20 µl. Stability indicating properties of the proposed method was proved through exposure of the analyte solutions to 4 different stress conditions of acidic, basic, oxidative and photo-irradiation conditions.

Results: Under optimized conditions the average recovery was ranged from 100.4–101.7%. The lower limit of quantification (LOQ) and the lower limit of detection (LOD) were 0.097 and 0.029 µg/ml, respectively. A linear correlation in the range of 1–200 µg/ml with the correlation coefficient (r^2) of ≥ 0.999 was obtained. Relatively high inter-and intra-day precisions were achieved, the percentage RSD values were lower than 2. The obtained results were validated according to USP validation parameters.

Conclusion: The proposed method was found to be not only a greener method but also faster and more convenient than the USP compendial method. Greener here means that the method is more eco-friendly as it avoids usage of toxic solvent and reagent and switch to more benign chemicals. In addition, allow for injection of urine samples directly into an analytical column without pretreatment due to micellar solubilization of the interfering components of the biological samples.

Keywords: Green analytical chemistry, Cephalexin, Sodium dodecyl sulfate, Micelle L. C, Human urine sample, Stability indicating method.

INTRODUCTION

Cephalexin (7*R*)-3-Methyl-7-(α-D-phenylglycylamino)-3-cephem-4-carboxylic acid monohydrate [1] (fig. 1), is a first-generation cephalosporin antibiotic it is a useful alternative to penicillin's in patients with penicillin hypersensitivity. It is excreted in urine as unchanged drug by renal tubular secretion and glomerular filtration [2]. Many analytical methods have been reported for the analysis of cephalexin in pure drugs, dosage forms and biological fluids including its relevant monograph in USP [3].

RP-HPLC methods for determination of cephalexin in plasma, serum and urine have been reported [4-6]. Other chromatographic, spectrophotometric and colorimetric methods have been developed for quantification of cephalexin [7-12]. At least 70–90% of a cephalexin dose is eliminated in urine within 8–12 hours in adults with normal renal function without change [13]. Micelle L. C has been introduced in several publications as more eco-friendly chromatographic technique in pharmaceutical testing [14-16].

The aim of this work is to develop and validate more eco-friendly (greener) stability indiating analytical methods, compared with the official one in USP without compromising efficacy, through minimizing usage of hazard organic solvents and switching to benign solvents in micellar liquid chromatography for determination of cephalexin in pharmaceutical dosage form and human urine as a biological samples.

MATERIALS AND METHODS

Materials and chemicals

All materials were supplied by Sigma pharmaceuticals corp.-Egypt. Including cephalexin USP compendia reference standard "CRS" of 100% purity, cephalexin pure material, commercial samples of starcef (cephalexin) 1g tablets and a placebo contains the same raw materials used in the production process including microcrystalline cellulose, povidone K 30, talc magnesium stearate, croscarmellose sodium and colloidal silicon dioxide, these materials are of pharmaceutical grade and are of the same type used for preparation of the drug product. All solvents and reagents are of HPLC grade. They were purchased from Sigma-Aldrish including sodium dodecyl sulphate (SDS), isopropanol IPA, and phosphoric acid.

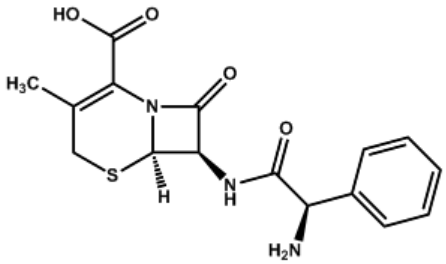

Fig. 1: Chemical structure of cephalexin

Apparatus

Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with SCL-10Avp system controller, LC-10ADvp pump, DGU-14A degasser, SIL-10ADvp auto sampler, CTO-10ACvp column oven, SPD-10Avp DAD detector, and Class-VP software was used to quantify the samples.

Reference HPLC method

According to the USP, the compendial HPLC method utilizing C_{18} (250×4.6 mm, 5 µm) separation column and mobile phase composed of water, acetonitrile, methanol and triethylamine in the ratio (850:

100: 50: 15) and 0.0985 % of sodium 1-pentansulfonate. The pH is to be adjusted with phosphoric acid to 3±0.1. According to the green analytical chemistry parameters, this method would be greener if the time of analysis could be reduced and if it utilize more environmentally benign solvents and chemicals (or ideally eliminate the usage of organic solvent) without compromising the efficiency of the analytical procedure.

