Impurities profiling of brands of ceftriaxone sodium injection marketed in Ibadan, Southwest Nigeria

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ABSTRACT

Background: Several factors can affect the effectiveness of a drug in therapy. One of such is the presence of impurities.

Objectives: Based on the current useful utility status of ceftriaxone injection as an antibacterial agent, a comprehensive impurity profiling of thirteen brands was carried out in order to provide a basis for specifying appropriate storage conditions.

Methods: The profiling was carried out by developing a new liquid chromatographic method with UV detection. Factors that could affect the separation method such as elution mode, pH, flow rate, detection wavelength and type of organic modifier were studied. Validation studies comprising calibration curve, LOD and LOQ determination, accuracy and repeatability were determined. The method was successfully applied to the determination of ceftriaxone and impurities in 13 brands of ceftriaxone injections.

Results: The developed method involved separation of ceftriaxone and major impurities within 15 minutes using gradient elution with KH_2PO_4 (pH 7.5) and methanol (flow rate, 1 mL/min). Separation was achieved on a C-18 column at 220 nm. Linearity was obtained within the range 7.8 – 250 µg/mL (r^2 = 0.9996) with LOD and LOQ as 74.56 and 225.9 ng/mL respectively. Relative errors from the intra- and inter-day assessment were generally less than 2%. All the brands complied with the content BP specification of 92-108% of ceftriaxone. However, the impurities content in all the 13 brands were far higher than the 0.2% specified by ICH for the dose of the drug.

Conclusion: There is need for measures to adequately control storage conditions of the injection in order to limit impurities content.

Keywords: Ceftriaxone, Impurities profiling, Liquid chromatography, Storage conditions

Profilage des impuretés des marques d'injection de sodium ceftriaxone commercialisées à Ibadan, sud-ouest du Nigéria

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RESUME

Contexte: Plusieurs facteurs peuvent affecter l'efficacité d'un médicament en thérapie. L'un d'entre eux est la présence d'impuretés.

Objectifs: Sur la base du statut d'utilité actuel de l'injection de ceftriaxone en tant qu'agent antibactérien, un profil d'impureté complet de treize marques a été réalisé afin de fournir une base pour spécifier des conditions de stockage appropriées.

Méthodes: Le profilage a été réalisé par le développement d'une nouvelle méthode de chromatographie liquide avec détection UV. Les facteurs qui peuvent affecter la méthode de séparation telles que le mode élution, le pH, le débit, la longueur d'onde de détection et le type de modificateur organique ont été étudiés. Des études de validation comprenant la courbe d'étalonnage, la détermination de limite de détection LD et de limite de quantification LQ, la précision et la répétabilité ont été déterminées. La méthode a été appliquée avec succès à la détermination de la ceftriaxone et des impuretés dans 13 marques d'injections de ceftriaxone.

Résultats: La méthode développée impliquait la séparation de la ceftriaxone et des impuretés majeures en 15 minutes en utilisant une élution par gradient avec KH_2PO_4 (pH 7,5) et du méthanol (débit 1 mL/min). La séparation a été réalisée sur une colonne C-18 à 220 nm. La linéarité a été obtenue dans la gamme de 7,8 à 250 µg/mL (r^2 = 0,9996) avec LD et LQ étant de 74,56 et 225,9 ng/mL respectivement. Les erreurs relatives de l'évaluation intra et inter-journée étaient en général inférieures à 2%. Toutes les marques ont respecté le contenu de la spécification BP de 92-108% de ceftriaxone. Cependant, le contenu des impuretés dans les 13 marques était beaucoup plus élevé que les 0,2% spécifiés par ICH pour la dose du médicament.

Conclusion: Il est nécessaire de prendre des mesures pour contrôler de manière adéquate les conditions de stockage de l'injection afin de limiter le contenu des impuretés.

Mots-clés: ceftriaxone, profilage des impuretés, chromatographie liquide, conditions de stockage

INTRODUCTION

Ceftriaxone is presented as ceftriaxone disodium and the salt chemically is named as (6R,7R)-7[[(2Z)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[[(2-methyl-6-oxido-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)sulphanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-carboxylate 3,5 hydrate with structural formula $C_{18}H_{16}N_8Na_2O_7S_2$. 3½ H_2O.[1] It is an almost white or yellowish, slightly hygroscopic, crystalline powder. It is freely soluble in water, sparingly soluble in methanol, and very slightly soluble in anhydrous ethanol. It has a melting point of >155 ^oC and melts with decomposition. It has a pH value of 6.0 to 7.5 in a 10% aqueous solution and it is light sensitive.

Ceftriaxone is a broad-spectrum cephalosporin antibiotic with a very long half-life and high penetrability to meninges, eyes and inner ears. It is used for the treatment of the infections (respiratory, skin, soft tissue, urinary tract infection, ear, nose and throat infections) caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, staphylococci, *Streptococcus pyogenes* (group A beta-hemolytic streptococci), *E. coli*, *Proteus mirabilis*, *Klebsiella species* and coagulase-negative staphylococcus. Some of the other organisms susceptible to ceftriaxone are enteric bacteria and other eubacteria, *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Salmonella typhi*.[2]

Ceftriaxone is one of the antibiotics currently utilized for the management of several health conditions in patients. There are several generic brands of the drug currently in circulation in Nigeria. Apart from the need to ascertain the possibilities of interchangeability of the generic brands a critical quality requirement is also the control of specific process- and synthesis-related impurities present in the formulation. The British Pharmacopoeia specifies five impurities and related substances with ceftriaxone sodium. These are; Impurity A - disodium (*E)*-(6R,7R)-7-[(2-amino-1,3-thiazol-4-yl) (methoxyimino)acetamido]-3-[[2,5-dihydro2-methyl-6-oxido-oxo-1,2,4-triazin-3-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (E-*isomer*); Impurity B - (*Z*)-2-(2-amino-1,3,-thiazol-4-yl)-*N*-[5a*R*,6*R*)-1,7-dioxo-1,4,6,7-tetrahydro-3*H*,5a*H*-aeto[2,1-*b*]furo3,4-*d*][1,3]thiazin-6-yl]-2-(methoxyimino)acetamido; Impurity C – 6-hydroxy-2-methyl-3-mercapto-1,2,4-triazin-5(2*H*)-one; Impurity D – S-(1,3-benzothiazol-2-yl) (*Z*)-(2-amino-1,3-thiazol-4-yl)methoxyimino)thioacetate and Impurity E – (6*R*,7*R*)-7-amino-3-[[2,5,-dihydro-6-hydroxy-2methyl-5-oxo-1,2,4-triazin-3-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene2-carboxylic acid. [1]

There is an ever increasing interest in impurities present in active pharmaceutical ingredients (APIs). Recently, not only purity profile but also impurity profile has become essential as per various regulatory requirements. In the pharmaceutical world, an impurity is considered as any other organic material, besides the drug substance, or ingredients, arising out of synthesis or unwanted chemicals that remain with APIs. The impurity may have probably been introduced either during formulation, or upon aging of both APIs and formulated APIs in medicines.[3] A good illustration of this definition may be identification of impurity in APIs like 1-(1, 2, 3, 5, 6, 7-hexahydro-s-indacen-4-yl)-3-4[-1-hydroxy-1-methyl-ethyl)-furan-2-sulphonylurea using Multidisciplinary approach.[4]

Impurities have no therapeutic value but are potentially harmful and hence need to be controlled. In order to set an impurity limit, some of the factors taken into considerations are; the toxicity potential of the impurities and the likely impurities that actually occur.[5]

The presence of these unwanted chemicals even in small amounts can significantly affect the efficacy and safety of the pharmaceutical products. The different pharmacopoeias (e.g. British Pharmacopoeia[1] and the United States Pharmacopoeia[6]), are slowly incorporating limits to allowable levels of impurities present in the APIs or formulations. The limits also include exclusion to the barest minimum such deleterious contaminants as heavy metals and pathogens.

