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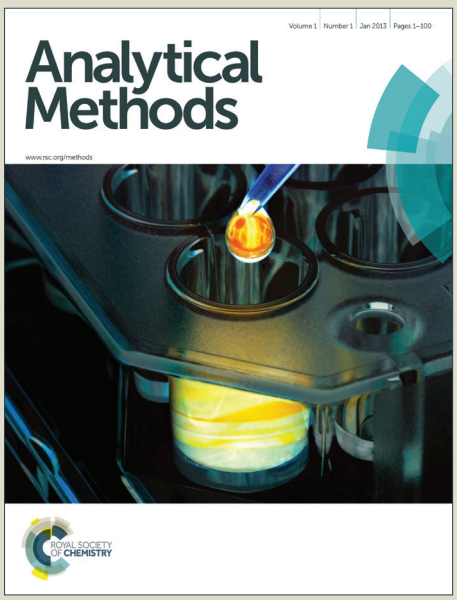
Accepted Manuscript



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Abstract

A stability-indicating gradient reversed phase liquid chromatography (RP-LC) method has been developed for the quantitative determination of cephalothin (CET), an antimicrobial compound, in the presence of its impurities and degradation products generated from forced degradation studies. The developed method is also applicable for the related substances determination in bulk drugs. The chromatographic separation was achieved on an Agilent Eclipse XDB-Phenyl, 250 mm x 4.6 mm, 5 µm column with mobile phase containing a gradient mixture of solutions A (aqueous ammonium phosphate buffer, pH 4.5) and B (acetonitrile) as mobile phase. The flow rate was 1.0 mL/min and the detection wavelength was 238 nm. The drug substance was subjected to stress conditions of hydrolysis, oxidation, photolysis, humidity and thermal degradation. Considerable degradation was found to occur in base, acid and oxidative stress conditions. In the developed HPLC method, the resolution between CET and its potential degradation products was found to be greater than 2.4, further, the peak purity of CET in all conditions were more than 99% proved the stability-indicating power of method. The less active metabolite of cephalothin, deacetylcephalothin (impurity B), was identified and is showed significant formation especially in basic condition. This method is capable to detect the degradation products of CET at a level of 0.05% with respect to test concentration of 500 µg/mL for a 10 µL injection volume. The HPLC method developed was validated with respect to linearity, accuracy, precision, specificity, limit of quantitation, limit of detection and robustness and system suitability. To our knowledge, a rapid stability-indicating LC method for CET has not been published elsewhere and this method can be applicable to evaluate the quality of product samples as well as in stability studies of cephalothin.

***Keywords*:** Cephalothin, Degradation products, Liquid chromatography, Stability-indicating, Validation

Stability-indicating LC method for the determination of cephalothin in lyophilized powder for injection

Karen de Souza Rugani [a,b]; Hérida Regina Nunes Salgado [b*]

[a] Departamento de Fármacos - Desenvolvimento e Validação, Bioagri Laboratórios – A Mérieux NutriSciences Company, Rodovia Rio Claro – Piracicaba, SP 127, km 24, CEP 13412-000 – Piracicaba, SP, Brazil

[b] Departamento de Fármacos e Medicamentos, Faculdade de Ciências Farmacêuticas, UNESP – Univ Estadual Paulista, Rod. Araraquara-Jaú, km 1, CEP 14801-902 Araraquara, SP, Brazil

* Corresponding author at: Universidade Estadual Paulista (UNESP), Faculdade de Ciências Farmacêuticas, Departamento de Fármacos e Medicamentos, Campus Universitário, 14801-902, Araraquara, SP, Brazil. Tel.: +55 33016967; fax: +55 33016960.

