EVALUATION OF CEFACLOR ORAL SUSPENSIONS STABILITY USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ANTIMICROBIAL DIFFUSION METHODS

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ABSTRACT

The effect of temperature stresses on Cefaclor suspensions under different storage conditions for a duration of 14 days was tested. The degradation of Cefaclor was determined on the 2nd, 7th and 14th day after reconstitution using a sensitive and precise Reversed phase High Performance Liquid Chromatographic (RP-HPLC) method. The RSD values for Forticef, Midocef, Ceclor, Cefabac and Cloracef, indicated a good precision of the RP-HPLC method. The limit of detection (LOD) and the limit of quantification (LOQ) were found 0.008 mg/ml and 0.03mg/ml respectively. The antimicrobial effect of Cefaclor suspension was also tested against pathogenic bacteria using the cylinder diffusion method. The RSD values range of the antimicrobial assay for all the Cefaclor compounds were 1.47-3.7%. The LOD and LOQ were 0.2mg/ml and 1mg/ml respectively. During the normal use of Ceclor, Midocef, and Forticef the loss of activity and the degradation were less than 5% on the 14th day of preservation at 4°C. However, the percentage of degradation for Cefabac and Cloracef on the 14th day reached 5 and 6%, respectively. Statistical multiple comparison between the effect of 4°C and 25°C indicated non significant mean differences ($P \geq 0.05$) for Forticef, Cefabac, Ceclor and Cloraf and significant effect for Midocef ($P \leq 0.05$). Significant effects were observed between (4°C and 37°C) and (25°C and 37°C) for all except Ceclor. Multiple comparisons between days of storage showed non significant mean difference values at 4°C except Cefabac. However significant results between days were found at 25°C and 37°C except for Midocef between (7th and 14th day)**.** It was found that the pediatric suspensions of Cefaclor in the Jordanian market were stable and contained the amount of active ingredient specified by the United States pharmacopoeias specification (USP) and the British Pharmacopoeias specifications (BP).

Keywords: Cephalosporin, Cefaclor, Forticef, Midocef, Ceclor, Cefabac , Cloracef and Jordan.

INTRODUCTION

Cefaclor is 7-[(2-amino-2-phenyl-acetyl) amino] - 3-chloro-8-oxo-5-thia-1-azabicyclo [4.2.0] Oct-2- ene-2-carboxylic acid monohydrate (US Pharmacopoeia and National Formulary 24th Ed. Philadelphia, PA, 2000), a chlorinated modification of a second generation, semi-synthetic and orally administrated cephalosporin antibiotic (Supperenant and Preston, 1985). It is indicated for the treatment of urinary tract infection, otitis media, skin infections and respiratory infections (Oberlin and Hyslop, 1990). *In vitro* studies have shown that this drug is highly active against *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis, Salmonellae, Shigellae, Haemophilus influenzae, Staphylococci* and group A beta-hemolytic *streptococcus* (Xiaoyan *et al*., 2003).

The chemical degradation of drugs could lead to the loss of potency of the product, therefore a knowledge of the chemical nature and the substance being handled is required. Thus, all the possible types and causes of degradation could be determined and suitable measures could be taken to retard these reactions (Deshpande *et al.,* 2004). Drugs could be degraded by various chemical reactions; the most common of which are oxidation, hydrolysis and racemisation. The rate of these reactions could be affected by a variety of factors, such as pH, temperature, carbon dioxide, oxygen, light and humidity. Thus, the stability of a medicine related to the various changes that occurred during preparation and storage, and to the effects of these changes on its fitness for use. Since drugs were degraded by various chemical reactions, their degradation could often be retarded by the judicious selection of containers and closures and by controlling storage conditions (Wade, 1980). Some of the major concerns expressed by healthcare practitioners with regard to drug stability were the effects of the environmental stresses on the drug product's integrity throughout its lifetime. Stability assessment started by the application of a suitable stressor or challenge to the medicine, and the measurement of the effects of such stressor on the physical and chemical properties of the medicine in the dispensed packages at appropriate time intervals. The principal tests used as part of stability

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studies are storage tests of the product, which are carried out under controlled stresses that represent the conditions that most likely to occur during storage (http://www. Online, 2004).The purpose of stability testing is to provide evidence of how the quality of the drug product varied with time under the influence of a variety of environmental factors, such as temperature. In spite of the recommendation of storage conditions, temperature had a pronounced effect on the rate of degradation of the active ingredient which usually doubled for every 10°C rise in temperature (Dawson, 1994).

It is particularly important that, during patient counseling, the patient should be given an advice how and where to store medication. However, while proper storage conditions should be informed to the patient during counseling, it was also recognized that proper controls beyond the supplier could be difficult (Orwa *et al.,* 2004).

The activity of Cefaclor as ß -lactam antibiotic could be detected with microbiological inhibition tests. A linear correlation was reported between the absolute quantity of a given antibiotic and the surface of an inhibition zone (Boisn *et al.,* 1995).The stability of an antibiotic solution could be evaluated be measuring the inhibition zones and drug residue analysis by HPLC at different times of preservation.