Several recent books[5,7] and journal reviews [8,9] address the topic of impurities and guidelines are available from US and international authorities.[10] In the synthesis of new chemical entities, impurities in pharmaceutical compounds or a new chemical entity (NCE) can originate during the synthetic process, from raw materials, intermediates, and / or by-products. Impurity profiling of tablets by GC-MS and MDMA (3, 4-Methylene dioxy methamphetamine) samples produced impurities in the intermediates *via* the reductive amination route.[11]

The determination of cephalosporins has received much attention from researchers and several methods exist in literature for the quantitation of brands of various cephalosporins. Some of the methods that have been described for the quantitative determination of cephalosporins include; spectrophotometry[12,13,14], spectrofluorimetry[15], HPLC[16,17,18,19,20,21,22], potentiometry[23] and voltammetry.[24] However, most of the HPLC methods were described as time-consuming, tedious, and dedicated to sophisticated and expensive analytical instruments. Some reviews regarding the analysis of cephalosporins are reported in the literature.[25, 26] El-

Shaboury in 2007 reported the analysis of cephalosporins antibiotics [27] and recently Jin *et al*. in 2014 reviewed HPLC and LC-(MS/MS) methods for analysis of third-generation cephalosporins in biological fluids.[26]

Likewise some reports exist in literature for the impurities profiling of cephalosporins. However, much attention has not been placed on the impurities profiling of ceftriaxone. To the best of the knowledge of available literature only two reports could be sourced reporting the impurities profiling of ceftriaxone formulations. Kumar *et al.* (2010) reported a stability indicating fast LC method for determination of ceftriaxone and tazobactam for injection related substances in bulk and pharmaceutical formulation.[28] Separation was achieved in isocratic mode using Kromasil, C18, 250 x 4.6 mm, 5 µm column with mobile phase A containing potassium dihydrogen phosphate buffer (pH adjusted to 6.5±0.05 with Orthophosphoric acid), Citric acid buffer (pH adjusted to 5.0±0.05 with NaOH solution) and Acetonitrile and mobile phase B containing tetradecyl ammonium bromide, tetraheptyl ammonium bromide and Acetonitrile at different time intervals as eluent at a flow rate 0.8 mL/min. UV detection was performed at wavelength of 230 nm. The method is simple, selective and stability indicating. The method was described as accurate with linearity ranging from 3.0289 to 9.0862 µg/mL. The method precision for the determination of related impurities was below 5.0% RSD. The Percentage recoveries of known related impurities from dosage forms ranged from 87.3 to 112.5%. LOD and LOQ of all related impurities of ceftriaxone and tazobactam was established and ranged from 0.119 - 0.552 µg/mL for LOD and 0.356 – 1.658 µg/mL for LOQ. In another reported procedure, Lu *et al.* (2014) reported a combination of reversed phase liquid chromatography and zwitterion exchange-reversed phase-hydrophilic interaction mixed-mode liquid chromatography coupled with mass spectrometry for the analysis of antibiotics and their impurities and included a description of impurities profiling for ceftriaxone.[29]

The current usefulness of ceftriaxone sodium injection powder and the proliferation of the generic brands demand a comprehensive study of the impurities profile. This will in the long run serve as a guide to ensuring the quality of the product, more especially in Nigeria where extremes of humidity, temperature and sunlight can catalyze the decomposition of the product. This research is aimed at providing a suitable liquid chromatographic method for the quantitative assessment of the impurities of ceftriaxone sodium.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (ACN), HPLC grade was obtained from Fisher Scientific (Leicestershire, UK) and methanol (BDH, Poole England). Potassium dihydrogen phosphate crystals (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). Phosphoric acid as obtained from BDH (Briare, France) and sodium hydroxide also from BDH. A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to purify further demineralised water.

Instrumentation and liquid chromatographic conditions

The liquid chromatographic system from CECIL CE Instruments comprising 4100 dual piston pump, 4200 UV-VIS variable wavelength detector and column oven (4601) were used for the analysis. The LC system is equipped with a workstation comprising of Powerstream® Chromatography System Manager ADEPT series CECIL CE 4900. An ultrasonicator from Branson Ultrasonics Corporation (Danbury, CT, USA) and a pH meter from Metrohm (Herisau, Switzerland) were used to dissolve the sample and measure the pH of the mobile phase, respectively. The column was kept in the column oven maintained at 40 ^oC.

Final chromatographic separations were achieved on a Lichrospher RP 18 column from HICHROM UK with dimensions of 125 mm x 4 mm with internal diameter of 5µm (LISPR P-18-5-125AF).

The final optimal mobile phase adopted was a gradient mixture of 0.067 M KH_2PO_4, pH 7.5 and methanol pumped at a flow rate of 1.0 mL/min. The gradient programme [time (min)/%methanol] was set as 0/30, 5/85 to 10/90 to 15/90. The gradient dwell volume of the system is 1.6 mL. The injection volume was 20 µL and UV detection was performed at 220 nm. The mobile phases were degassed by ultrasonication process.

Methods

Samples and Preparation of standard solutions

The drug substance utilized for method development was an old expired ceftriaxone (CEFT) sample (expired for over two years). This old sample was used in order to include as much as possible the impurities of ceftriaxone. The ceftriaxone which has passed its shelf-life was stored at ambient laboratory environment. For assay, ceftriaxone reference substance was adopted and passed through the optimized procedure prior to utilization to verify the presence of the impurities prior to its utilization as a reference standard.

The stock solution consisted of a 1 mg/mL ceftriaxone in a diluent mixture of KH_2PO_4: Methanol (50:50 % v/v).
Thirteen different commercial CEFT injection powder samples were sourced from retail pharmacies in Ibadan, Nigeria. For investigation, a similar stock of 1 mg/mL solution of each brand of CEFT was made in the solvent mixture.

Robustness study

A robustness study was performed by means of an experimental design involving small changes in some of the critical parameters that can affect the chromatographic separations. The factors were each investigated in turn and the influence of each factor in providing clear-cut elution of the six peaks (ceftriaxone and its 5 impurities) as well as resolution of the late eluting peaks was studied. Some of the factors investigated include effect of organic modifiers (methanol and acetonitrile), effect of percentage content of organic modifier, effect of flow rate on separation, effect of detection wavelength and effects of change in pH of the buffer utilized.