Miceller liquid chromatography

Preparation of stock solutions

200 µg/ml stock standard solution of cephalexin was prepared in water, then calibrators were prepared by serial dilutions have been made using the mobile phase as solvent to prepare a series of the calibration standard solutions having concentration ranging from 1-200 µg/ml.

Tablets sample preparation

Randomly selected 20 tablets were powdered and mixed; an accurately weighed portion of powder samples equivalent to 20 mg of cephalexin was dissolved and diluted to the appropriate volumes with water. Solutions were filtered through nylon sample filter (Whatman, 0.22µm).

Urine sample preparation

Blank human urine samples were collected freshly from health adult volunteers just before administration of starcef 1g tablets (zero time), other samples were collected at 6 and 8 hours intervals for quantitative determination of the drug.

Urine samples were injected directly without dilution or extraction. They were only

filtered through nylon sample filter (Whatman, 0.22µm).

Stress degrdation of cephalexin under acidic, basic, oxidative and photo-irradiation conditions

In order to determine the stability-indicating capacity and specificity of the proposed green HPLC method; forced degradation studies were performed at various stress conditions. Alkaline hydrolytic condition was applied by reflux a solution of 1 mg/ml of cephalexin solution with 0.1 N and 0.01 N sodium hydroxide solutions for 30 minutes. For Acidic hydrolytic condition a solution of the same concentration was refluxed with 0.1 N hydrochloric acid solution. After reflux with acid or alkali solutions were cooled to room temperature, neutralized and diluted with water to the appropriate concentration. Reflux for one hour with 30 % hydrogen peroxide of a solution of 1 mg/ml cephalexin has been performed to achieve the oxidative hydrolysis condition, finally in photo-irradiation conditions cephalexin stock solution of 1 mg/ml was irradiated under a CAMAG 200-W high-pressure mercury lamp for 10 hrs. The distance between the light source and the sample was maintained at 25 cm.

Method development and chromatographic conditions

The chromatographic conditions were selected on the basis of green analytical chemistry parameters. Micellar mobile phase composed of 0.1M (SDS) and 10 % IPA, pH was adjusted to 3±0.05 with phosphoric acid, the flow-rate was 1.0 mL/min, the UV detector was set at 254 nm and the injection volume was 20 µl. The performance of two different separation columns was compared in terms of the system suitability parameters. Superficially porous particle (SPP) or "Core–Shell" packed column-Kinetex C_{18} 75×4.6 mm, 2.6 µm and the conventional totally porous particle packed column (TPP)-Zorbax eclipse plus C_{18} 100×4.6 mm, 5 µm.

RESULT AND DISCUSSION

Optimization of the chromatographic conditions

Considering green chemistry fundamentals [17], various solvent systems as mobile phases were compared for the development of a green and environmentally benign RP-HPLC method trough avoiding toxic solvent and reagents and use more benign ones. During the method development, different surfactants have been investigated at their critical micelle concentrations (CMC) such as Brij-L23, tween 80 and SDS; the initial separation time with all of them was too long (more than 15 minutes) with inappropriate system suitability parameters as the peak shape was distorted with tailing factor more than 1.8.

SDS (CMC = 8.27 mM) [18] was found to be the most appropriate surfactant for the separation. The SDS concentration has been increased gradually till 0.1 M to achieve the optimum chromatographic separation.

Different organic modifiers such as ethanol and ethyl acetate were investigated but asymmetric peaks with unaccepted tailing factor and low number of theoretical plates were obtained. The most appropriate organic modifier was found to be IPA.

The optimum mobile phase composition was found to be 0.1M SDS: IPA (90: 10) at pH 3 which gave excellent peak shape with asymmetry factor less than 1.3 and number of theoretical plates greater than 4000. The total run time was less than 10 min. as shown in fig.2 & fig. 3. The detection wavelength for the analysis of cephalexin was set at 254 nm as reported in USP [8].