In Jordan, as one of semi-arid country, there are geographical areas with different temperatures that exceed 40^{o}C in the Jordan Valley, 42^{o}C in the desert area, 37^{o}C in Aqaba gulf and 25^{o}C in the mountings area. These temperatures were considered stress conditions for storing suspension antibiotics. Therefore, the aim of this study was to determine whether Cefaclor oral suspension preparations in the Jordanian market will still be stable after reconstitution for 14 days at 4, 25 and 37^{o}C. It also aimed to determine their stability after dispensing by subjecting all the suspensions to simulated patient storage conditions for a duration of 14 days.

MATERIALS AND METHODS

Patient storage conditions

The effect of different temperature stresses on reconstituted Cefaclor suspensions, under patient storage conditions was investigated for the duration of 14 days. A mini survey of 200 patients was conducted in pediatric clinic and pediatric ward of Al-Karak governmental hospital which serves the population in Karak city, the rural areas around and Jordan Valley.

Cefaclor oral suspensions

Cefaclor suspensions manufactured by five different companies were bought from the local Jordanian market. Four of them were local (Forticef/Ram Company, Cefabac/The Arab Pharmaceutical Manufacturing Co. Ltd, Cloracef/Dar Al-Dawa Company, Midocef/ Midpharma Company) and one was imported (Ceclor/Eli Lilly, Italy). Cefaclor concentration in all these suspensions was 25 mg/ml. The granules were reconstituted according to manufacturing instructions either with HPLC water (Tedia Company, USA) or with sterilized distilled water (P.S.I Jeddah, Kingdom of Saudi Arabia) according to use (Hammad *et al.,* 2002).

Cefaclor standards

In the course of HPLC experiments analysis, four replicates were used for both standard and tested antibiotics preparations. Cefaclor USP standard solution for HPLC experiment was prepared by dissolving 0.015 gm of Cefaclor USP (Eli Lilly, USA) powder in HPLC water to obtain 0.03 mg/ml. Standard Cefaclor caliberation curve was done to ensure the method validation. For the linearity study, four replicates of each of the following different concentrations were assayed 0.0075mg/ml, 0.015mg/ml, 0.03mg/ml, 0.045 mg/ml and 0.06mg/ml. Standard curve was obtained by plotting the standard concentration (mg/ml) versus the average peak area. Standard for the antimicrobial assay was prepared by dissolving 25 mg of Cefaclor USP standard in 25 ml sterilized distilled water to obtain (1 µg/µl) and consequently a 10 µl volume was used to obtain 10 µg of Cefaclor USP standard. To ensure a valid and measurable zone of inhibition which will be used for the comparison during 14 days of storage and to ensure that there will be a decrease in the inhibition zones diameter by decreasing the antibiotic concentration, USP standard calibration curve was done for each strain of bacteria by using four replicates of the following concentrations 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/10 µl. Standard calibration curve was obtained by plotting the standard concentration (µg/10 µl) versus the average inhibition zone.

SAMPLE PREPARATION

HPLC Samples

Aliquots of the prepared suspensions were suitably diluted with HPLC water (0.6ml of the suspension containing 15mg Cefaclor diluted 500 times v/v with HPLC water) to obtain a concentration 0.03mg/ml. Four replicate samples for each company were then filtered through sterile syringe filter 0.2µm, then 20 µl samples was injected to the chromatographic system.

Antimicrobial samples

Aliquots of the prepared suspensions were suitably diluted with sterilized distilled water to obtain a concentration 1 µg/µl and 10 µl of this solution was taken for the assay.

Apparatus and HPLC conditions

All the chromatographic separations were performed at room temperature using liquid chromatographic pump

model LC-10 AT (Shimadzu, Japan) which was used to deliver the mobile phase to the analytical column, eusrospher 100-5 C_{18}, Length × ID was 250 × 4.6 mm with pre-column (Knauer-ASI-Advanced Scientific Instrument, Germany). The mobile phase was prepared daily and consisted of monobasic sodium phosphate (Sigma-Aldrich) dissolved in HPLC water to obtain 12.5 mmol/L and mixed with methanol (Tedia Company, USA) in a ratio 85:15 respectively (Quanyun and Lawrence, 1999). The pH was adjusted to 2.6 with concentrated phosphoric acid. The solution was filtered through 0.45 µm membrane filter then was sonicated for degassing prior to use (United States Pharmacopoeia and National Formulary 24th Ed. Philadelphia, PA, 2000). A 20 µl of sample was injected into a six way injection port (Rheodyne, USA). Detection was achieved by UV-visible detector SPD-10 AVP (Shimadzu, Japan) at wavelength 265 nm and flow rate 1.5 ml/min. Integrator C-RAG chromatopac (Shimadzu, Japan) was used for determination of the eluted peaks. The integrator set at attenuation 3 and speed 5. The retention times for all chromatograms were around 22 minutes (USP and National Formulary 24th Ed. Philadelphia, PA, 2000). Quantitative analysis of Cefaclor in samples was based on chromatograms peak area relative to standard chromatograms peak area.