Validation studies

Following optimization of the chromatographic conditions, a comprehensive validation protocol was carried out for the assay of CEFT and its impurities from the innovator product and its generic brands.
A 2-day calibration curve was prepared using the range 7.8 – 250 µg/mL of CEFT in the diluent mixture giving a six data point calibration curve. The best fitting calibration range was arrived at as the range with the lowest intercept, highest slope and best coefficient of determination (r^2).
The limit of detection (LOD), limit of quantitation (LOQ) at 95% confidence intervals of slope and intercept were estimated from the calibration curve according to current ICH guidelines.[10]
In addition to the foregoing, a 2-day assessment of the accuracy and repeatability of the new HPLC methodology for the quantitation of CEFT and its impurities was carried out at concentration levels of 20, 50 and 200 µg/mL representing the low, mid and high region of the calibration curve. In each instance four replicates samples were prepared and determined.
Assay of ceftriaxone and its impurities in generic brands
For each of the brands, 10 mg quantity of the sample was weighed and dissolved in 10 mL of the solvent mixture (KH_2PO_4: MeOH; 50:50%v/v) and solution filtered. A final 200 µg/mL solution was prepared for injection into the HPLC. Each sample (20 µL) was injected into the chromatograph using the optimized mobile phase gradient. Four replicate analyses of each brand were carried out.
The amount of ceftriaxone was calculated from the calibration curve while the level of each of the 5 impurities was estimated by comparison of the peak area with that of the ceftriaxone main peak. The value of each of the impurities was estimated by a consideration of the threshold value relative to daily intake as prescribed by the International Conference on Harmonization (ICH) of technical requirements for pharmaceuticals for human use.[10]

RESULTS

Method development and optimization

Optimization of mobile phase

The first mobile phase adopted is a combination of 0.067 M KH_2PO_4 and methanol (98:2 % v/v) as the mobile phase on a C-18 column in a oven at 40 ^oC and in the isocratic mode with detection wavelength of 254 nm.
The resulting chromatogram obtained is presented in Figure1. As evident from this figure, there was separation of two main peaks and two minor peaks. Further modification of the mobile consisted of use of KH_2PO_4: MeOH in a ratio of 97:3 %v/v. The result obtained is presented in Figure 2. The late eluting peak gave a broad band signaling a co-elution of the peaks.The mobile phase combination was further readjusted to KH_2PO_4:MeOH (95:5 %v/v) as presented in Figure 3.

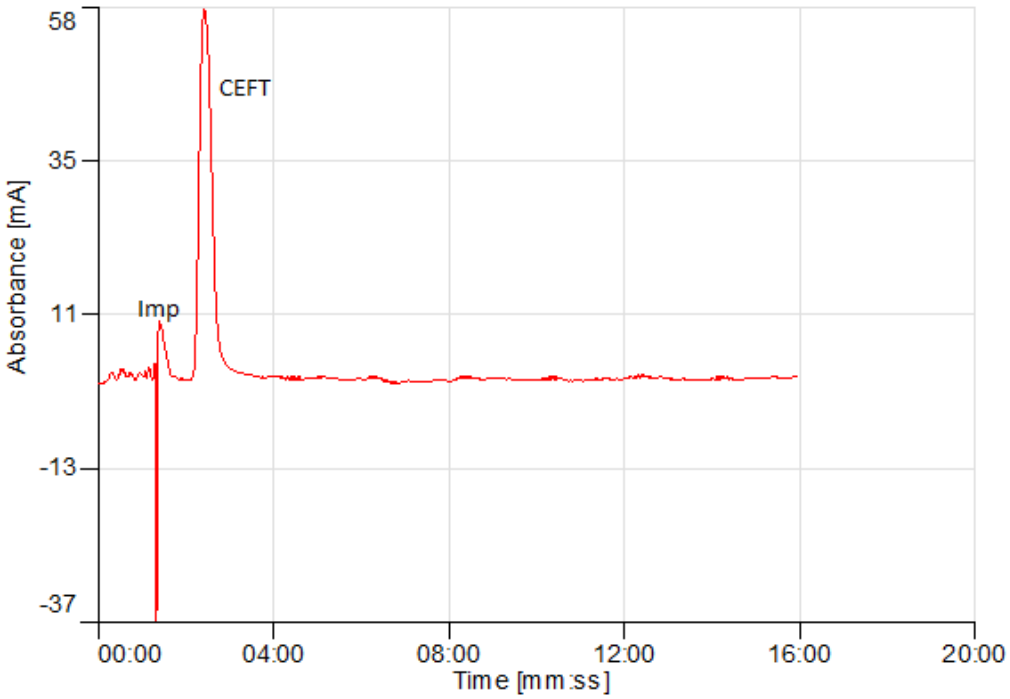


Figure 1: Chromatogram for the separation of ceftriaxone and impurities (KH_2PO_4:MeOH 98:2%v/v)

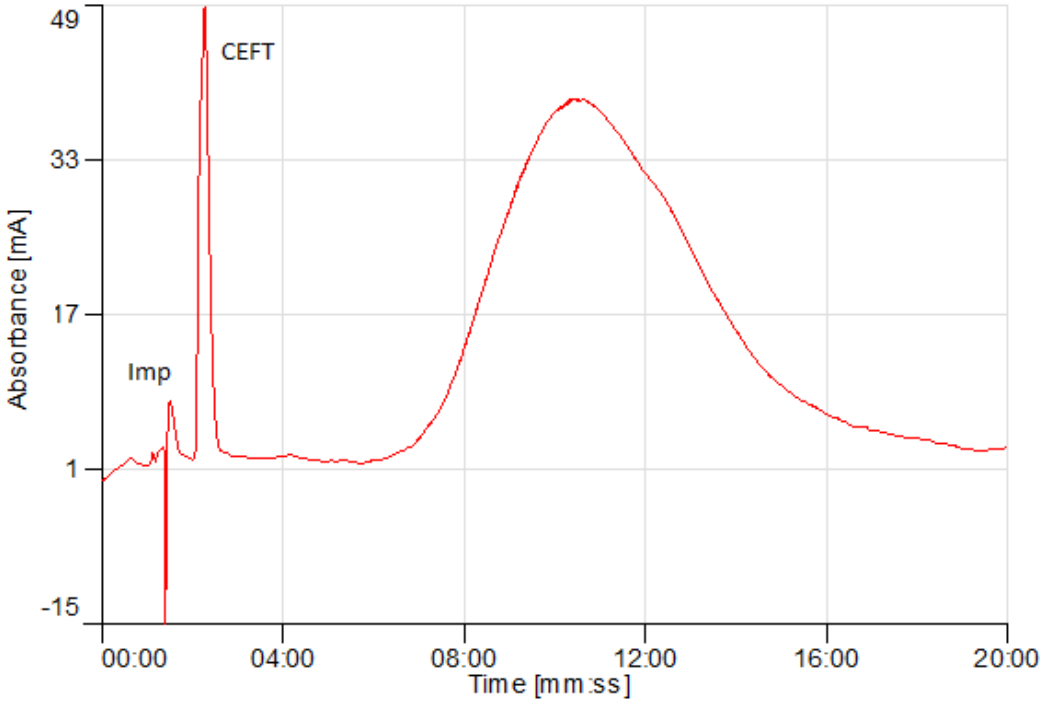


Figure 2: Chromatogram for the separation of ceftriaxone and impurities (KH_2PO_4: MeOH 97:3%v/v)