E-mail address: salgadoh@fcfarunesp.br **(H.R.N. Salgado).**

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found to occur in base, acid and oxidative stress conditions. In the developed high performance liquid chromatography (HPLC) method, the resolution between CET and its potential degradation products was found to be greater than 2.4, further, the peak purity of CET in all conditions were more than 99% and proved the stability-indicating power of method. The less active metabolite of cephalothin, deacetylcephalothin (impurity B), was identified and showed significant formation especially in basic condition. This method is capable to detect the degradation products of CET at a level of 0.05% with respect to test concentration of 500 µg/mL for a 10 µL injection volume. The HPLC method developed was validated with respect to linearity, accuracy, precision, specificity, limit of quantitation, limit of detection and robustness. To our knowledge, a rapid stability-indicating LC method for CET has not been published elsewhere and this method can be applicable to evaluate the quality of product samples as well as in stability studies of cephalothin.

***Keywords*:** Cephalothin, Degradation products, Liquid chromatography, Stability-indicating, Validation

1. Introduction

Cephalothin (USAN, United States Adopted Names), also named cefalotin (INN, International Nonproprietary Name), is a first-generation cephalosporin antimicrobial chemically described as (6R,7R)-3-[(acetyloxy)methyl]-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicy-clo[4.2.0]oct-2-ene-2-carboxylic acid (Figure 1), it has an empirical formula of $C_{16}H_{16}N_2O_6S_2$, a molecular weight of 396.44 g mol^{-1} [1,2] and a pk_a value of 2.22 [3]. Cephalothin is given as the sodium salt by injection and 1.06 g of cephalothin sodium is equivalent to about 1 g of cephalothin [2].

Cephalosporins act by inhibiting synthesis of the bacterial cell wall and present a larger spectrum of activity than penicillins due to be more stable to many bacterial β-lactamases [4,5]. Cephalothin was the first cephalosporin available for clinical use in the U.S. and it is most active against Gram-positive cocci, including oxacilin-susceptible staphylococci and *Streptococcus* spp and has moderate activity against some Gram-negative bacilli [6]. It is indicated for treatment of infections of skin and soft tissue [7]. Cephalothin remains the antibacterial of choice for treatment of uncomplicated celullits [8].

Cephalothin is given by intramuscular (IM) and intravenous (IV) route, however, the IV is preferred because the IM is very painful. It is widely distributed in body tissues and fluids except the brain and cerebrospinal where the concentrations achieved are low and unpredictable. About 70% of the dose is bound to plasma proteins and the plasma half-life varies from about 30 to 50 minutes. Approximately 20 to 30% of CET is rapidly deacetylated in the liver (hepatic biotransformation) and about 60 to 70% of a dose is excreted in the urine as cephalothin and the less active metabolite, deacetylcephalothin [2,9].

Cephalothin has been determined by spectrophotometry [BP, 2010] and by high-performance liquid chromatographic (HPLC) with ultraviolet detection [11-13]. Analysis of cephalothin and its deacylated metabolites in human urine [14-15], in human plasma by LC [16,17], and its determination in rat blood [18] were also reported in the literature. However, there is no reported stability-indicating LC method for the determination of cephalothin in its lyophilized powder for injection dosage form.

Several analytical methods are described in the literature for analysis of cephalosporins in raw material and finished product. Among these are found titrimetry [19]; high performance liquid chromatography [20-22]; ultraviolet spectrophotometry [21,23,24] and

visible spectrophotometry [25]; infrared spectrometry [26,27], agar diffusion [28-31] and turbidimetry bioassay [32].

In quality control of pharmaceutical products, the identification and quantification of the active substance and its impurities are very important due to reasons of safety and efficacy. The impurities and possible degradation products which may be present in the medicines may change their chemical, pharmacological, and toxicological properties [33,34].

The forced degradation studies are performed to facilitate the development of analytical methods to obtain a better understanding of active ingredient and stability of the product, and to provide information on routes of degradation and formation of degradation products [34,35]. In this context, the stress test (forced degradation) is defined as a stability test performed with the finished product subjected to conditions that exceed those used in accelerated stability studies [36]. According to the ICH Q1A-R2 [33], stress testing (forced degradation) performed with the addition of drug can help identify the likely degradation products, contribute to the definition of the intrinsic stability of the molecule, and also validate the ability of the analytical procedure used to be a stability-indicating method.

