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A comparative validation study of Cefprozil diastereoisomers determination in human plasma by HPLC-MS/MS and HPLC-UV methods: application to bioequivalence pilot study

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A SIMPLE, sensitive and reliable HPLC-MS/MS and HPLC-UV methods were developed and validated for *Cis*- and *Trans*-Cefprozil diastereoisomers determination in human plasma. The plasma samples subjected to protein precipitation after the addition of cephalexin as internal standard. Chromatographic separation was achieved using Waters Xbridge C18 column 5µm, 4.6×150mm maintained at 25° C. The HPLC-MS/MS method utilized MRM transitions; 390.1 to 208.1 and 348.1 to 158.2 for Cefprozil and cephalexindetection respectively, while the wavelength 292 nm was used for the UV detection. Both methods provided good linearity for *Cis*- and *Trans*-diastereoisomers with in the ranges of 0.05-10.00 and 0.02-1.00 µg/ml respectively. Methods were validated and applied successively to study the bioequivalence of two Cefprozil pharmaceutical products. The maximum plasma levels detected (C_{max}) of Cefprozil for the brand and generic products were, respectively; 10.0 and 9.9 µg/ml using the HPLC-MS/MS method compared to 10.5 and 10.6 µg/ml using the HPLC-UV method. The pharmaceutical products were found to be bioequivalent after analysis using both methods. The reference product pharmacokinetics data were statistically compared over the two methods and insignificant P-values were obtained. This comparison considered as extra prove for both methods reproducibility, reliability and ability to quantify Cefprozil diastereoisomers in human plasma.

Keywords: Cefprozil diastereoisomers, HPLC-MS/MS, HPLC-UV, determination, pharmacokinetics, and Bioequivalence.

Introduction

Cefprozil (CEF) is a semi-synthetic glycyl-cephalosporin used as a broad-spectrum antibacterial drug_ENREF_1 because ofits ability to inhibit cell-wall synthesis [1,2]_ ENREF_2_ENREF_2. It is prescribed to treat skin and respiratory infections by susceptible microorganism's strains like *streptococci* [1,2]. It composes from a mixture of two biologicallyactive diastereoisomers, *Cis*- (CFZ) and *Trans*- (CFE)diastereoisomers in approximated 9; 1ratio respectively, Figure 1. Proper estimation ofCEF bioavailability should be conducted to avoid prospective side effects such as the generation of microbial resistance [3]_ENREF_5. Liu *et al.* (2016) suggested that the bioequivalence conductance based on CFZ only can be enough[4]. However, the FDA draft guidance for CEF determination is still regulating total CEF determination for bioequivalence conductance. According to this regulation, the individual determination of each diastereoisomers is essential for bioequivalence conductance.

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Method development for CEF diastereoisomers determination in the biological sample is a critical process due to the stereo-isomeric structure[5]. Several methodshave been reported for CEF determination in plasma samples[4,6-12]_ ENREF_2_ENREF_2. The recent published paper by He *et al*. (2018) show proper sensitivity and simplicity[12], however,itsuffer reliability which will be illustrated later.In this vein, a primary objective for this work was to introduce a simple, valid and reliablemethods for CEF diastereoisomers determination in human plasma.

Chromatographic systems, especially equipped with MS or UV detection,are widely used for such analytical purposes. A common argument spread over researchers which are interested in API determination is related towhich technique is suitable for my application and which one is more reliable. In this vein, a secondary objective for this work was to introduce an accepted answer for this argument related to the quantified concentration reliability.

Herein, weintroduce two simple, validated and reliable HPLC-MS/MS and HPLC-UV methodsfor CEF diastereoisomers determination in human plasma. Both methods validated according to EMA guidelines for bioanalytical methods validation. The validated methods were used to evaluate the pharmacokinetics and bioequivalence of two CEF pharmaceutical products, brand and generic, in human plasma. Moreover, the obtained pharmacokinetics data were statistically compared to give a proper answer for the mentioned argument. The pharmacokinetics data for the brand product and also for the ratio of Brand to generic (B/G) products were used for this comparison. P-values obtained from the t-test were used to study the presence of significant differences between the pharmacokinetics dataor not.

Material and Methods

Chemicals and reagents;

CFZ(895 µg/mg, lot number H0H115) and CFE(938 µg/mg, lot no. IOH203) werepurchased from USP ROCKVILLE, USA. Cephalexin (CPH; 998.8 µg/mg lot no. B383290) was provided by DSM Anti-infective Schemferm, S.A. HPLC grade methanol, Perchloric acid (PCA), extra pure formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Scharlau, Spain. All other reagents used were of analytical grade. Drug-free plasma sampleswere supplied by Shabrawishi Hospital in Cairo, Egypt. Ultrapure water ASTM grade I was prepared using Barnstead Water Purification System - Thermo-Scientific, USA.