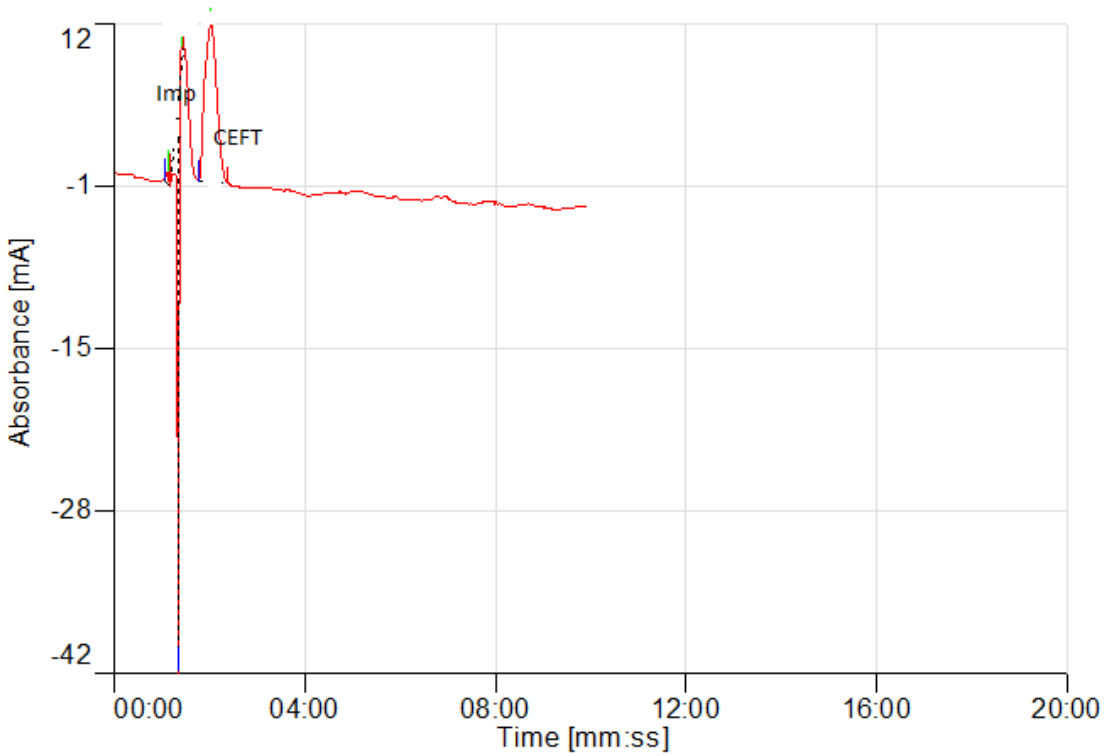


Figure 3: Chromatogram for the separation of ceftriaxone and impurities (KH_2PO_4: MeOH 95:5%v/v)

Optimization of chromatographic technique

Isocratic elution

The efficiency of the chromatographic procedure to elute the ceftriaxone peak alongside the 5 major impurities was evaluated by the adoption of isocratic elution mode for the mobile phase. The results obtained for the combination of KH_2PO_4: MeOH and KH_2PO_4: ACN are presented in Figures 4 and 5 respectively.

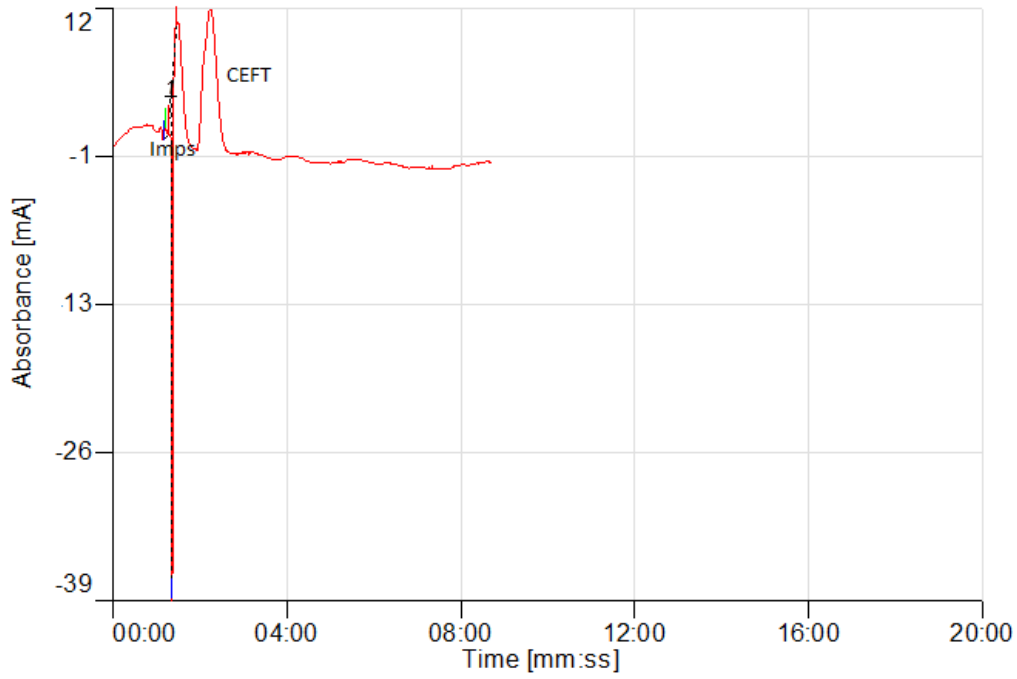


Figure 4: Isocratic elution mode for ceftriaxone determination using KH_2PO_4: MeOH (97:3%v/v)