Antimicrobial Cylinder –plate test conditions

The growth method is performed as follows: Each over night bacterial strain grown on nutrient agar plates, was inoculated into 5 ml of nutrient broth medium and incubated for 2-6 hr at 37°C until the turbidity of the culture reached 0.5 McFarland standard (0.1 OD at 600nm). This gave a suspension containing approximately 1 x 10^8 CFU/ml (Jorgensen *et al.,* 1999). Then 100 µl of the suspension was taken and spread evenly over the entire surface of the Müeller-Hinton agar plate (25 ml Müeller-Hinton agar in 100 mm standard plate) (Macfaddin, 1985).

The media was inoculated with, either *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, or *Klebsiella. Pneumoniae* ATCC 10031. Any excess of surface moisture was allowed to be absorbed before applying the drug. The plates were then incubated within 15 min at 37°C for 24 hours (Jorgensen *et al.,* 1999).

For the assay we used stainless steel cylinders, 8 mm diameter outside and 6 mm diameter inside, carefully cleaned and autoclaved after each use (USP and National Formulary24th Ed. Philadelphia, PA, 2000). Diameters of inhibition zones were measured at the opening day, 2nd day, 7th and 14th day and then the decline in Cefaclor activity substances was monitored. This test was standardized every day by using 10 µl of Cefaclor USP solution containing 1 µg/µl used as a control for each bacterial strain.

STATISTICAL ANALYSIS

The collected data were analyzed according to one way ANOVA followed by post hoc comparison, using SPSS program**.** Values were considered significant when ($P < 0.05$).

RESULTS

A mini survey of 200 patients in Al-Karak Hospital, was conducted. The results of the mini survey indicated that 42% of patients stored their antibiotic suspension in the refrigerator, while the remaining 58% stored their suspensions in other common areas of the house with a temperature range from 25-45°C in Aqaba Gulf, the Jordan Valley and other areas (fig. 1).

A comparative analysis of the average label percentage of Cefaclor between companies on the opening day using HPLC residue analysis was done to estimate the concentration of each product of Cefaclor. The results demonstrated that the average label percentage (n = 4) of

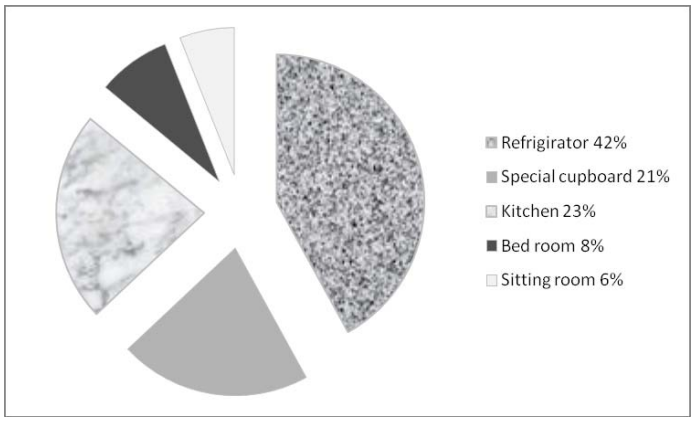
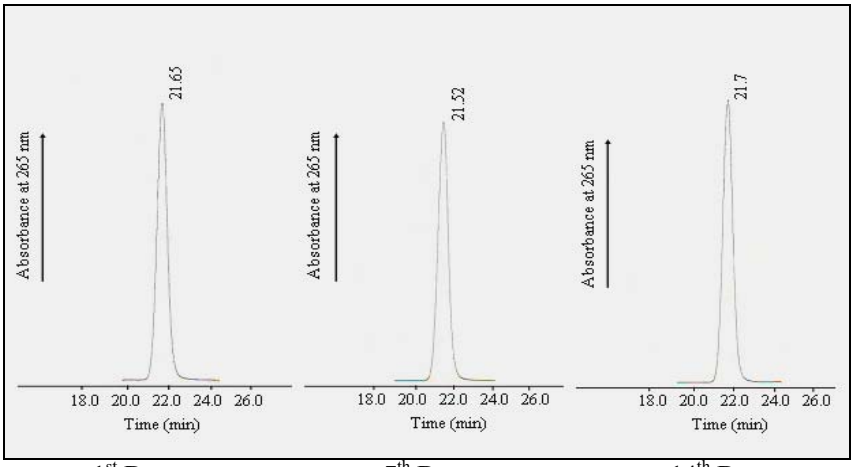


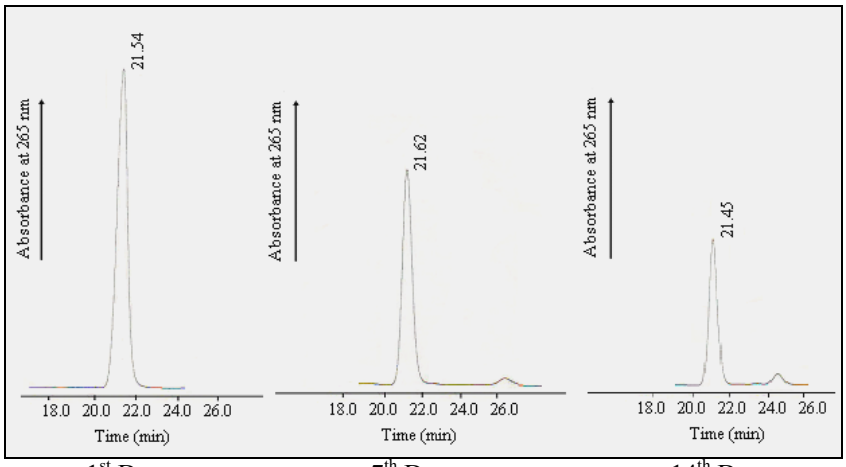
Fig. 1: Cefaclor Patient storage conditions. The storage areas of antibiotic suspensions among 200 patients included in the survey. 42% store the antibiotic in the refrigerator, 58% store it in other areas of the house at the ambient temperature.