Stock standards and working standards preparation;

Stock solutions of 1.0 mg/mL for CFZ, CFE, and CPH were prepared in methanol. Working solutions of 20 and 100 µg/mL CFZ and 5, 50, and 200 µg/mL CFE were prepared from their respective stock standard solutions in 5% methanol. A working solution of 100 µg/mL was prepared for CPH inDI-water and labeled as CPH-IS-WS. Appropriate volumes from working solutions were used togetherto prepare human plasma calibrators at concentration of; 0.05, 0.1, 0.5, 1, 2.5, 5 and 10µg/mL for CFZ and 0.02, 0.05, 0.1, 0.15, 0.3, 0.6, and 1 µg/mL for CFE. Quality controls (QCs) concentration were0.15, 4, and 8 µg/mL for CFZ and 0.06, 0.4, and 0.8µg/mL for CFE. All Stock and working solutions were stored at 4□ whereas biological samples and calibrators were stored at -80□ and brought to room temperature (RT) immediately before use.

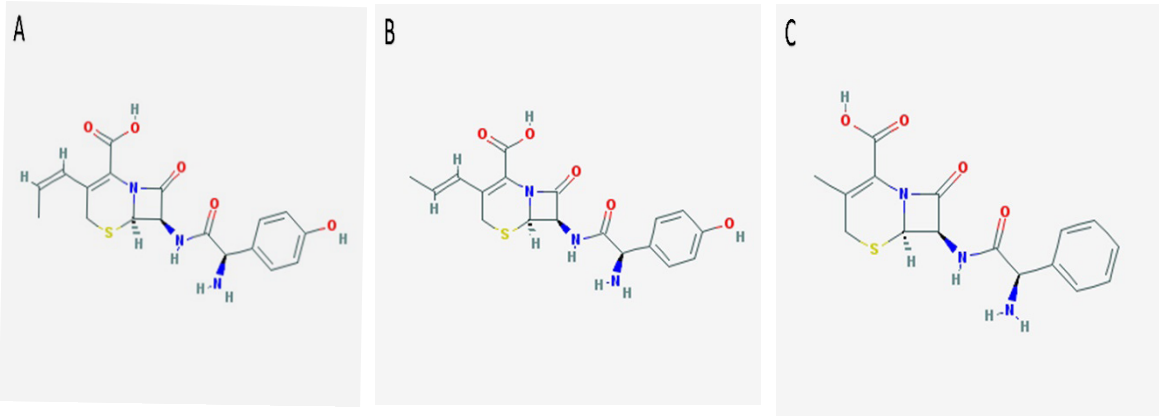

Fig. 1. Chemical structure of CEF isomers; CFZ (A) and CFE (B), and CPH (C).

Matrix effect (ME) and recovery (REC) standard working solutions;

ME was assessed only for the LC-MS/MS assay, where the REC was assessed for both methods. For the following preparations, the mobile phase buffer used is the buffer used for chromatographic separation. Both of ME and REC were evaluated at three levels; Low, medium and high. To preparea low-level standard solution - CEF-ME_REC-STDL –mix 75 µl from CFZ3 and 120 µl from CFE4 and complete to 10 ml by the appropriate mobile phase buffer. For Medium-level standard solution - CEF-ME_REC-STDM – Mix 40 µl from CFZ1 and 20 µl from CFE2 and complete to 10 ml by the appropriate mobile phase buffer. To prepare the high-level standard solution - CEF-ME_REC-STDH – mix 80 µl from CFZ1 and 40 µl from CFE2 and complete to 10 ml by the appropriate mobile phase buffer. Finally, for the working solutions preparations, a 50 µl from CPH-WS plus 30 µl from the appropriate mobile buffer (instead of the extraction solvent) were added to 0.15 ml from CEF-ME_REC-STDL, M or H to prepare CEF-ME_REC-WL, M or H respectively.

Sample preparation;

Appropriate volumes (0.15 ml) of human plasma samples and calibrators were dispensed into 1.5 ml Eppendorf plastic containers followed by addition of 50 µL from CPH-IS-WS. For the HPLC-MS/MS method, 30 µL from 30% TFA wasadded, whereas, 30 µL from 10% PCA wasadded forthe HPLC-UV method, to the samples. The samples were vigorously mixed for 2min followed by centrifugation at 14000 rpm for 10 min. Finally, the supernatants were separately transferred to a new glass vial (2ml) to be injected. Injection volumes used were 10 µL for the HPLC-MS/MS method and100 µL forthe HPLC-UV method.

Instrumentalanalysis conditions;

HPLC-MS/MS system consisted from Agilent 1200 Series (binary pump) coupled to AB SCIEX API 4000 triple quadrupole. CEF and CPH transitions were 390.1 to 208.1 and 348.1 to 158.2 respectively. Ion spray voltage, ion source temperature, de-clustering potential, CAD, Curtain gas, gas1, and gas2 parameters were ramped and their optimal values were 5500, 600, 55, 9, 15, 50, and 50 respectively. Collision energies 14 and 12 were used for CEF and CPH respectively. Waters Xbridge C18 column, 5µm 4.6×150mm, maintained at 25° Cand pumped for 5 min at 1ml/min using methanol; 0.05% FA in the ratio 35%; 65%. Forthe HPLC-UV method; the analysis was performed using Agilent 1260 Infinity series equipped with a quaternary HPLC pump and coupled with a variable wavelength detector operated at 292 nm. Equivalently, Waters Xbridge C18 column, 5µm 4.6×150mm, maintained at 25° C and pumped for 7min at flow rate 1ml/min using acetonitrile; 0.05M potassium phosphate monobasic adjusted topH3 in the ratio 13%; 87%.