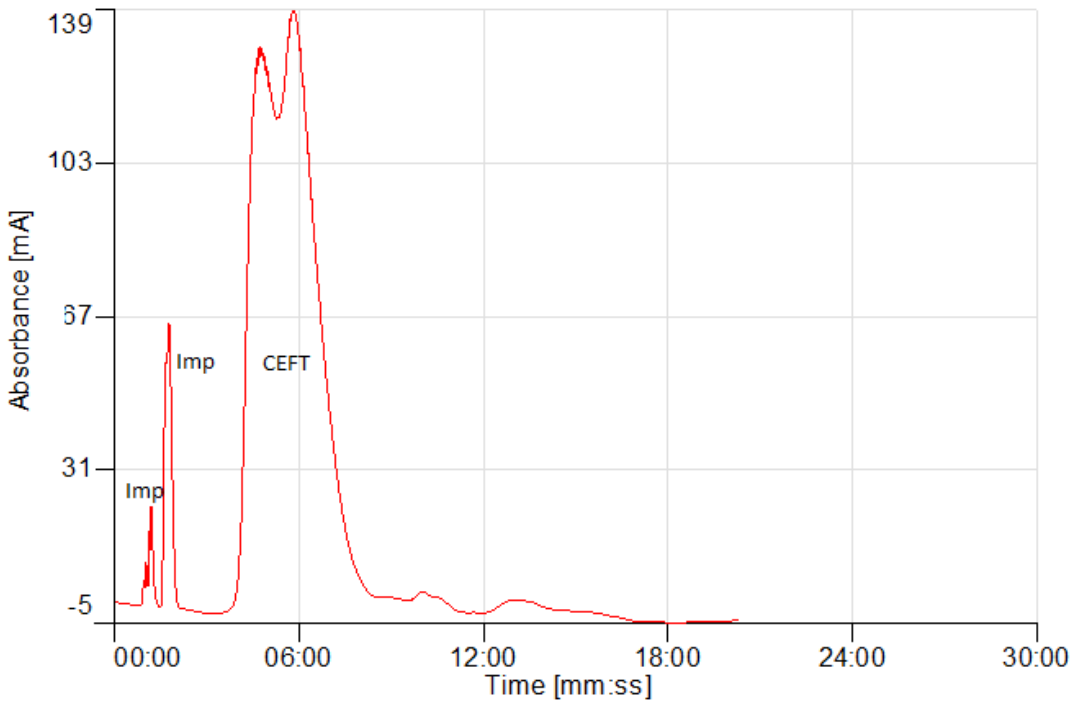


Figure 5: Isocratic elution mode for ceftriaxone determination using KH_2PO_4: ACN (97:3%v/v)

Gradient elution

The separation observed for the isocratic mode was not satisfactory hence a gradient elution mode was attempted. The best gradient elution mode optimized and adopted is presented in Table 1. The resulting chromatogram for this optimized gradient elution mode is presented in Figure 6.

Table 1: Gradient Elution Mode for the separation of ceftriaxone and its impurities

Time (mins)	% A (KH_2PO_4)	% B (MeOH)
0	70	30
5	30	70
10	10	90
15	10	90

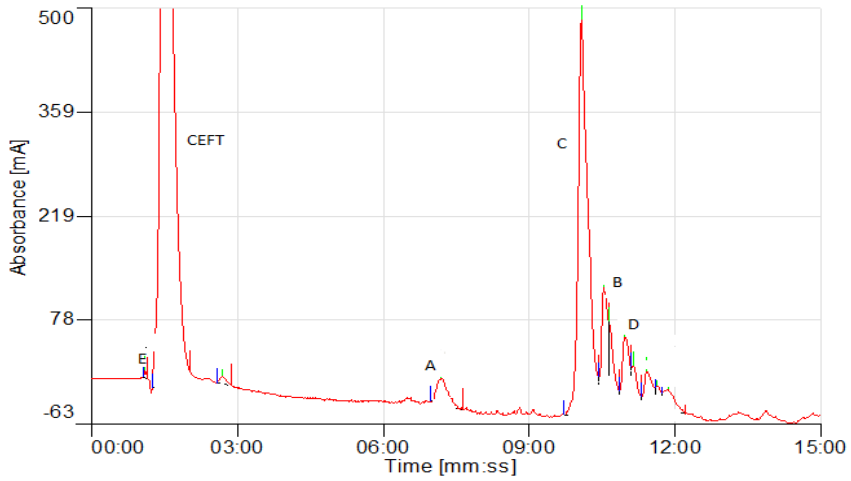


Figure 6: Optimized chromatogram for ceftriaxone and its impurities (E, A, B and D)

Robustness study

Prior to and following the optimization of the mobile phase gradient for the separation of ceftriaxone and its impurities, several small changes in the chromatographic conditions were carried out in order to study their effects on the chromatographic patterns. This procedure is commonly referred to as robustness and it is a major parameter commonly used in assessing the suitability of a method for analysis of a sample.

Effect of diluent for sample preparation

The effect of diluent used in preparing the sample was studied by using various combinations of solvents known to effect complete dissolution of ceftriaxone. The diluents adopted are the mobile phase (KH_2PO_4: MeOH 98:2%v/v) as well as methanol: mobile phase (50:50 % v/v). The appropriate solvent mixture between the two mixtures attempted was found to be the combination of methanol and the mobile phase in a 50:50 %v/v combination and this solvent mixture was adopted for subsequent determinations.

Effect of stock concentration

The effect of the stock concentration of ceftriaxone prepared for the chromatographic analysis was studied by using 1 mg/mL and 0.5 mg/mL stock solutions in the diluent. The effect observed was that the main peak of ceftriaxone gave a large band in the higher concentration while the impurities were not well resolved. This was the observation under the isocratic mode. For the 0.5 mg/mL stock solution, the main peak was still prominent but the impurities were really very low and the chromatographic peaks were not well resolved.

However, on the adoption of the gradient elution modes, the use of 1mg/mL was sufficient to separate both the main ceftriaxone peak and its 5 impurities.

Effect of organic modifier

The organic modifier adopted in the British Pharmacopoeia for the determination of the related substances and assay of ceftriaxone injection is tetraheptylammonium bromide and tetradecylammonium bromide. Apart from the cost of these organic modifiers, the need to use readily available organic modifier and one less toxic informed the use of common solvents such as methanol and acetonitrile.

In the isocratic mode, the effect of organic modifier was studied by the use of methanol in various percentages; 2, 3 and 5% in combination with the phosphate buffer. The organic modifier was thereafter changed to acetonitrile and the various combinations were again attempted.

For the separations with methanol, sharper and well-resolved peaks were observed than when acetonitrile was adopted as the organic modifier. For subsequent attempt under the isocratic and gradient elution modes, methanol was adopted as the organic modifier.

Effect of flow rate

The effect of flow rate was carried out by investigating the rate of flow of mobile phase using 1.0, 1.1 and 1.2 mL/min. Best separation was obtained with the use of 1.0 mL/min in both the isocratic and gradient elution modes. For the higher flow rates, although faster run time was obtained, the resolution of the late eluting peaks containing majority of the impurities was compromised.

Effect of detection wavelength

The chromatographic separation started with the adoption of the general detection wavelength of 254 nm. This is also coupled with the fact that this is the wavelength specified in the British Pharmacopoeia[1] for the assay of ceftriaxone and determination of its related substances. The separation using 254 nm showed that better peak was obtained at 254 nm for the principal peak relative to the impurities.

In order to capture the separation of the impurities properly, the chromatographic separations were further carried out at 220 and 266 nm. These are peaks at which ceftriaxone has significant absorption bands from a previous spectrophotometric analytical study.[30] Best separation was observed for the principal peaks and the impurities at 220 nm and this was thereafter adopted for subsequent work in the gradient elution mode.

Effect of pH of buffer

The effect of the pH of mobile phase comprising of 0.067 M KH_2PO_4 was investigated at various pH levels of 3.5, 5.5 and 7.5. Best separations were obtained at pH 7.5 for both ceftriaxone and its impurities. The chromatograms were characterized by sharp peaks though with co-elution of the late eluting peaks. The results of sharp peaks led to the adoption of gradient elution mode at pH 7.5.

Validation studies

Following the optimization of the separation of ceftriaxone and its impurities, validation studies were carried out involving preparation of calibration curve, accuracy and repeatability assessments.

Preparation of calibration curve

A 2-day calibration plot of ceftriaxone in the diluent was done using the peak area obtained in the optimization step as a guide to the minimum and maximum data point on the calibration range. The results obtained are presented in Table 2.The range adopted and the linear regression equations obtained are presented in Table 3. The best line of fit was obtained at the concentration range of 7.8 – 250 µg/mL.