Validation of the green RP-HPLC method

The proposed green RP-HPLC method was validated according to the USP guidelines in terms of system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness, specificity and selectivity.

The system suitability was evaluated through repeated injections of cephalexin standard solution. The SPP short column offered excellent system suitability parameters including repeatable peak area, high column efficiency, low tailing factor, short and stable retention time and thus low consumption of the mobile phase which is in a good agreement with to the recommended green analytical chemistry parameters as shown in table 1. The linearity of the proposed method was evaluated by the linear least square analysis. Calibration curves were constructed form the series of dilutions of cephalexin standard solutions, in the range of 1–200 µg/ml for spiked drug matrix samples and urine samples. Calibrators were injected in triplicates and peak areas were plotted against concentrations. The equation for the calibration curves was calculated and the correlation coefficients were found to be R^2>0.999 in all cases. The limits of detection LOD and limits of quantitation LOQ were considered as three and ten times of the noise level, respectively [19] as indicated in table 2.

The accuracy was determined as percent recovery for spiked samples injected in triplicate of placebo solutions and urine samples at concentration levels ranged from 10–50 µg/ml. The percentage recoveries were ranged from (100.4–101.7%) with % RSD within 2.0 % which indicated the accuracy of the proposed method as shown in table 3.

The precision of an analytical procedure expresses the closeness (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions; Inter-day precision was evaluated by analysing freshly prepared samples of drug-matrix and spiked urine sample in triplicates at three different concentration levels of 10, 30 and 50 µg/ml. Intra-day precision was studied using freshly prepared similar solutions (n=6) at three different days. In all cases the RSD of the obtained results was lower than 2, these results are summarized in table 4. The ICH guidelines defined robustness of an analytical procedure as its capability to remain unaffected by small but deliberate variations in the method parameters [20-22]. Robustness was determined by monitoring the effect of slight changes in the separation conditions as illustrated in table 5. The percent recoveries of cephalexin were good under all studied conditions and did not show significant change (RSD = 0.47%) on slight change of the critical parameters.

The specificity of the proposed green RP-HPLC method was proved by excellent chromatographic resolution of the target peak and different degradation product peaks under different forced degradation conditions where the resolution factor was greater than 2 in all cases. The peak purity check indicated that the determined

purity factor was lower than the calculated threshold as shown in fig. 4. Cephalexine stock solution was exposed to various stress conditions. On treating cephalexin with 0.1 N NaOH complete degradation has been observed, when treated with 0.01 N NaOH for an hour, three degradation product peaks at 2.4, 3.7 and 4 minutes were observed with extensive degradation to 90 % of the peak of interest (fig. 5). Cephalexin was stable on treating with 0.1 N HCl but degrades to considerable extent on boiling in 0.1 N HCl for one hour, the height of peak was reduced by about 50 % and one new peak was observed at 2.5 minutes before peak of interest (fig. 6). Cephalexin was found to degrade more rapidly in oxidative conditions. Upon reflux with 30 % hydrogen peroxide for 30 minutes, peak height was reduced dramatically with the appearance of new degradation peaks at 2.5 and 4.7 minutes (fig. 7). No significant changes in peak height under photo-irradiation conditions (Fig.8) which indicate photostability.

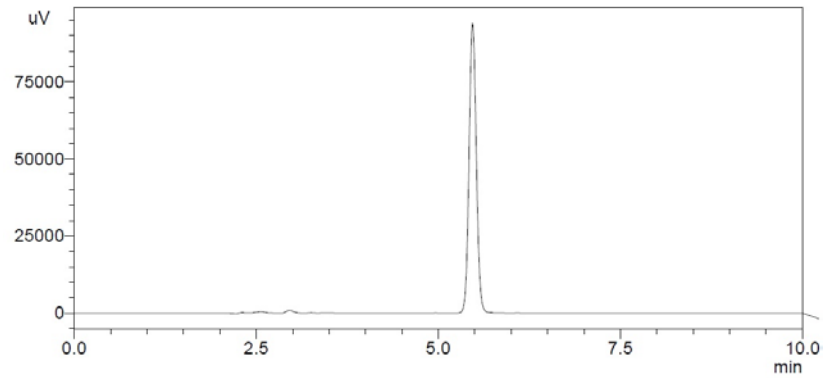

Fig. 2: Cephalexin reference standard solution Under proposed green chromatographic conditions