Accordingly, the goal of the present work was to develop simple, sensitive and selective LC stability-indicating method for the determination of CET in its lyophilized powder for injection dosage form. Moreover, the present manuscript describes the degradation behavior of cephalothin under acidic, basic, oxidative, thermal, humidity and photolytic conditions with the identification of the main degradation product: deacetyltcephalothin. Stability-indicating methods are traditionally performed using gradient elution, in order to ensure that degradation products of several chemical compositions are all separated. Optimization of LC conditions to separate the CET and its

degradation products included the identification of impurity B (deacetylcephalotin), on a reversed phase Phenyl column, and method validation will be discussed in Results, section 3.1.

The system suitability method acceptance criteria set in each condition of CET samples were: tailing factor ≤ 2.0, resolution between CET and any of the components > 2.0 and theoretical plates >2000. Hence, this method was successfully validated according to the ICH guideline Q2 (R1) [37].

2. Materials and Methods

2.1. Materials

Cephalothin sodium salt reference standard was assigned purity 99.2% and supplied by Sigma–Aldrich (Germany). Cephalotin for impurity B identification reference substance was supplied by European Pharmacopoeia (Strasbourg, France). All reagents used in the experimental procedure were of analytical grade. Acetonitrile, hydrogen peroxide (H_2O_2), phosphoric acid and concentrated hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Ammonium phosphate dibasic was supplied by Synth (Diadema, Brazil) and sodium hydroxide pellets (NaOH) was supplied by Vetec (Rio de Janeiro, Brazil). The cephalothin sodium lyophilized powder for injection, which was claimed to contain 1 g of active drug (cephalothin), was kindly donated by União Química Pharmaceutical Industry (Pouso Alegre, Brazil). Ultrapure water was obtained from a Milli-Q™ Plus apparatus (Millipore™, USA) and was used to prepare all solutions. Millex PVDF membrane filters with a pore size of 0.45 μm and diameter of 33.0 mm (Millipore™, Tullagreen, Carrigtwohill, Ireland) was used. All other chemicals were of analytical grade.

2.2. Chromatographic system

An Agilent model 1200 DAD/UV LC system, consisting of a quaternary pump (G1311A), a degasser (G1322A), a column thermostat (G1316A), a diode array detector (DAD) (G1315A), and an thermostatted autosampler (4 C) (G1329A), was used. The chromatographic data were recorded using a computer system with ChemStation data acquiring software (also from Agilent). The peak purity was determined using a diode array detector (DAD).

Chromatographic separation was achieved on a Eclipse XDB - Phenyl column (250 mm × 4.6 mm, 5 µm particle size) – Agilent™ applying gradient elution based on a mobile phase A (20 mM ammonium phosphate buffer, pH 4.5) and mobile phase B (acetonitrile). The system was programmed at 35ºC for the column oven, 238 nm for detection wavelength and 10 µL for injection volume in a flow rate of 1.0 mL/min. The LC gradient program (Time (in min) / % mobile phase B) was set as 0.0/10; 2.0/10; 15.0/30; 15.1/10 and 30.0/10.

2.3. Preparation of sample for LC analysis

2.3.1. Standard preparation

The cephalothin standard (10 mg) was accurately weighted and transferred into a 20 mL volumetric flask, diluted with purified water, and sonicated by 10 minutes to prepare the standard solution at concentration of 500 µg/mL. Solutions were filtered through a 0.45 µm PVDF membrane filter (Millipore, Ireland) prior to injection.

2.3.2. Sample preparation

For the analysis of cephalothin, an amount of powder equivalent to 10 mg of CET was transferred to a 20 mL volumetric flask with ultrapure water and sonicated for 10 min, followed by the dilution to volume with ultrapure water obtained the concentration of 500 µg/mL. These solutions were filtered through a 0.45 µm PVDF filter before injections.