Table 2: Selection of calibration range for the determination of Ceftriaxone

Sample no.	Concentration (µg/mL)	Day 1 Peak Area	Day 2 Peak Area	Average Peak Area
1	7.8	207	234	220.5
2	15.6	412	412	412
3	31.3	1004.1	773.6	888.85
4	62.5	1804.1	1568	1686.05
5	125.0	3431.8	3765.5	3597.23
6	250.0	7625.9	7050.6	7338.25
7	500.0	14752.6	14689.5	14721.05

Table 3: Linear regression data for the determination of Ceftriaxone

Range (µg/mL)	Day 1			Day 2			Average			
	m	c	r^2	m	C	r^2	M	C	r	r^2
7.8125–500	29.6855	-30.8718	0.9992	29.426	-100.483	0.9992	29.556	-65.898	0.99996	0.9999
7.8125 – 250	30.623	-68.333	0.9970	28.679	-51.942	0.9972	29.4696	-60.2823	0.9998	0.9996
7.8125 – 125	27.409	44.1667	0.9970	30.172	-110.846	0.9914	28.779	-33.044	0.9994	0.9988

m = slope, c = intercept, r = correlation coefficient, r^2 = coefficient of determination

Estimation of LOD and LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the calibration curve using equations 1 and 2 respectively.

$$LOD = \frac{3.3\sigma}{slope} \text{---------(1)}$$

$$LOQ = \frac{10\sigma}{slope} \text{----------(2)}$$

Where σ is the standard deviation of the blank signals, which gave a value of 0.6658. At this value the LOD was calculated as 74.56 and LOQ as 225.9 ng/mL.

Estimation of 95% Confidence intervals

The 95% confidence intervals of slope and intercept were estimated using Microsoft excel® at a probability level of 0.05 and was found to be 29.47±1.96 and -60.28±8.17 respectively.

The calibration and analytical parameters for the assay of ceftriaxone using this new HPLC method are presented in Table 4.

Table 4: Calibration and Analytical parameters for the assay of ceftriaxone

Parameter	Value
Range (µg/mL)	7.8 – 250
Slope	29.47
95% Confidence interval of slope	1.96
Intercept	-60.28
95% Confidence interval of intercept	8.17
Correlation coefficient (r)	0.9998
Coefficient of determination (r^2)	0.9996
LOD (ng/mL)	74.56
LOQ (ng/mL)	225.9

Accuracy and Repeatability

The results of the 2-day assessments of accuracy and repeatability are presented in Table 5 (intra-day assessment) and Table 6 (inter-day assessment).

Table 5: Intra-day accuracy and Repeatability of the new LC method for Ceftriaxone

Assessment	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	Recovery (%)	RSD (%)[b]	Relative error (%)
Intra-day[a](Day 1)	20	19.938	99.94	1.48	0.31
	50	49.126	98.25	1.25	1.75
	200	197.836	98.92	1.05	1.08
Intra-day[a](Day 2)	20	20.146	100.73	1.39	0.73
	50	49.591	99.08	2.59	0.88
	200	199.39	99.70	0.65	0.31

[a]n=4 for each concentration level; [b]RSD = Relative Standard Deviation

Table 6: Inter-day accuracy and repeatability of the new LC method for Ceftriaxone

Assessment	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	Recovery (%)	RSD (%)[b]	Relative error (%)
Inter-day[a]	20	20.02	100.15	1.47	0.10
	50	49.33	98.67	1.95	1.34
	200	198.61	99.31	0.91	0.70

[a]n=8 for each concentration level; [b]RSD = Relative Standard Deviation

Assay of CEFT impurities in dosage forms

The results obtained for the assay of the impurities in the 13 brands of ceftriaxone injection powder are presented in Table 7. As it is the convention, the impurities are calculated as percentages of the active pharmaceutical ingredients. The ranges for most of the impurities as obtained for all the brands are in 2 digits signifying high content of the impurities especially for impurities A, B, C and E in most of the brands.

Table 7: Impurities content of ceftriaxone brands

Ceftriaxone brand	Impurities*				
	Imp. A	Imp. B	Imp. C	Imp. D	Imp. E
1	9.45±0.42	10.53±1.95	8.32±0.91	0.71±0.05	12.54±0.37
2	18.70±0.36	14.53±1.90	13.36±1.36	0.76±0.03	18.70±0.36
3	15.51±1.02	14.93±0.82	15.28±1.20	0.82±0.02	15.92±1.16
4	10.46±0.09	15.74±1.15	13.36±0.69	0.55±0.11	13.76±0.09
5	16.82±1.26	8.13±0.55	6.09±1.15	0.71±01.0	11.58±1.48
6	13.32±0.97	15.59±1.09	7.61±0.62	1.12±0.24	16.80±1.82
7	6.02±0.96	11.80±1.61	10.59±1.88	0.79±0.06	20.67±1.89
8	5.11±0.83	15.12±0.77	6.45±0.78	1.18±0.06	23.29±0.58
9	5.24±0.42	15.82±1.65	9.92±0.52	1.13±0.24	22.47±0.61
10	7.86±0.88	17.86±1.32	15.28±0.73	0.65±0.09	19.32±1.20
11	4.87±1.06	13.79±1.95	11.81±0.55	0.87±0.14	20.49±1.59
12	6.55±0.21	10.95±0.12	5.40±0.23	0.87±0.17	20.26±0.92
13	5.01±1.20	21.95±1.13	14.06±1.33	1.10±0.09	20.68±1.18

**calculated as % of CEFT; n=4*

DISCUSSION

The goals set for the new method development include; use of readily available and environmental friendly reagents as opposed to the reagents utilized by the Pharmacopoeias and some of the previously reported methods for impurities profiling of cephalosporins, separation of CEFT from its 5 main impurities and utilization of mobile phase combinations that will ensure shorter analysis time without compromising resolution. A major part of the goals were accomplished in the research work.

Method Development and Optimization steps

The attempt at developing a new method stems from the desire to provide a relatively simple, cost-effective and safe procedure for the quantification of the various impurities found in the drug ceftriaxone. Ceftriaxone has found great usefulness in the management of several clinically important situations and there are at present a good number of generic brands available in Nigeria. However, only twelve brands in addition to the innovator product were available in Ibadan, the site of this study.

The method development commenced from the utilization of a reversed phase HPLC technique involving the use of a C-18 column and with a mobile phase consisting of a phosphate buffer (KH_2PO_4) and methanol. The presence of several ionizable groups in CEFT and its impurities accounted for the utilization of a buffer and the adoption of methanol also provides a means of ensuring solubility of the compounds as they move through the column. Due to the hydrophilic nature of CEFT Na and some of its impurities, the reversed phase mode of HPLC was adopted as that will ensure that the compounds do not ionize unduly to cause tailing of the peaks which can compromise resolution.