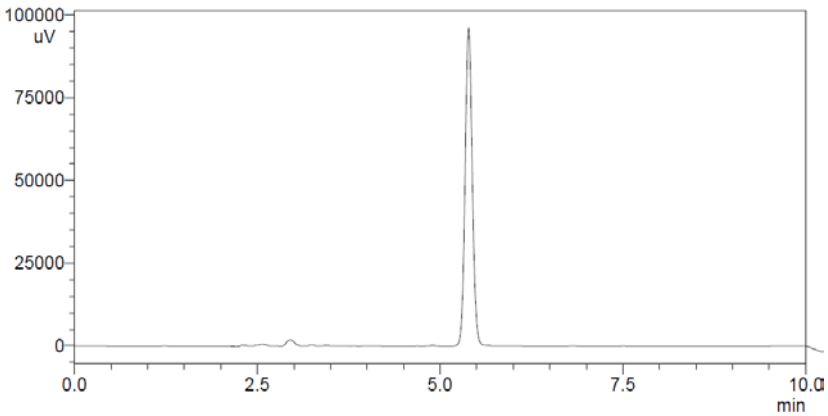

Fig. 3: Cephalexin test solution Under proposed green chromatographic conditions

Table 1: System suitability parameters for comparison between the performance of SPP and TPP Columns (n=5)

Parameter	Kinetex C18 (75×4.6 mm, 2.6 µm)–SPP column	Zorbax eclipse plus C18 (100×4.6 mm, 5 µm)-TPP column
% RSD in retention time	0.048%	0.056%
% RSD peak area	0.278%	0.315%
tailing factor	1.25±0.05	1.55±0.05
retention time±SD (min)	5.5±0.05	6.73±0.05
HETP	56.6	67.17

Table 2: Linearity of the calibration curve for cephalexin in standard preparations and in drug-matrix preparation. Number of points in the regression line is 3 for each case

Variable	Value (cephalexin in standard preparation)	Value (cephalexin in drug-matrix)	Value (cephalexin in urine sample)
Linearity range (µg/ml)	1–200	1–200	5–200
Correlation coefficient (r2±SD)	0.999±0.0004	0.999±0.0005	0.999±0.005
Slope	12619	12837.3	12167.5
95% confidence interval for the slope	11988.05-13249.95	12195.44-13479.16	11559.13-12775.87
Intercept	7298	10075.42	51762.48
95% confidence interval for the intercept	6933.1-7662.9	9571.649-10579.19	49174.35-54350.6
Regression equation	y = 12619x+7298.	y = 12837.3 x+10075.4	y = 12167.5 x±51762.5
LOD (µg/ml)	0.029	0.06	0.11
LOQ (µg/ml)	0.097	0.2	0.37

Table 3: Estimation of the accuracy in standard solution, drug matrix solution or urine sample (n=3)

Quantity added (µg/ml)	Drug–matrix preparation			Urine sample preparation		
	Measured conc. (µg/ml)±SD	RSD (%)	% Recovery	Measured conc. (µg/ml)±S.D	RSD (%)	% Recovery
10	10.13±0.16	1.58	101.3	10.16±0.17	1.67	101.6
30	30.24±0.43	1.42	100.8	30.50±0.51	1.67	101.7
50	50.54±0.88	1.74	101.08	50.68±0.98	1.93	101.4

Table 4: Precision data for the proposed HPLC method

Time	Tablet sample preparations			Urine sample preparations		
Inter-day precision						
Sample concentration (µg/ml)	10	30	50	10	30	50
Mean % recovery	99.76	100.38	99.18	101.12	99.62	100.34
% RSD	0.05	0.11	0.09	0.29	0.18	0.23
Intra-day precision						
Day-1						
Sample concentration (µg/ml)	10	30	50	10	30	50
Mean % recovery	100.39	98.98	99.91	99.11	101.87	100.33
% RSD	0.23	0.17	0.08	0.14	0.28	0.22
Day-2						
Sample concentration (µg/ml)	10	30	50	10	30	50
Mean % recovery	98.57	99.94	99.48	100.45	99.85	99.34
% RSD	0.38	0.27	0.15	0.08	0.13	0.06
Day-3						
Sample concentration (µg/ml)	10	30	50	10	30	50
Mean % recovery	100.34	99.91	99.58	100.35	99.99	99.31
% RSD	0.03	0.11	0.35	0.54	0.39	0.67