2.4. Forced degradation studies

Forced degradation conditions used are more severe than accelerated stability testing such as temperature (50°C or 60ºC); relative humidity (≥75%); hydrolysis at high and low pH; oxidation and photolysis [37]. When performing forced degradation study, the ideal range of degradation of the active ingredient is 5-20%, in order to cause degradation but not so intense that generates secondary products [35,38]. The stress solutions were prepared to obtain a final concentration of 500 µg/mL of CET. Prior to injection, samples were withdrawn at appropriate time, neutralized (in case of acid and alkali hydrolysis), diluted with purified water and passed through Millex 0.45 µm membrane filter. The total chromatographic run time was about two times higher than the retention time of the drug peak in order to allow the elution of all possible degradation products formed. In all stress conditions the peak area of CET and its degradation products were obtained. The stress samples were assayed against a qualified cephalothin reference standard and the peak purity tests were carried out for the cephalothin and its degradation products peaks by using DAD-UV detector. The identification of the impurity B (deacetylcephalothin) was carried out.

2.4.1. Acid and base hydrolytic degradation

The cephalothin sample was treated separately with in 0.1 M HCl and 0.01 M NaOH. The acid solution was exposed to a temperature of 60°C for 40 minutes and the basic solution to a room temperature for 5 minutes. The forced degradation in acidic and basic media was performed in the dark. The solutions were neutralized and then diluted with purified water.

2.4.2. Oxidative degradation

To study the oxidative degradation on the drug sample, a solution of 3% H_2O_2 was added and kept at room temperature for 20 minutes in the dark. The resultant solution was diluted with purified water.

2.4.3. Humidity degradation

The dry powder of the drug sample was placed at 30ºC / 75% RH (in a climate chamber) for 10 days to study humidity degradation. After, the sample was dissolved and diluted with purified water.

2.4.4. Thermal degradation

The dry powder of the drug sample was placed in oven at 60°C for 10 days to study dry heat degradation. Powder was then dissolved and diluted with purified water.

2.4.5. Photochemical degradation

The photochemical stability of the drug was studied by exposing the dry sample powder to ultraviolet (UV) and cool white fluorescence (VIS) lamps for 10 days at 25ºC.

The illumination used was not less than 1.2 million lux hours (VIS) neither 200 watt h/m^2 (UV). The sample powder was then dissolved and diluted with purified water.

2.4.6. Standard preparation: Cephalothin for Impurity B identification

A standard solution containing 220 µg/mL of cefalotin for Impurity B identification (deacetylcephalothin) was prepared in purified water and analyzed in the forced degradation test. This solution was filtered through a 0.45 µm PVDF membrane filter (Millipore, Ireland) prior to injection.

2.5 Analytical method validation

The developed chromatographic method was validated for the following parameters: specificity, linearity, range, precision, accuracy, detection limit, quantitation limit and robustness [37].

2.5.1. Specificity

The specificity was conducted during the forced degradation studies (section 2.4). Placebo was also evaluated under the same conditions. Peak purity tests were used to show that the CET chromatographic peak is only attributable to this component.

2.5.2. Precision

The precision of the method was evaluated by injection of six independent preparations of cephalothin sample at a concentration of 500 µg/mL quantified against a

reference standard; the % RSD of assay was calculated. The intermediate precision of the method was evaluated using two different analysts in different days.

2.5.3. Detection limit (LOD) and Quantitation limit (LOQ)

The LOD and LOQ were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions of CET with known concentrations.

2.5.4. Linearity

Linearity standard solutions were diluted from cephalothin stock solution prepared in purified water (1250 µg/mL) at five concentration levels from 50 to 150% of assay analyte concentration (250, 375, 500, 625 and 750 µg/mL). Each concentration was analyzed three times and the calibration curve was constructed using analyte standard peak area ratio *versus* concentration of analyte. Linearity was evaluated by linear least-squares regression analysis.

2.5.5. Accuracy

The accuracy of the method was evaluated in triplicate at three concentration levels (375, 500 and 625 µg/mL) by standard addition technique. The percentage of recoveries was calculated at each level.