As presented in Figure 1, the adoption of this mobile phase was only able to separate two components, a peak at about 1.29 mins and CEFT. Although the two peaks were clearly resolved, the other minor impurities were not eluted. This led to the utilization of higher concentrations of methanol as the organic modifier. The use of 3% and 5% methanol produced the chromatograms shown in Figs 2 and 3. The adoption of 3% appears optimal as it was able to give a broad band at around 8-16 mins. This shows that there are other minor peaks in the sample which were co-eluted together. Increasing the composition of methanol (Fig. 3) completely eliminated the broad band. Two things may be happening here. One is that the concentration of methanol reduced or repressed the ionization of whatever components may be giving rise to the broad band or that the presence of excess methanol is not required for elution. Appearance of late eluting peaks in RP-HPLC implies the presence of components that are avidly bound to the non-polar column and thus requiring longer time for separation. Since methanol has 50% polarity, its presence may not be able to carry out elution of non-polar compounds.

Further optimization steps adopted involved the use of isocratic and gradient modes of elution. Under the isocratic mode, two organic modifiers utilized gave different separation patterns. For the adoption of

methanol, two closely eluting peaks were obtained within the first 4 mins (Figure 4) and there appears that some minor peaks were present. On changing the organic modifier to acetonitrile, there was no improvement except for elution of well resolved peaks. Acetonitrile, however, appears to favour the elution of CEFT compared to other impurities present in the sample.

The gradient elution was attempted as it gives the opportunity of varying the composition of the mobile phase as the elution progresses. The local concentration of the mobile phase will profoundly affect the distribution of the components and at the same time allow for more solute-solvent interactions that can promote separation of multi-component samples. The ability to subtly change the composition of mobile phase over a narrow time range is a great advantage of gradient elution and it has been severally adopted by many workers for the determination of impurities in pharmaceuticals.

Several gradient combinations of mobile phases A (0.067 M KH_2PO_4, pH 7.5) and B (methanol) were attempted. The target was to get a mobile phase combination that will separate all the six components of interest (CEFT and the five impurities A, B, C, D and E). All the gradients were sufficient to give a separation of all the components. However, best separation of all the components was obtained with the gradient programme [time (min)/%methanol] was set as 0/30, 5/85 to 10/90 to 15/90 that effectively separated all the six components. This separation was accomplished in less than 15 mins and this portrays a great advantage over reported method for ceftriaxone and its impurities. In the report presented by Kumar *et al.* (2010)[28], about 30 mins was required to fully separate all the impurities in ceftriaxone-tazobactam injection formulation.

From the gradient elution programme, it appears increasing concentration of methanol was required for the elution of late peaks. However, gradient elution was far better than isocratic elution in carrying out this optimal separation. As observed earlier, presence of high amounts of methanol appears important for dislodging the bound hydrophobic impurities on the column. The solvating power of methanol seems good enough to allow the hydrophobic components to be carried into solution. The mobile phase combination reported in this work is simple and it is less complicated than previous ones reported by Kumar *et al.* (2010)[28] and Lu *et al.* (2014)[29] and it is also less expensive and the reagents in preparing them are readily available in most analytical laboratories. The objective of developing a new method that can be adopted in any laboratory setting was thus accomplished.

Robustness studies

A wide range of factors that can affect the separation of CEFT and its impurities was identified and the effects of small changes in the factors were studied in order to further optimize the separation. Some of the factors studied include; effect of diluent for sample preparation, effect of stock concentration of sample, effect of organic modifier, effect of flow rate, effect of detection wavelength and effect of pH of mobile phase A (0.067 M KH_2PO_4).

The diluent was optimized to be a 50:50%v/v combination of mobile phase and methanol. This readily dissolved the samples without leaving sediments and in addition it gave chromatographic separation that was devoid of any baseline noise drift.

The effect of the stock concentration provided an opportunity to effectively quantify the presence of impurities while not allowing too much tailing of the CEFT main peak. The advantage of the gradient elution became apparent once again as there was no need to reduce the 1 mg/mL stock solution.

The effect of flow rate was studied both for isocratic and gradient elution modes. In all instances, the resolution of the peaks was better at a flow rate of 1 mL/min as opposed to the use of 1.1 and 1.2 mL/min.

The detection wavelength was carefully studied judging from the previous reports on the determination of ceftriaxone. The three wavelengths adopted (220, 254, and 266 nm) gave differing results. While CEFT main peak was well resolved at the 3 wavelength values, only 220 nm gave optimal resolution of the impurities. Thus, 220 nm was adopted as the wavelength of detection of the impurities profiling of ceftriaxone developed in this method. In the previous studies,[28,29] detection wavelengths of 238 and 270 nm respectively were utilized as the detection wavelengths for CEFT and its impurities.

The effect of pH of the phosphate buffer was studied by varying the pH (3.5, 5.5 and 7.5). The best separation obtained was at pH 7.5 and this pH produced the sign of eluting all the impurities of CEFT under the isocratic mode. Thus, pH 7.5 was adopted for the gradient elution studies. The ionization constants (*pKa*) of CEFT are 3, 3.2 and 4.1. Thus at the pH values of 3.5 and 5.5, CEFT and alongside impurities A, B, C and E with residual cephalosporin moieties will be unionized due to closeness of the pH to the pKa values. The insufficient nature of the buffers at pH 3.5 and 5.5 to separate the impurities substantially may thus be due to lack of ionization giving them opportunity to remain bound to

the hydrophobic stationary phase. However, it appears only a minor proportion of the buffer is required for the ionization to take place as separation proceeded with higher concentrations of methanol as opposed to the buffer. Using the Henderson-Hasselbach equation (equation 3), when there is a wide separation between the pH of a medium and the pKa, a large fraction of the species exist in the ionized form and this makes transfer into a polar phase possible and better than when a large fraction exist in the unionized form. This therefore explains why pH 7.5 must have been effective in carrying out the separation of the six components.

$$pH = pKa + Log \frac{[Ionized]}{[Unionized]} \text{----------(3)}$$

Validation studies

In the current method for the determination of CEFT and its impurities, the validation parameters determined are linearity and range, accuracy and repeatability, LOD, LOQ as well as the confidence intervals of slope and intercept.

The linear range adopted for the determination of CEFT in this work compares favorably with those previously reported by Kumar *et al.* (2010) [28] and Lu *et al.* (2014) [29] and the calibration range offers the advantage of being able to determine minute quantities of the compound. The calculations leading to the selection of the best range for the determination of CEFT is presented in Table 3. The best range is given as one which produces the highest slope, least intercept and highest correlation coefficient. From Table 3, the range 7.8 – 250 µg/mL was selected since it gives a good intercept, slope and correlation coefficient. The range extending to 500 µg/mL was not selected as there is not much advantage to be gained in the sensitivity (slope) for the 2-fold concentration increase from 250 - 500 µg/mL. The limits of detection and quantitation were estimated respectively as 74.56 and 225.9 ng/mL. These values point to the sensitive nature of this new method for the determination of CEFT and its impurities.

The assessments of the intra- and inter-day accuracy and repeatability are presented in Tables 5 and 6. A cursory look at the tables shows that the new method was sufficient enough to carry out an excellent determination of CEFT in the quality control samples. The relative errors obtained for both the intra-day and inter-day accuracy and repeatability were generally less than 2% lending credence to the suitability of the new method for the analysis of CEFT.