Validation Methodology

Selectivity;

Six different human plasma samples from six different lotswere extracted and injected to test the ability of both methods to differentiate the tested analytes from the potential plasma endogenous matrix.

Linearity and Lower limit of quantification (LLOQ);

Freshly prepared and extracted plasma calibrators were used to evaluate methods linearity on three different days. Regression and correlation coefficients were calculated and the best weighing was selected. The peak area ratios of the analytes to IS were plotted against analytes concentration.The LLOQ was validated based on the Signal-to-noise ratio of the analytes to the background signals ratio which should be more than five_ENREF_13. The manufactures' software was used for this purpose.

Within- and between-run precision and accuracy;

The previously described three levels of QCs were used to evaluate within- and between-run precision and accuracy for both methods. Six replicate for each QC level were extracted and analyzed using both methods within the same day and used to evaluate within-run precision and accuracy. Six sets of QCs were analyzed independently in six different days using both methods and used to evaluatebetween-run precision and accuracy.

Matrix effect andrecovery;

The ME was evaluated only for the HPLC-MS/MS assay as following; A 200 µl from the freshly prepared ME working solutions were dispensed and lyophilized using nitrogen dryer. Parallel, a six drug-free plasma samples from six different donors were extracted in triplicates using the described sample preparation without addition of drug or internal standard, instead 50 µl

DI-Water were added. A proper volumes (200 µl) from the extracted plasma supernatants were used to reconstitute the lyophilized working standard followed by strong agitation to insure proper re-solvation. The working solutions and the reconstituted lyophilized-samples were injected using the LC-MS/MS assay and the detector responses were recorded and compared. RECwas evaluated by comparing the peak areas of freshly extracted QCsplasma samples against the peak areas for working solutions.

Stability;

Analytes stabilities were evaluated in the native biological matrix and after sample processing. Biological matrix stability is not dependent on the method; therefore, it was evaluated using only HPLC-MS/MS method, while processed samples stability is method dependent and evaluated for both methods. The stabilities of all analytes in biological matrices were evaluated by analyzing QCs aliquots at the following storage conditions; At RT for 6h and 24h, at 4° C for 24h, after three successive freeze-thawing (FT) cycles, and after long storage (LTS) at -80° C for 1 month. Processed samples stabilities were evaluated during injection (auto-sampler stability; ASS) by comparing freshly extracted samples vs extracted samples spent 24h in the auto-sampler. The stock solutions standardsstabilities (SS) were evaluated by analyzing old prepared stock standards vs a freshly prepared ones. The stock solutions were diluted to get final concentration equal to QCM and injected using the HPLC-MS/MS chromatographic conditions. These standards were evaluated at three different storage conditions; At RT for 6h and 24h, at 4° C for 24h, and after LTS at -20° C for 1 month.

Study design;

The current study was performed according to the Egyptian ethics and guidelines for generic drug registration. Two different CEF suspension (500 mg / 5ml) products were used to assess their bio-equivalency through10 healthy volunteers (>18 years) in a cross-over study.Suspensions' dose volumeswere 5ml and the washout period was ten days. Blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 5, 6, 8, 10 and 12 h post-dose administration. Plasma aliquots resulted from blood centrifugation at 4000 rpm for 5 min were separated and stored at -80°C until analysis. All volunteers' samples were extracted and analyzed using the HPLC-MS/MS and the HPLC-UV methods. Their concentrations were used to evaluate the pharmacokinetics parameters; C_{max}, area under the concentration-time curve from 0 min to the last measured concentration (AUC_{0-t}), area under the concentration-time curve from 0 min to infinity ($AUC_{0-\infty}$), mean residence time from 0 min to time t (MRT_{0-t}) and to infinity ($MRT_{0-\infty}$), the time to reach maximum concentration (T_{max}, time taken to eliminate half the initial dose ($T_{1/2}$), and clearance (CL). Bioequivalence conducted at 90 % Confidence and the range 80 – 125 % was used to evaluate the log-transformed values. The calculated pharmacokinetics from both methods were further compared usingstudent-t test for the brand product and for the individual B/G ratios. Obtained p-values were used toevaluate the impact of determination methods on the pharmacokinetics parameters. The results obtained can be considered as extra testing for methods reproducibility.

Data analysis;

For system control and data acquisition and analysis, Agilent *Chemstation B.04.01* software was used for the HPLC-UV method and Sciex *Analyst®1.6.2* software was used for the HPLC-MS/MS method. *Kinetica* 5.0 was used to calculate pharmacokinetics, whereas, *GraphPad prism* was used for student t-test and p-value statistical analysis.