Forticef, Ceclor, Cloracef, Cefabac and Midocef were 111.62, 113.22, 111.76, 100 and 100% respectively. These results showed that all the products were within the United States Pharmacopoeias specification (USP) 90% - 120% (USP, 2004) and the British Pharmacopoeias specifications (BP) 80%- 120% (British Pharmacopoeia,



1st Day 7th Day 14th Day

Fig. 2a: Representative Cefaclor standard USP chromatograms. The freshly prepared Cefaclor standard was estimated on the 1st, 7th and 14th day.



1st Day 7th Day 14th Day

Fig. 2b: Representative Cloracef chromatograms. The degradation of Cloracef was estimated on the 1st, 7th and 14th day at 37°C storage.

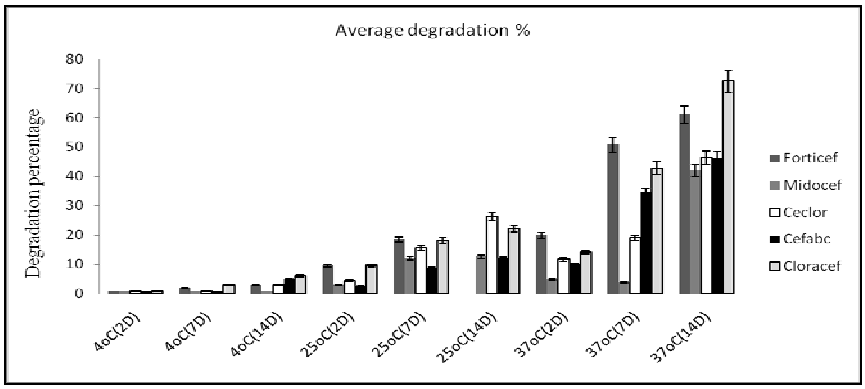


Fig. 3: Cefaclor average degradation percentage. A comparison analysis of the average degradation percentage of Forticef, Midocef, Ceclor, Cefabac and Cloracef using Rp-HPLC method at 4°C, 25°C and 37°C and on the 2nd ,7th and 14th day of storage. The limit of detection (LOD) was 0.008g/ml and the limit of quantification (LOQ) was 0.03mg/ml. the values are the average of replicates (n = 4).

Table 1: A comparative analysis of the average label percentage of Cloracef, Cefabac, Ceclor, Forticef and Midocef suspensions using HPLC at 4, 25 and 37°C temperatures on 2nd, 7th and 14th day of storage. The standard deviation (SD) and the relative standard deviation (RSD%) were calculated [n=4].

Company Name	Temperature storage 4°C						Temperature storage 25°C						Temperature storage 37 °C					
	2nd Day		7th Day		14th Day		2nd Day		7th Day		14th Day		2nd Day		7th Day		14th Day	
	Average Label% + (SD)	RSD%	Average Label% + (SD)	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%	Average Label% + (SD	RSD%
Forticef	110.62± (0.48)	0.43	109.62± (1.60)	1.46	108.60± (0.95)	0.87	101.94± (0.02)	0.02	93.14+± (0.288)	0.3	84.38± (1.10)	1.3	91.70± (0.24)	0.26	60.86± (0.08)	0.13	50.74± (0.32)	0.6
Midocef	99.19± (1.52)	1.5	99.20± (1.35)	1.3	99.20± (0.82)	0.82	97.24± (0.28)	0.28	88.00± (0.76)	0.86	87.60± (0.12)	0.14	95.28± (2.64)	2.77	66.36 ± (0.24)	0.36	58.20± (0.36)	0.61
Ceclor	112.22± (1.85)	1.6	112.22± (1.68)	14	110.22± (2.19)	1.9	108.74± (0.96)	0.88	97.66± (0.04)	0.4	86.94± (0.48)	0.55	101.46± (0.24)	0.24	94.26± (0.16)	0.16	66.94 ± (0.12)	0.18
Cefabac	99.26± (0.80)	0.8	99.26± (1.62)	1.6	95.26± (1.60)	1.67	97.59± (0.28)	0.28	91.54± (0.64)	0.69	88.34± (0.72)	0.82	90.38± (0.04)	0.04	65.94± (0.08)	0.12	54.18± (0.12)	0.22
Cloracef	110.70± (1.54)	1.39	108.76±2.12)	1.9	105.72± (2.33)	2.2	102.08± (0.48)	0.47	93.64± (0.86)	0.92	89.64± (0.44)	0.49	97.64± (0.12)	0.12	69.00± (0.16)	0.3	39.40± (0.28)	0.7

*Deg% (Degradation percentage): Average label% on the opening day – Average label% in the named day

UK, 2001). For HPLC assay, the linearity was evaluated and established by four replicate injections of each of the following USP Cefaclor standard concentrations (0.0075, 0.015, 0.03, 0.045 and 0.06 mg/ml). The average peak area for the four replicate injections (n=4) generated linear results over the investigated concentration range for Cefaclor from 0.0075 to 0.06 mg/ml (y=6E+06x+2895; R^2 = 0.9994), where y is average peak area, x is the concentration of USP Cefaclor standard and R is the correlation coefficient.