Table 5: Effect of experimental parameters on the percent recoveries of cephalexin

Parameter	Modification	(%) recovery of cephalexin
Mobile phase	0.1M SDS: IPA	
	93: 7	100.1
	90: 10	99.35
	87: 13	101.1
Type of column	SPP	100.5
	TPP	100.2
pH	3.1	100.1
	3.0	100.3
	2.9	100.2
Flow rate (ml/minute)	0.9	100.1
	1.0	99.95
	1.1	99.87
Wavelength (nm)	260	100.1
	254	99.85
	245	99.90
Filtration system	Nylon	100.1
	PTFE	100.7
	Centrifuge	99.4
Column temperature	25 °C	100.1
	27 °C	100.3
RSD (%)		0.47 %

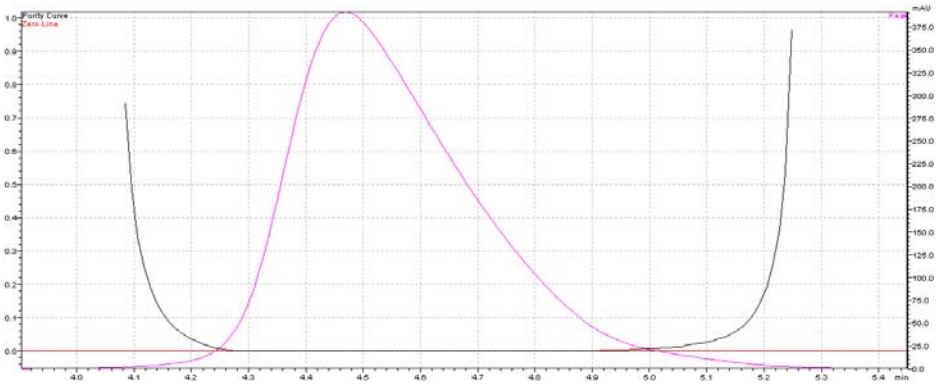

Fig. 4: Cephalexin peak purity by DDA detector, the purity factor was lower than the calculated threshold indicating the high selectivity of the proposed method