Results and Discussion

Sample preparation;

As mentioned above, CEF determination in biological samples is a challenging process because of its hydrophilic and amphoteric natures[13]_ENREF_15. These natures were concerned during methods development and several strategies were examined. The hydrophilic nature of CEF minimizedthe available sample preparation choices, either we had to undergo protein precipitation orto implement expensive SPE analysis [14-16]_ENREF_21. The choices with potential benefits belonged to protein precipitation with reagents like TFA and PCA. The selected reagents have proved abilityto; precipitate the proteins effectively,provide supernatant enriched bythe hydrophilic content and produce limited hydrophobic contentcompared to the organic precipitants like methanol oracetonitrile [17]. These selections were essential to avoid column overload with sticky hydrophobic components, especially if we designed to use mobile phase with low organic modifier content. Protein precipitation was investigated for both methods. Both reagents had

a comparable recovery, but they were different in the interfering matrix. For the HPLC-MS/MS method, TFA reagent was selected due to its ability to eliminate the matrix effect. Where as, for the HPLC-UV method, PCA reagent was found to have supernatant with lower background interference compared toTFA supernatant and therefore it was selected.

Chromatographic conditions and detection;

Both TFA and PCA supernatants have extremely low pH value (<1) and can easily hydrolyze the stationary phase ether bond of C18 columns[18]_ENREF_16. To avoid suchcolumn deterioration, Waters Xbridge C18 column was selected for CEF diastereoisomers separation because of its known resistance to extremely low pH. MS spectral optimization was tested in both positive and negative modes. CEF amphoteric nature required acidic (pH < 4) or basic (pH > 8.5) mobile phase conditions to force most molecules to had only one charge, positive or negative, on its surface[19,20]. Therefore, during signal optimization (tuning) in positive mode, Q1 for CEF couldn't be observed before diluting CEF diastereoisomers in acidified solution (0.1% FA). The acidified solution allowed pH value below the carboxyl group pKa and hence overall positive clear signal. MS1 and MS2-fragmentation spectra for CEF and CPH were presented in Fig. 2. Base peaks 208 and 158 were selected as a quantitative transition for CEF and CPH respectively, while m/z 184 was selected as a conformational transition for CEF. *He et al.* (2018) utilized 391.2 as Q1 for CEF detection[12], this m/z is unreliable, where; the theoretical m/z for CEF in the adduct form [M+H] is 390.1 (389.1 monoisotopic mass plus 1.0 for hydrogen adduct), this theoretical m/z compiled with the observed m/z in our method and also with the recorded m/z within thereference spectrumfounded at*mass bank of North America* database. These unreliable transitions for CEF within *He et al.* developed methodis uncommon in HPLC-MS/MS methods generation and hardly can be accepted. For negative mode, it required pH >8 to achieve a suitable MS signal capable to retrieve the required LLOQ.Besides,the matrix effect was found to be easily eliminated in the positive mode than in the negative mode. As a result the positive mode was the candidate mode.

For the HPLC-UV method, the UV spectrum was screened for each isomer to test the wavelength with maximum absorption. Each isomer had its own absorption maximum; 280 nm for CFZ (the most abundant isomer) and 292 nm for CFE. Accordingly, 292 nm was used for both isomers determinationto enhance the CFE signal and to lower background interference. The selected wavelength was tested for both diastereoisomers reproducibility.

For chromatographic separation, CEF diastereoisomers shown better resolution in acidified mobile phase than alkaline one. FA was used as an acidic volatile buffering agent for the HPLC-MS/MS method while phosphate buffer (adjusted to pH 3) resolution was found to be better than phosphoric acid or FA for the HPLC-UV method. For the HPLC-MS/MS method, methanol had higher intensities compared with acetonitrile, while for the HPLC-UV method, acetonitrile had a higher resolution between analytes and interfering matrix.

Methods validation

Selectivity;

Drug-free plasma samples were extracted and injected using both methods HPLC-MS/MS and HPLC-UV. Fig. 3A and 4A show a typical blank plasma chromatogram for both methods respectively. No endogenous interfering peaks were observed in the analytes retention time's regions for both methods. The background intensities were below 20% for CFZ- and CFE-LLOQ and below 5% for CPH_ENREF_13 and both methods can be considered selective for all analytes. The chromatograms in Fig. 3B and 4B represent extracted plasma samples containing CFZ, CFE, and CPH for both methods HPLC-MS/MS and HPLC-UV respectively. The HPLC-MS/MS analytes retention times were 3, 3.6 and 3.7 min for CFZ, CFE, and CPH respectively. The HPLC-UV analytes retention times were 4.7, 6.1 and 5.2 min for CFZ, CFE, and CPH respectively.