2.5.6. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the retention time and area of cephalothin were assessed. The flow

rate of the mobile phase was changed to 0.9 and 1.1 mL/min, the column temperature was studied at 30ºC and 40°C, the effect of the percent organic strength was studied by varying initial proportion of acetonitrile by 8% and 12% and the pH of 20mM ammonium phosphate buffer was changed to 4.4 and 4.6. Only one parameter was changed while the others were kept constant.

3. Results

3.1. Optimization of chromatographic conditions

The main objective of the chromatographic method is to separate cephalothin from its degradation products. During the development of this method, different stationary phases such as C18, C8, phenyl and cyano as well as different compositions of mobile phase were tested (potassium phosphate buffer, triethylamine 0.1% (v/v), triethylamine 0.1% + acetic acid 0.1% (v/v), sodium perchlorate buffer, acetonitrile, methanol and ethanol). The chromatographic separation was achieved on an Eclipse XDB-Phenyl (250 mm × 4.6 mm, 5µm) column using mixture of aqueous 20 mM ammonium phosphate buffer - pH 4.5 and acetonitrile as a mobile phase in a gradient mode. The flow rate of the mobile phase was 1.0 mL/min, the column temperature was 35 ºC, at 238 nm, and the peak shape of the cephalothin was found to be symmetrical. In optimized chromatographic conditions CET and its degradation products were separated with resolution greater than 2. The retention time of CET was found to be 14.7 min (Figure 2).

3.2. System suitability

System suitability tests are used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters measured were tailing factor, capacity factor, theoretical plates, retention time and repeatability as %R.S.D. of peak area for six injections of a standard solution of a 500 µg/mL of CET (100% concentration). The tailing factor showed less than 2, the capacity factor was more than 2 and the theoretical plates were more than 2000. The average of retention time was 14.8 minutes and the %RSD of peak area was 0.71%.

3.3. Linearity

The linearity for detector response was observed in the concentration range of 250-750 µg/mL for cephalothin. The calibration curve was constructed with concentration against peak area. The regression equation for the calibration curve was found to be $y=19.61312x + 32.45824$ and the correlation coefficient (r^2) of 0.99999 was obtained. Good linearity was found between the peak area and analyte concentration.

3.4. Precision

The repeatability of the method was assessed by six determinations of the concentration of 500 µg/mL representing 100%. The intermediate precision was carried out by a second analyst in a different day. The % RSD of cephalothin during the repeatability and the intermediate precision was 0.548% and 0.547%, respectively, confirming good precision of the method.

3.5. Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ were determined experimentally for cephalothin and results were found to be 0.0760 μg/mL and 0.2532 μg/mL, respectively.

3.6. Accuracy

The accuracy of the method was evaluated by recovery studies and was carried out by standard addition method at three different levels (75%, 100% and 125%) described in table 1. The percentage of recoveries for cephalothin and percent RSD were found to be, respectively, 99.41% and 0.54%.

3.7. Forced degradation studies (Specificity)

The specificity of the developed method was determined by injecting sample solutions (500 μg/mL) which were prepared by stress conditions. Degradation in cephalothin sample was observed when subjected to stress conditions like acid hydrolysis, base hydrolysis and oxidation. Under oxidative conditions, photodegradation and humidity conditions, in 10 days, cephalothin sample showed stable. The summary of forced degradation results is given in Table 2 and 3. Typical chromatograms of stress testing are shown in Figure 3 and Figure 4. The placebo was also evaluated (Figure 5) and peak purity test results confirmed that the cephalothin peak is homogenous and pure in all the analyzed stress samples (Figure 6). The impurity B (deacetylcephalothin) was identified by comparative analysis with a reference standard solution (Figure 7).

Accounting for the fact that additional peaks of degradation products appeared in the chromatograms in addition to the impurity B, the proposed method demonstrated excellent selectivity and resolution between CET and the other compounds (minimum

resolution of 2.4), being, therefore, applicable for routine cephalothin analysis, including samples from stability studies.