Limit of detection (LOD) was done by analyzing serial dilutions of USP Cefaclor standards, corresponding to the concentration at the lower end of the calibration curve (0.008 mg/ml) and the limit of quantification (LOQ) was also determined as the lowest reproducible measurement of peak area (0.03mg/ml). The statistical results demonstrated low relative standard deviation (RSD) calculated relative to the average of four replicates of each assay. Precision of HLPC method was demonstrated by the low RSD (%). The calculated RSD% at all the

Table 2: Multiple comparison of concentration of Cefaclor compounds between days of storage (2nd, 7th and 14th) at the different storage temperature (4, 25 and 37°C). The mean difference is significant at ≤ 0.05 level.

Company	Tm	(I) Day	(I) Mean	(J) Day	(J) Mean	(I-J) Mean Difference	**Sig
Forticef	4°C	1	28.11	7	27.88	0.43	NS
				14	27.76	0.35	NS
		7	27.88	14	27.79	0.34	NS
	25°C	1	27.85	7	23.75	4.1	S
				14	21.62	6.23	S
		7	23.75	14	21.62	2.13	S
	37°C	1	28.75	7	16.16	12.59	S
				14	13.63	15.12	S
		7	16.16	14	13.62	2.53	S
Cefabac	4°C	1	25.21	7	25.98	0.77	NS
				14	23.82	1.39	NS
		7	25.98	14	23.82	2.16	S
	25°C	1	25.36	7	23.18	2.18	S
				14	22.38	2.98	S
		7	23.18	14	22.38	0.8	S
	37 °C	1	25.50	7	16.92	8.58	S
				14	13.98	11.52	S
		7	16.92	14	13.98	2.94	S
Ceclor	4°C	1	28.02	7	28.88	0.86.	NS
				14	27.46	0.56	NS
		7	28.88	14	27.46	1.42	NS
	25°C	1	2.45	7	22.52	3.93	S
				14	22.38	4.07	S
		7	22.52	14	22.38	0.14	S
	37°C	1	26.32	7	21.58	4.74	S
				14	14.75	14.75	S
		7	21.58	14	14.75	6.83	S
Cloracef	4°C	1	28.08	7	28.34	0.26	NS
				14	26.33	1.75	NS
		7	28.34	14	26.33	2.01	NS
	25°C	1	28.52	7	23.99	4.53	S
				14	22.99	5.53	S
		7	23.99	14	22.99	1.00	S
	37°C	1	27.38	7	16.69	10.69	S
				14	9.29	18.09	S
		7	16.69	14	9.29	7.40	S
Midocef	4°C	1	24.78	7	25.81	1.03	NS
				14	25.73	0.95	NS
		7	25.81	14	25.73	0.08	NS
	25°C	1	23.7	7	20.65	3.05	S
				14	20.55	3.15	S
		7	20.65	14	20.55	0.10	NS
	37°C	1	24.04	7	15.58	8.46	S
				14	13.54	10.5	S
		7	15.58	14	13.54	2.04	S

** Significance: (NS) non significant, (S) significant. The mean difference is significant ≤ 0.05 level.

storage temperatures (4°C, 25°C and 37°C) and on all day of analysis, were shown in (table 1).

The percentage of degradation for each tested sample was calculated and compared to 5% limit of degradation (Lemke, 1992). Any value above 5% was considered

Table 3: Multiple comparison of concentration of Cefaclor compounds of the same company at the different storage temperature(4, 25 and 37°C) . The mean difference is significant at ≤ 0.05 level.

Company	(I)Tm	(I)Mean	(J)Tm	(J) Mean	(I-J) Mean difference	**Sig
Forticef	4°C	27.90	25 °C	24.85	3.05	NS
			37°C	19.96	7.94	S
	25°C	24.85	37°C	19.96	4.89	S
Cefabac	4°C	25.07	25 °C	23.71	1.36	NS
			37°C	18.87	6.02	S
	25°C	23.71	37°C	18.87	4.84	S
Ceclor	4°C	26.31	25 °C	21.99	4.33	S
			37°C	19.07	7.24	S
	25°C	21.99	37°C	19.07	2.92	S
Cloracef	4°C	27.95	25 °C	25.53	2.42	NS
			37°C	18.15	9.80	S
	25°C	25.53	37°C	18.15	7.38	S
Midocef	4°C	25.05	25 °C	21.18	3.87	S
			37°C	17.33	7.72	S
	25°C	21.18	37°C	17.33	3.85	S

**Significance: (NS) non significant, (S) significant. The mean difference is significant ≤ 0.05 level.

Table 4: Effect of different Cefaclor commercial preparations on the growth of *E. coli, S. aureus* and *K. pneumoniae* estimated on the opening day (1st day) before storing them at different temperatures**.** The standard deviation (SD) and the relative standard deviation (RSD%) were calculated [n=4].