The Lower limit of quantification (LLOQ);

The current methods offer 0.05 ug/ml LLOQ for CFZ and 0.02 µg/mL for CFE. The Signal-to-noise ratio for the HPLC-MS/MS method was greater than 100 and 30 for CFZ and CFE respectively, Fig. 3C. The Signal-to-noise ratio for the HPLC-UV method was greater than 10 for CFZ and CFE, Fig. 4C. LLOQ for CFZ and CFE concentrations were below 1/20 of their C_{max} [21]. heir calculated concentrations precision and accuracy were validated and confirmed to be within internationally accepted limits (< 20%) [21]_ENREF_13. The Signal-to-noise for both methods was greater than 10_ENREF_5.

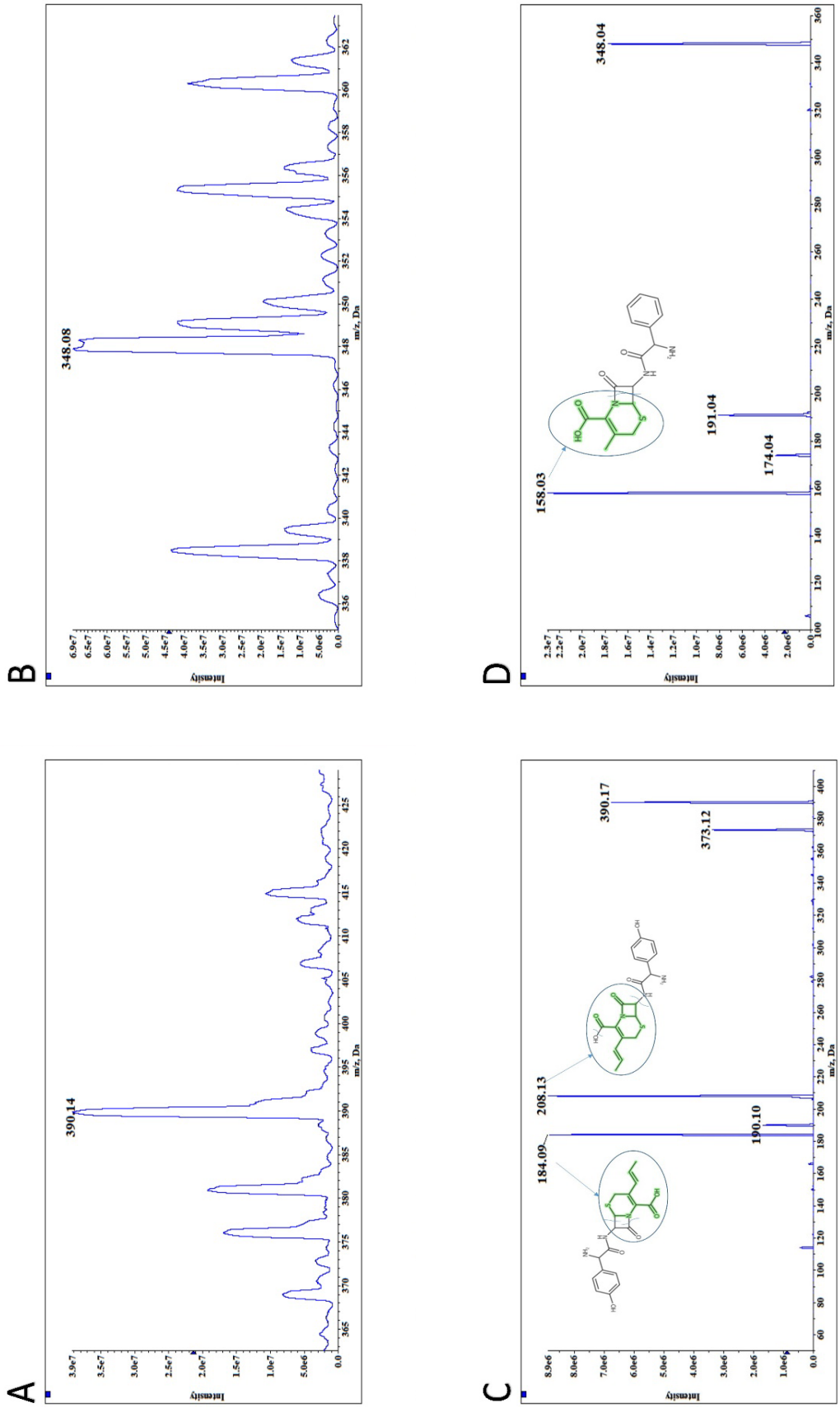

Fig. 2. MS spectrum of CEF (A), MS spectrum of CPH (B), MS/MS spectrum of CEF (C) and MS/MS spectrum of (CPH)