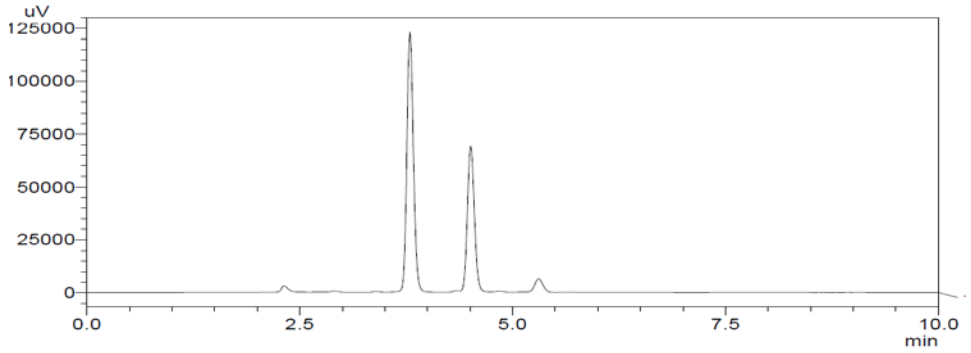

Fig. 5: Cephalexin solution under alkaline hydrolysis conditions

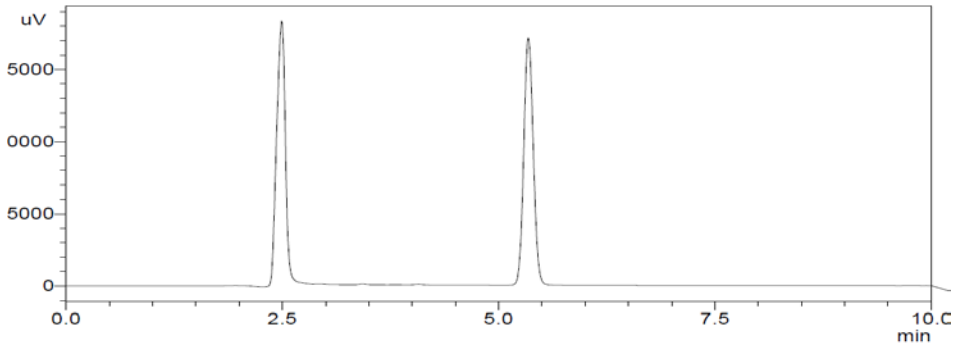

Fig. 6: Cephalexin solution under acidic hydrolysis conditions

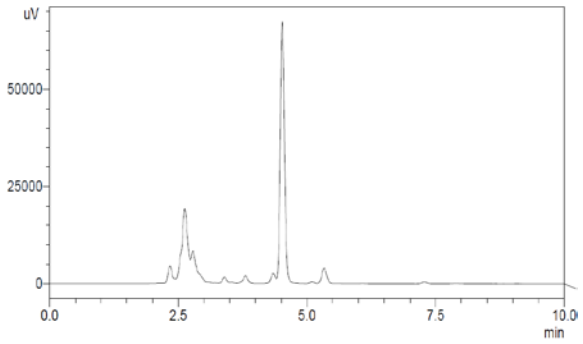

Fig. 7: Cephalexin solution under oxidative hydrolysis conditions

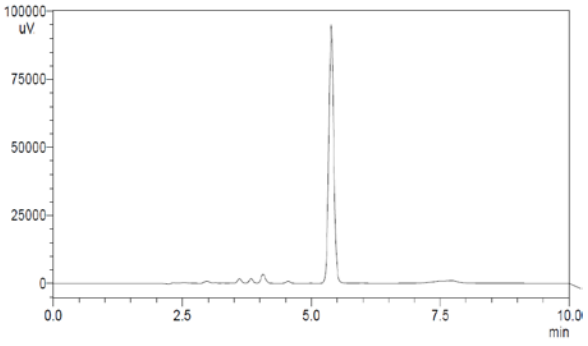

Fig. 8: Cephalexin solution under photo-degradation conditions

Application of the proposed method

The proposed method was found to be rapid, selective, sensitive and suitable for the quantitative determination of cephalexin in pure form, in finished drug form and in the urine sample. The last application (fig.9) is one of the major advantages of micellar chromatography due to the ability to directly inject physiological fluids because micelles have an ability to solubilize proteins, which enables MLC to be useful in analyzing untreated biological fluids such as plasma, serum, and urine [23].

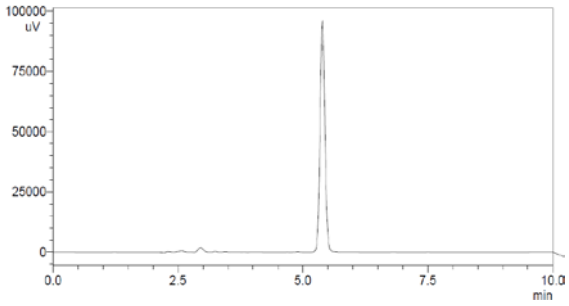

Fig. 9: Cephalexin in urine sample injected as it is after 8 hours of oral administration

CONCLUSION

This study focus on micellar RP-HPLC tech which is considered greener than conventional HPLC technique as it utilize more eco-friendly solvents and chemicals at the same time it is faster and could be used for analysis of biological fluids directly without compromising the efficiency. A valid and fast stability indicating HPLC method was developed. The proposed method ensures precise and accurate determination of cephalexin in tablet dosage form as well as human urine samples. No interference from the tablets excipients or interfering molecules in the urine samples. With the proposed method satisfactory separation of cephalexin from the degradation products was achieved and high recovery of the analyte was obtained.

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CONFLICT OF INTERESTS

Declared None

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