Bacterial strain	Average of the inhibition zones diameter ± SD (mm) n = 4									
	Forticef	Forticef RSD%	Midocef	Midocef RSD%	Ceclor	Ceclor RSD%	Cefabac	Cefabac RSD%	Cloracef	Cloracef RSD%
E. coli	24.50 ±(0.54)	2.2	21.66 ±(0.81)	3.7	24.50 ±(0.54)	2.2	22.16 ±(0.75)	3.3	24.66 ±(0.51)	2.06
S. aureus	34.50 ±(0.83)	2.4	30.16 ±(0.98)	3.2	34.66 ±(0.51)	1.47	30.00 ±(0.63)	2.1	34.83 ± (0.75)	2.15
*K.pneumonia*e	30.66 ±(0.81)	2.6	28.00 ±(0.89)	3.1	31.66 ±(0.51)	1.6	29.00 ±(0.98)	3.3	31.16 ±(1.16)	3.7

significant degradation and the sample was thus considered unstable (Naidong *et al.*,2003). The freshly prepared Cefaclor standard concentrations were estimated on the 1st, 7th and 14th day using HPLC method (fig. 2a). A representative chromatogram for the degradation of Cloracef is shown in (fig. 2b).

During the normal use and storage at (4°C), The statistical analysis of the HPLC results indicated no significant degradation occurred except for Cefabac and Cloracef degradation which reached on the 14th day 5 and 6% respectively (P<0.05) (table 1). A comparison analysis of the cefaclor average degradation percentage using Rp-HPLC is shown (fig. 3). Multiple comparison of concentration of cefaclor compounds between days of storage at the different temperature is shown (table 2). Similar multiple comparison between the different temperature is also shown (table 3).

For the microbial assay, the linearity was also evaluated by four replicates of ten USP Cefaclor standard concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20µg/10 µl). The average inhibition zone for the four replicates used against each bacterial strain generated linear results over the investigated concentration range for Cefaclor from 2-20µg/10 µl: For Cefaclor against *S. aureus* (y= 2.878x+1.133; R^2 = 0.996), for *E. coli* (y =2.221x-0.933; R^2 = 0.996) and for *K. pneumonia* (y = 2.784x+0066; R^2 = 0.996). The limit of detection (LOD) of inhibition zone was done by analyzing serial dilution of USP Cefaclor standards, corresponding to the concentration at the lower end of the calibration curve (0.2 mg/ml) and the limit of

Table 5: Effect of different commercial Cefaclor preparations on *E. coli, S. aureus and K. pneumonia*e growth at different storage temperature (4°C, 25°C and 37°C). The standard deviation (SD) was shown on the table and the relative standard deviation (RSD%) was given in the result section [n=4].

Tm	Drug	Average diameter of inhibition zones ± SD (mm) n=4								
		E. coli			*S. aureus*			*k. pneumoniae*		
		2nd day	7th day	14th day	2nd day	7th day	14th day	2nd day	7th day	14th day
4°C	Foricef	23.0± (0.89)	24.2± (0.78)	22.2± (0.75)	33.7±(0. 81)	34.3± (0.1.21)	32.5± (0.83)	30.16± 0.75)	29.50± (1.22)	28.66± (0.81)
	Midocef	22.0± (1.1)	21.7± (0.51)	21.7± (0.81)	30.5±(1. 64)	30.2± (0.75)	30.8± (0.75)	27.50± (0.83)	27.16± (0.98)	27.83± (0.98)
	Ceclor	24.2± (0.8)	24.6± (0.51)	23.0± (0.89)	35.7±(0. 51)	34.2± (0.98)	34.0(± 0.89)	30.66± (0.51)	31.66± (0.51)	31.50± (0.83)
	Cefabac	22.5± (0.54)	23.3± (0.51)	22.0± (0.63)	29.8± (0.40)	29.5.0± (0.54)	28.7± (0.51)	28.66± (1.03)	29.50± (0.54)	27.50± (0.83)
	Cloracef	24.5± (0.89)	24.5± (0.83)	22.83± (0.98)	34.8± (0.98)	28.7± (0.51)	32.5± (0.54)	31.50± (0.54)	31.00± (0.63)	30.83± (0.98)
25°C	Foricef	23.7± (0.70)	21.6 ± (0.70)	20.33 ± (0.57)	34.00± (0.00)	31.33± (0.57)	29.33± (0.57)	30.33± (0.57)	28.66± (0.57)	26.66± (0.57)
	Midocef	21.0± (0.00)	20.3± (0.57)	19.33± (0.57)	30.00± (0.00)	28.33± (0.57)	27.33± (0.57)	28.33± (0.57)	26.66± (0.57)	25.66± (0.57)
	Ceclor	24.3± (0.57)	22.3± (0.57)	21.66± (0.57)	32.66 ± (0.57)	31.66± (0.57)	29.66± (0.57)	31.66± (0.57)	30.33± (0.57)	27.00± (1.00)
	Cefabac	21.3± (0.57)	19.3± (0.70)	18.33± (0.70)	30.66 ± (0.57)	28.66± (0.57)	26.66± (0.57)	28.00± (0.00)	27.66± (0.57)	25.00± (0.00)
	Cloracef	22.3± (0.57)	21.3± (0.57)	19.33± (0.57)	32.50 ± (0.57)	29.66± (0.57)	28.33± (0.57)	29.66± (0.57)	28.66± (0.57)	26.66± (0.57)
37°C	Foricef	22.3± (0.57)	19.7± (0.57)	15.66± (0.57)	30.33± (0.57)	28.33± (0.57)	22.66± (0.57)	29.66± (0.57)	26.33± (0.57)	21.33± (0.57)
	Midocef	19.3± (0.57)	16.7± (0.57)	14.33± (0.57)	28.33± (0.57)	23.00± (0.00)	20.33± (0.57)	26.66± (0.57)	21.66± (0.57)	19.66± (0.57)
	Ceclor	22.7± (0.57)	20.3± (0.57)	17.33± (0.57)	31.66± (0.57)	27.33± (0.57)	21.00± (0.00)	29.33± (0.57)	25.66± (0.57)	23.33 ± (0.57)
	Cefabac	19.7± (0.57)	16.7± (0.57)	14.66± (0.57)	28.33± (0.57)	23.33± (0.57)	19.66± (0.57)	25.66± (0.57)	21.66± (0.57)	18.33± (0.57)
	Cloracef	21.3± (0.57)	16.7± (0.57)	13.33± (0.57)	29.66± (0.57)	25.33± (0.57)	20.00± (1.00)	27.33± (0.57)	22.66± (0.57)	19.66± (0.57)