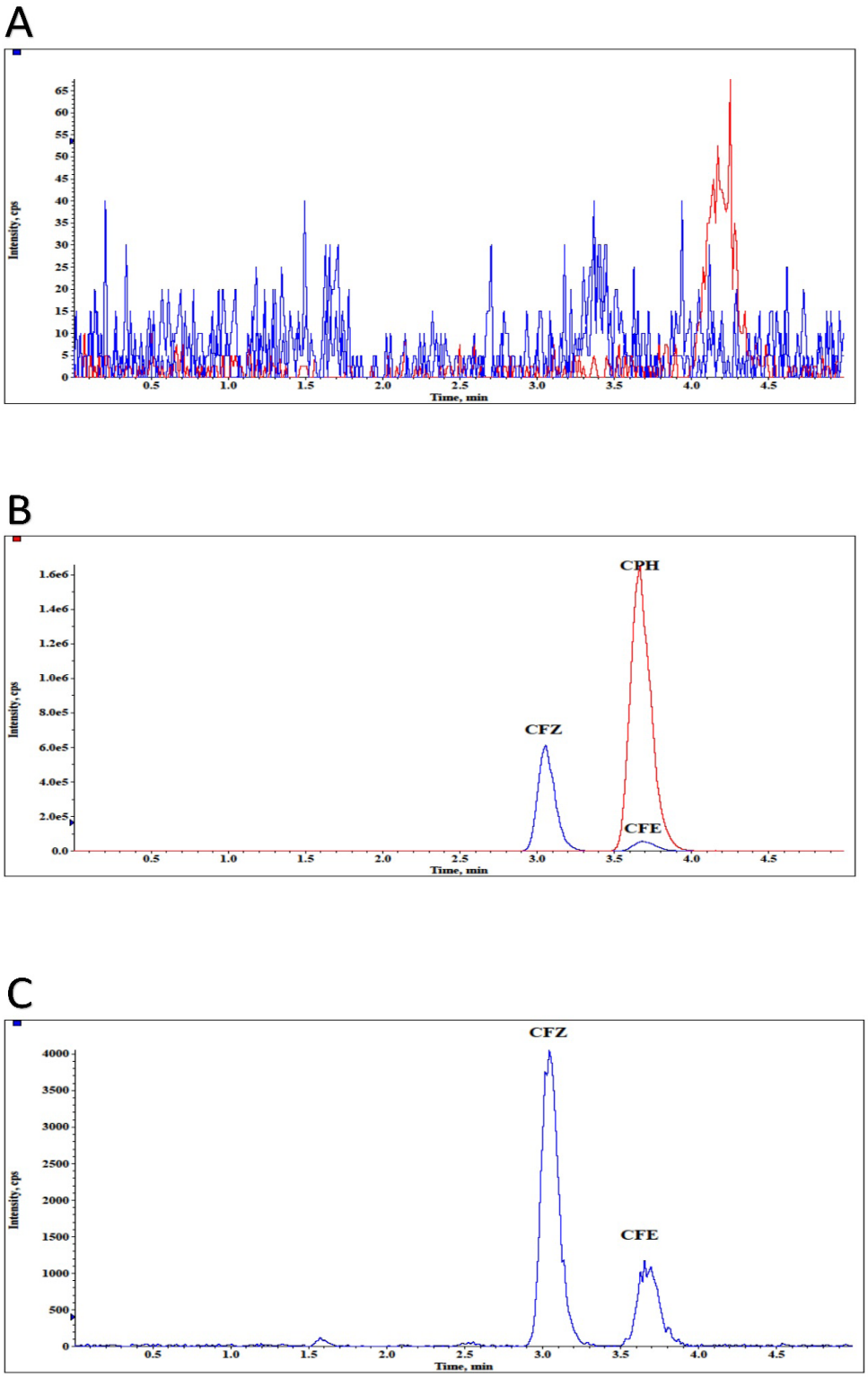

Fig. 3. HPLC-MS/MS representative chromatograms for the injected blank plasma extract (A), injected plasma extract spiked with CEF-diastereoisomers and CPH (B)and injected LLOQ plasma extract (C).