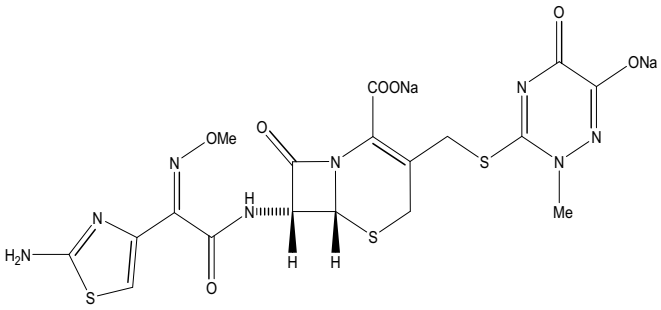
Assay of CEFT impurities

The results obtained for the assay of CEFT in terms of drug content show that in all the brands adequate amount of CEFT are present. However, the quality of a finished pharmaceutical product is not judged by the content of active ingredients alone but also by the presence of closely-related substances and other impurities. This is the main thrust of this research work. The impurity profiling of the CEFT brands were carried out with a view to making judgments about their suitability. Cephalosporins are susceptible to environmental degradation. In particular, in the tropics where Nigeria belongs, extremes of humidity, sunlight and temperature can give rise to the presence of impurities in cephalosporins and other susceptible pharmaceuticals.

Following the successful development of the new method for the impurity profiling of CEFT and in the absence of chemical reference substances for the impurities, the partition coefficients (Log P) of CEFT alongside that of the BP-specified impurities were estimated using ChemDraw® Ultra software v. 8.0.[31] The results for the prediction of Log P for the six compounds are presented in Table 8.

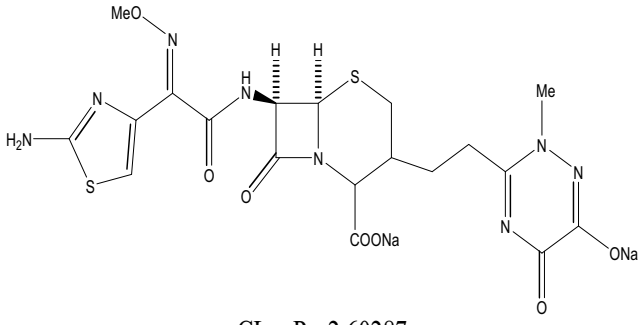
Table 8: ChemDraw® Prediction of Log P for CEFT and impurities

Ceftriaxone



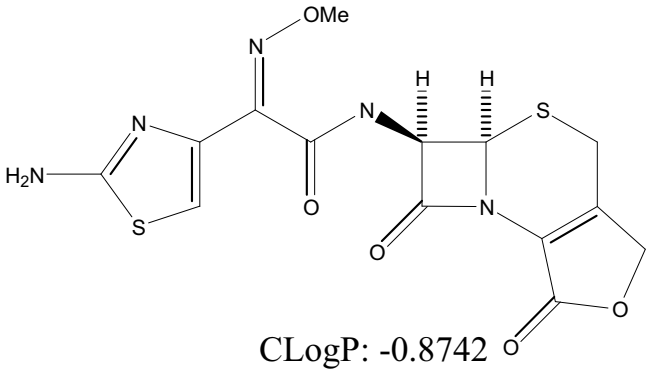
CLogP: -3.09983

Impurity A



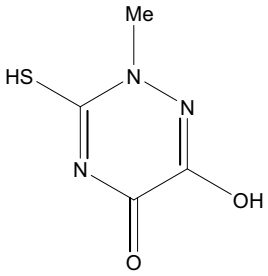
CLogP: -2.60287

Impurity B



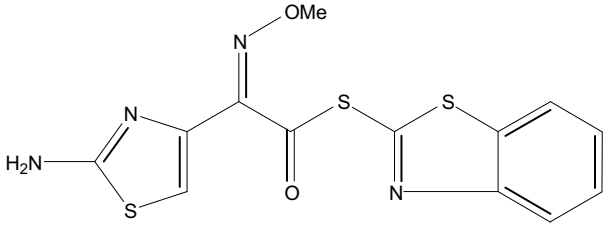
CLogP: -0.8742

Impurity C



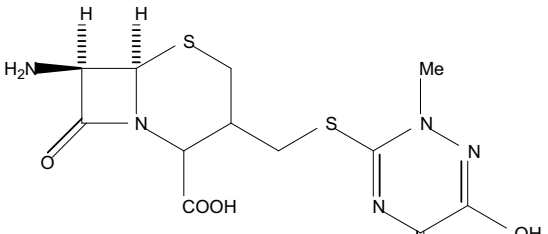
CLogP: -1.14314

Impurity D



CLogP: 2.496

Impurity E



CLogP: -4.88421

The ranking of hydrophilicity as predicted from the Log P values is E>ceftriaxone>A>C>B>D, i.e. based on the estimated Log P values, the elution of the peaks follows the order E, ceftriaxone, A, C, B and D. Thus compounds with high Log P have higher solubilities in the oil interphase relative to the water phase. When negative values are obtained, it implies the compound has very high solubility in the water interphase. In recent times, adoption of LC with mass spectrometric detection has proven useful in identifying impurities whose chemical reference substance is not available. With this ranking it became possible to detect the five impurities in the ceftriaxone preparations. The results obtained are as presented in Table 7.

In estimating the relevance of each impurity, the ICH recommends that the threshold of impurities for a drug taken at greater than 100 mg – 2 g daily dose is 0.2%. From the results obtained in this work, all the impurities were present at levels exceeding this threshold. In addition to the foregoing, majority of the brands

contain more than 2 or 3 other unidentifiable impurities. However, majority of the generic brands contain very low quantities of impurity D as detected by the new chromatographic technique. However, of greater concern is the high level of the *Z*-isomer of ceftriaxone in the preparations. Although there has not been any report of the therapeutic or toxic effect of this isomer in ceftriaxone usage, caution is required so that the level is not exaggerated. Since biological systems are known to discriminate between stereoisomers, it may be of importance in no distant time.

The presence of majority of the impurities appear to be due to unsuitable environmental conditions, the need to prescribe appropriate storage conditions for ceftriaxone cannot be over-emphasized. It thus behooves to recommend that; the labeling instruction to store the drugs away from sunlight and at temperatures not exceeding 25 ^{o}C which appeared not to have been adhered to leading to the exaggerated levels of impurities in the ceftriaxone brands should be stringently followed. Also regular monitoring of generic brands should be done to ascertain their stability and adequate and appropriate protection against heat, humidity and light are recommended in order to control the levels of impurities in the ceftriaxone brands.

A major limitation in this study was that the ceftriaxone brands sampled for the work were all from Ibadan, Southwest Nigeria. Further studies will require that samples from other parts of the Country be included.

CONCLUSION

A new method for the determination of ceftriaxone injection powder was developed.The new method was applied to assay of ceftriaxone in generic brands of ceftriaxone sourced from retail outlets in Ibadan, Nigeria. In addition, the method was suitable enough to quantify the five main impurities in the ceftriaxone formulations and in all the brands the level of impurities found exceeded the threshold set by ICH for the dose of the compound. There is an urgent need to adhere to the recommended storage conditions for ceftriaxone.

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