quantification (LOQ) was determined as the lowest reproducible measurement of the inhibition zone area (1mg/ml) used against each bacterial strain generated linear results over the investigated concentration range for Cefaclor from 2-20µg/10 µl: For Cefaclor against *S. aureus* (y= 2.878x+1.133; R^2 = 0.996), for *E. coli* (y =2.221x-0.933; R^2 = 0.996) and for *K. pneumonia* (y = 2.784x+0066; R^2 = 0.996). The limit of detection (LOD) of inhibition zone was done by analyzing serial dilution of USP Cefaclor standards, corresponding to the concentration at the lower end of the calibration curve (0.2 mg/ml) and the limit of quantification (LOQ) was determined as the lowest reproducible measurement of the inhibition zone area (1mg/ml).

According to the antimicrobial assay results, the decrease in the zones diameter was not going dramatically with the degradation results which were obtained from the HPLC results. The Cloracef results showed 70% degradation on the 14th day at 37°C (table 1). However, the inhibition zone for *K. pneumoniae* decreased from 31.16 on the opening day (table 4) to 19.66 on the 14th day at 37°C reflecting 38% decrease (tables 4 and 5). The antimicrobial test based on the diameter of the inhibition zones showed that there was no significant change except at the 14th day of real use and storage, the inhibition zones were decreased to a lesser extent (table 5).

DISCUSSION

Cefaclor is widely used to treat an array of bacterial infections, for both Gram negative and Gram positive bacteria. The aim of antibiotic treatment is to maximize antibacterial activity to prevent any recurrence of infection and the creation of resistance pathogens (Lemke, 1992).

The basic criterion for the clinical efficacy of ß-lactam antibiotics, is the length of time in which serum concentration exceeds the minimum inhibitory concentration (MIC). The serum concentration of ß-lactam antibiotics must be taken in consideration in order

to get the antimicrobial and therapeutic efficacy. The dosing schedules for ß-lactam antibiotics should maintain serum concentrations above the MIC for the bacterial pathogen for at least 50% of the dosing interval to achieve therapeutic efficacy and prevent the development of resistance (Essack, 2001). To achieve this, a certain defined quantity of a chosen antibiotic is given over a period of time to enable the attainment of levels higher than the MIC.

Therefore, the use of ß-lactam antibiotics that have experienced ring breakage and hydrolysable side groups due to fluctuations in the external environment can result in reduced antibacterial activity. The same drug given over a standard period may thus attain only levels lower than the MIC. This concentration may not be high enough to completely eradicate the pathogenic bacteria; leading to an increase in the number of recurrent infections and the development of antibiotic resistance (Auckenthaler, 2002).

The statistical results of both HPLC and antimicrobial assays demonstrated low relative standard deviation (RSD) calculated relative to the average of four replicates of each assay. Precision of HLPC method was demonstrated by the low RSD (%). The calculated RSD% at all the storage temperatures (4^{o}C, 25^{o}C and 37^{o}C) and on all day of analysis, were shown in (table 1). The results of the effects of the different Cefaclor compounds on the inhibition zone of the three bacterial strains (*E. coli, S. aureus* and *K. pneumoniae*) at the opening day showed also low RSD% (table 4). At 4^{0}C, The RSD% ranges at the different storage temperatures 4^{o}C, 25^{o}C and 37^{o}C for all the Cefaclor compounds were found as shown in the results 0-4.7, 0-3.8 and 0-4.2 respectively. The statistical data indicated repeatability of the results. The antimicrobial results were in accordance and supported the HPLC results. The antimicrobial assay results of 10 µg/10 µl indicated that the Cefaclor which had a high label % between (111-113) such as Forticef, Ceclor and Cloracef gave large zones diameter of inhibition and the drugs which had a label% around 100% like Midocef and Cefabac gave inhibition zones similar to the standard inhibition zones (table 4).