3.8. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, and in all the varied chromatographic conditions (flow rate, column temperature, composition of organic solvent and pH value), no significant change was observed and %RSD values showed less than 2, confirming the robustness of the method.

4. Discussion

The liquid chromatographic procedure was carried out to develop a stability-indicating method to characterize degradation products from the studied cephalosporin. Chromatographic conditions were elected after different mobile phases with different organic solvent ratios were tested. Moreover, different brands of chromatographic columns were also tested to optimize the separation, and the effects of organic solvent proportions and compositions were examined.

The low LOD and LOQ values found are indicative of the high sensitivity of the method. Besides, an analytical method can be considered specific, when it can demonstrate drug separation and quantitation from the bulk physical mixture, finished product, its degradation products and excipients. Our results obtained in the stress degradation are in accordance with the current literature about the structural modifications of cephalothin.

5. Conclusions

HPLC methods have been widely used by pharmaceutical laboratories and they have gained popularity in stability studies due to their sensitivity, specificity and high-resolution capacity. A validated stability-indicating LC method was developed for quantitative determination of cephalothin and also is applicable for the related substances determination. The proposed LC method is found to be sensitive, precise, accurate, linear, robust, specific and convenient for the separation of cephalothin in the presence of its degradation products, including deacetylcephalothin, its less active metabolite. The method was fully validated showing satisfactory data for all the method validation parameters tested. Hence, the developed LC method can be used for the quality control and also for analysis of stability samples. The HPLC method developed is very simple and results confirm suitable precision, accuracy, and specificity. Therefore, the method could be useful for both routine analytical and quality control assay of cephalothin in pharmaceutical form and it could be a very powerful tool to investigate chemical stability of this cephalosporin.

Acknowledgements

We thank the laboratory União Química Pharmaceutical Industry (Pouso Alegre, Brazil) for the kind donation of medicines. This work was supported by Bioagri – a Mérieux NutriSciences Company (Bioagri Laboratórios (Piracicaba, Brazil) and H.R.N. Salgado is grateful to the PACD-FCF-UNESP (Araraquara, Brazil) and CNPq for financial supports.

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Table 1

Results of recovery for cephalothin.

	Amount of Standard Cephalothin added (µg/mL)	*Total Found (µg/mL)*	*Recovery (%)**	*Mean Recovery (%)*
R1	123.15	123.07	99.93	99.41
R2	246.30	243.50	98.86	
R3	369.46	367.44	99.45	

* Mean value of three replicates

Table 2

Summary of forced degradation results of cephalothin.

Stress condition	*Time*	*% Degradation*	*Purity factor*
Acid hydrolysis (0.1 M HCl, 60 °C)	40 minutes	16.10	99.92
Base hydrolysis (0.01 M NaOH, 60 °C)	5 minutes	14.92	99.91
Oxidation (3% H_2O_2, room temperature)	20 minutes	20.24	99.98
Photodegradation (UV/VIS)	10 days	0.10	99.98
Thermal (60 °C)	10 days	-0.81	99.98
Humidity (30 ºC / 75% RH)	10 days	-1.32	99.91

Table 3

Summary of degradation products.

RRT	*Acid hydrolysis (%) area*	*Basic hydrolysis (%) area*	*Oxidation (%) area*
0.26	ND	ND	0.13
0.29	ND	0.11	ND
0.37	ND	0.06	ND
0.39	ND	ND	0.13
0.44	0.73	2.86	1.54
0.49	ND	ND	0.25
0.58	0.23	ND	ND
0.60	ND	ND	1.80
0.72 (impurity B)	3.05	10.49	0.12
0.76	ND	ND	9.50
0.77	ND	ND	1.26
0.82	0.04	ND	ND
0.87	1.54	ND	ND
0.89	0.13	0.16	0.14
0.92	1.80	ND	ND
1.04	ND	0.05	ND
1.18	ND	0.05	ND
1.28	ND	0.04	ND

RRT: Retention relative time; ND: not detected