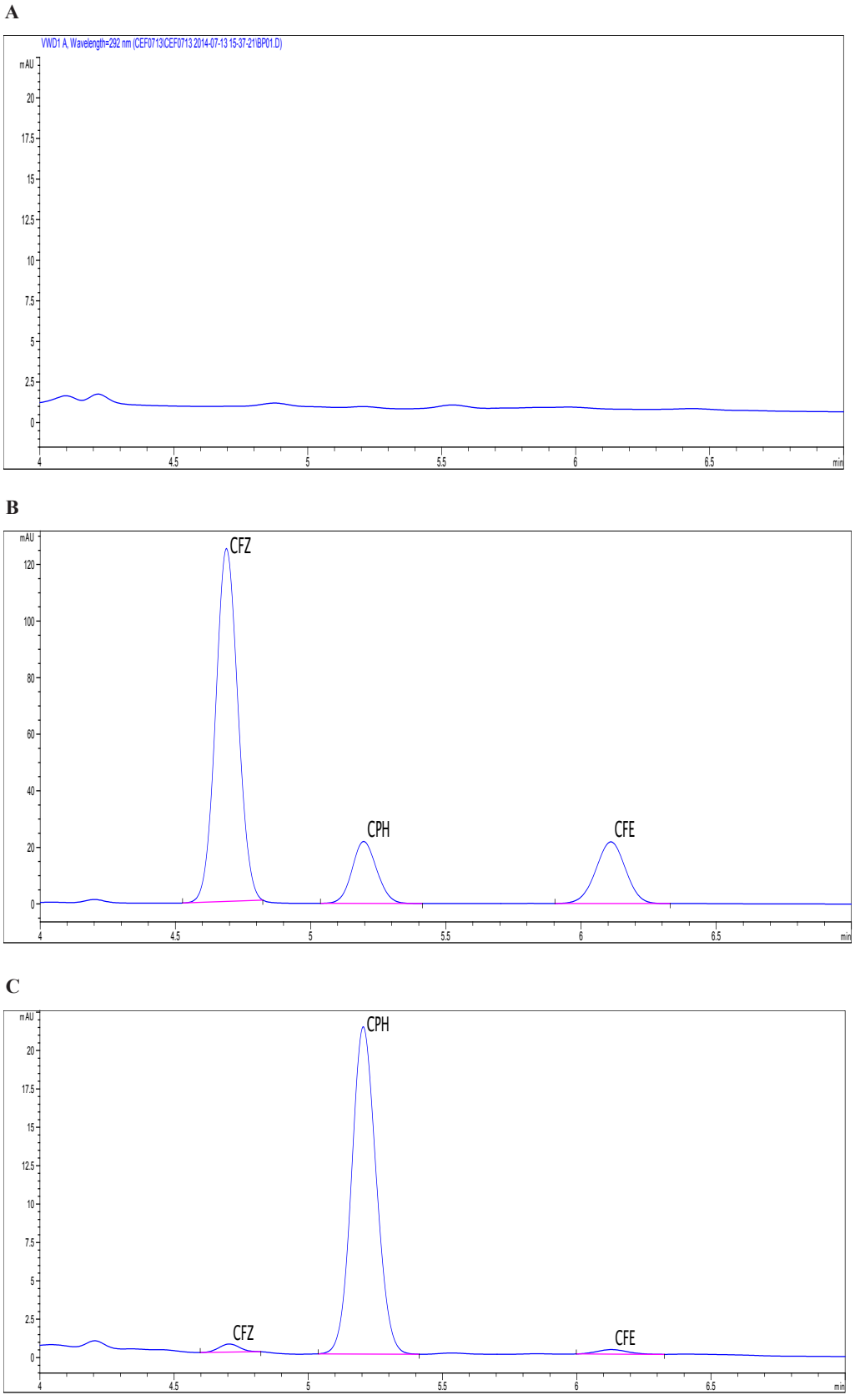

Fig. 4. HPLC-UV representative chromatograms for the injected blank plasma extract (A), injected plasma extract spiked with CEF-diastereoisomers and CPH (B) and injected LLOQ plasma extract (C).

Matrix effect (ME) and Recovery (REC);

As summarized in Table 1, for the HPLC-MS/MS method; MEs were 90.5, 96.0, and 99.2 % for CFZ QCs levels 0.15, 4, and 8µg/mlrespectively. MEs were 93.8, 103.8, and 111.3 % for CFE QCs 0.06, 0.4, and 0.8 µg/ml respectively and it was 96.9 % for CPH-IS. As discussed previously, ME is the direct relation between the analytes intensities in the matrix and the pure working standard solutions. Overall ME percentages were greater than 90%, i.e. the effect of the matrix in the analytes intensities was below 10%. Both methods RECs determined at the three QCs levels were greater than 80% for all target analytes. The achieved REC and precisions were necessary to maintain both methods linearity and LLOQ within the acceptance limits. HPLC-MS/MS and HPLC-UV methods average REC were 88.2 and 91.2 for CFZ, 82.4 and 85.2 for CFE, and 89.6 and 99.3 for CPH.

Linearity and range;

Within the three linearity days, a regressionswith R^2> 0.99 andwith 1/x weighing were observed during the daily analysis for theplasma calibrators in the ranges of 0.05 to 10 µg/mL for CFZ and 0.02 to 1µg/mL for CFE. CFZ and CFE concentrations were calculated through the general equation; [(Area ratio of CFZ (or CFE)/CPH) - (average linear intercept)]/ (average linear slope).

One of the major benefits resulted from these methods - compared to the other published papers - is using weighing 1/x instead of $1/x^2$. 1/x regression is usually referring to linear regression while $1/x^2$ refers to semi-quadratic regression[22]_ENREF_3. Table 2 summarized the four linearity equations with their regressions used to calculate CFZ and CFE concentrations for both methods.

Precision and Accuracy;

Both diastereoisomers within-and between-run precisions (RSD) for all QCs levels were below 9.68 % for the HPLC-MS/MS method and below 5.94 % forthe HPLC-UV method (Table 3). The relative errors (RE) for the detected concentrations werebelow 13 % for the HPLC-MS/MS method and below 11 % for the HPLC-UV method.The obtainedresults were within the acceptable limitsand criteria for precision and accuracy. The HPLC-UV method had a relative higher precision than the HPLC-MS/MS method which reflects the general higher stability of UV detector signal reproducibility than ESI-MS/MS detectors[23]_ENREF_11.