The percentage of degradation for each tested sample was calculated and compared to 5% limit of degradation (Lemke, 1992). Any value above 5% was considered significant degradation and the sample was thus considered unstable (Naidong *et al.,*2003). The freshly prepared Cefaclor standard concentrations were estimated on the 1st, 7th and 14th day using HPLC method (fig. 2a). A representative chromatogram for the degradation of Cloracef is shown in (fig. 2b). During the normal use and storage at (4^{o}C), The statistical analysis of the HPLC results indicated no significant degradation occurred except for Cefabac and Cloracef degradation which reached on the 14th day 5 and 6% respectively (P<0.05) (table 1). The antimicrobial test based on the diameter of the inhibition zones showed that there was no significant change except at the 14th day of real use and storage, the inhibition zones were decreased to a lesser extent (table 5).

For Cefaclor suspensions stored at room temperature (25^{o}C) a significant degradation (9.68%) occurred from the 2nd day for Forticef and Cloracef and (15.56%, 8.72% and 12.2%) from the 7th day for Ceclor, Cefabac and Midocef respectively (P<0.05). Multiple comparison between the concentration of Cefaclor compounds of the same company at the different storage temperature using statistical (SPSS) analysis indicated non significant results between 4^{o}C and 25^{o}C. However, Midocef showed significant mean difference between 4^{o}C and 25^{o}C. Significant mean differences were obtained for all Cefaclor compound between (4^{o}C and 37^{o}C) and (25^{o}C and 37^{o}C) except Ceclor showed no significant difference between (25^{o}C and 37^{o}C) (table 3). The statistical data of the comparison of the concentration between days at the different storage temperatures showed non significant differences at 4^{o}C between all days of storage and significant results at 25^{o}C and 37^{o}C (P≤ 0.05) (table 2). These results demonstrated that higher temperatures affect the stability of Cefaclor compounds. The higher percentages of degradation occurred between the 7th and 14th day at 25^{o}C and 37^{o}C (fig. 3). The degradation percentage do not differ significantly from one company to another (P>0.05) except for Midocef and Cefabac which gave lower degradation at room temperature as compared with other products (12%) (fig. 3).

At temperature 37^{o}C the degradation started significantly on the 2nd day and reached 40-70% degradation on the 14th day (P<0.05). The degradation of Midocef, Cefabac and Ceclor products ranged from 42-46%, as compared to 60 and 70% degradation occurred in Forticef and Cloracef, respectively (fig. 3). In the HPLC results a small peak was noticed in addition to the normal peak, of Cloracef which represented another degradable products estimated in the HPLC chromatogram (fig. 2b).

In parallel the zone of inhibition in the antimicrobial assay gave clear supportive evidence for these results, which mean a significant indicator to an increase in antibiotic bacterial resistance for these drugs and treatment failure if they are inappropriately stored (tables 4 and 5). For most antibiotics, some of the variation could not be explained by loss of activity due to a longer time of storage. Keto-enol tautomerisation, might affected the variation observed with these antibiotics, which depends on pH and temperature (Naidoo *et al.,* 2006; Cherlet *et al.,* 2006; Okerman *et al.,* 2007). The variation of results was low with fresh solutions, suggesting that the method is suited for stability testing.

The decrease in the zone of antimicrobial inhibition was not in accordance with the results of degradation obtained with the HPLC. The Cloracef showed 70% degradation on the 14th day at 37°C (table1). However, the the hnhibition zone for pneumonia decreased from 31.16 0n the opening day (table 4) to 19.66 on the 14th day at 37°C reflecting 38% decrease (tables 4 and 5). The reason for this might be due to the effect of the degradable products of Cefaclor on the bacteria. The understanding of the degradation of ß-lactams as well as the nature of their breakdown products has provided trust for developing newer ß-lactams which may posses an increased antibacterial activity towards the future resistant strains. It is well known that ß-lactam antibiotics are susceptible to different forms of breakdown in aqueous solutions (Boisn *et al.,* 1995).

In addition the inappropriate use of antibiotic suspension demonstrated by the mini survey pointed to a decrease in antimicrobial activity to these drugs if they were inappropriately stored especially in these areas.

Furthermore, the reconstitution and dispensing of a 14-day supply of Cefaclor suspension should be discouraged due to the instability of the drug on the 14th day, even when it was stored between 2 and 8°C.

It was interesting to note that the higher label % produced by the manufacturers may be related to the raw material which has a high degradation rate, and this will be considered an advantage, since if the product's degradation occurs during the storage as dry powder, the product active ingredients may still within the pharmacopoeias specifications.

In conclusion preservation of oral suspensions of Cefaclor at 4°C for 14 days after reconstitution is required to prevent rapid degradation of the antibiotic. The dispensing of a 14-day supply of Cefaclor suspension, should be discouraged, even if the drug is stored in this temperature range. However, It is not recommended to store the Cefaclor at 25 and 37°C because the degradation started from the second day of their preservation at 37°C. The pediatric suspensions of local and foreign Cefaclor in the Jordanian market were stable and contained the amount of the active ingredient, which was specified by the USP and BP.

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