Stability;

CFZ and CFE were found to be stable in the biological matrix at RT for 6h and 4°C for 24h. They also found to be stable after three F & T cycles and after storage for 30 days at -80°C (Table 4). CFZ and CFE had limited stability in the biological samples, therefore biological samples exposure RT should be minimized. SSs for CFZ, CFE, and CPH were found to be stable in all storage conditions as shown in table 5. CFZ, CFE, and CPH were found to be stable in the processed samples for 24 h for both methods conditions (Table 4).

Pharmacokinetics and bioequivalence evaluation:

For the HPLC-MS/MS method, the mean ± SD values of CEF-C_{max} for the brand and generic products were 9.97 ± 1.3 and 9.2 ± 1.5 µg/L respectively. The Same results were obtained for the HPLC-UV method, which has C_{max} for the brand and generic products 10.6 ± 1.4 and 10.6 ± 1.7 respectively. The obtained data from both methods are comparable with the FDA's access data for Cefprozil which states that mean plasma concentrations achieved after oral administration of 500 mg to fasting subjects were approximately 10.5 µg/L obtained within 1.5 hours after dosing. The average plasma total concentrations (±SD) for cefprozil concentration measured by both methods for the brand and generic products are listed in table 6 and graphically represented in Fig. 5.

The average values (± SD) for pharmacokinetic parameters; maximum concentration C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, MRT_{0-t}, $MRT_{0-\infty}$, T_{max}, $T_{1/2}$, and CL were summarized in Table 7.

P-values for the differences between C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for the same product "brand" or even for the brand/generic ratio obtained from both methods were all greater than 0.05. These results demonstrate that both methods were similar and there was no effect for them in the actual measured concentration.

TABLE 1. CFZ, CFE and CPH average recovery and matrix effect results.

		HPLC-UV		HPLC-MS/MS		HPLC-MS/MS	
Analyte	**Actual Concentration (ug/ml)**	**REC[a] % (Mean[b] ± SD)**	**RSD (%)**	**REC[a] % (Mean[b] ± SD)**	**RSD (%)**	**ME[c] % (Mean[d] ± SD)**	**RSD (%)**
CFZ	0.15	87.80 ± 0.28	0.32	84.36 ± 3.56	4.21	90.48 ± 4.05	4.47
	4.00	93.59 ± 0.03	0.04	90.48 ± 1.07	1.18	96.01 ± 2.38	2.47
	8.00	92.32 ± 0.15	0.16	89.66 ± 1.43	1.60	99.21 ± 2.27	2.29
CFE	0.06	88.33 ± 0.36	0.41	80.62 ± 2.72	3.37	93.78 ± 2.33	2.48
	0.40	83.75 ± 0.01	0.01	83.17 ± 0.28	0.34	103.76 ± 3.24	3.13
	0.80	83.61 ± 0.20	0.23	83.34 ± 1.55	1.86	111.29 ± 10.36	9.31
CPH	100	99.27 ± 0.11	0.11	89.61 ± 12.68	14.15	96.92 ± 2.25	2.32

[a] REC=Recovery
[b] n=3 for each level
[c] ME=Matrix effect
[d] n=6 for each level

TABLE 2. CFZ and CFE linearity equations and regressions

Analyte		**HPLC-UV**	**HPLC-MS/MS**
CFZ	Concentration =	(Area Ratio - 0.00121) / 0.485	(Area Ratio - 0.00104) / 0.03
	R^2 =	1.000	0.999
CFE	Concentration =	(Area Ratio + 0.00733) / 0.986	(Area Ratio - 0.00017) / 0.028
	R^2 =	0.998	0.992

TABLE 3. CFZ and CFE within-run (intra-day) and between-run (inter-day) precision[a]and accuracy[a] and processed samples stability[b]

HPLC-UV	Actual Concentration (µg/ml)	Within-run calculated concentration			Between-run calculated concentration			Autosampler concentration after 24 h at 4°C		
Analyte		**Mean ± SD**	**RSD (%)**	**RE (%)**	**Mean ± SD**	**RSD (%)**	**RE (%)**	**Mean ± SD**	**RSD (%)**	**RE (%)**
	0.15	0.13±0.002	1.50	10.75	0.15 ± 0.006	4.07	2.83	0.14 ± 0.001	0.80	5.36
CFZ	4.00	4.04±0.12	2.88	1.10	3.98 ± 0.13	3.32	0.44	4.39 ± 0.1	2.28	9.64
	8.00	8.01±0.17	2.08	0.15	7.98 ± 0.25	3.10	0.27	8.65 ± 0.11	1.29	8.18
	0.06	0.06±0.002	3.14	6.40	0.06 ± 0.003	5.94	6.74	0.05 ± 0.000	0.64	12.27
CFE	0.40	0.44±0.01	2.68	10.13	0.43 ± 0.01	2.68	7.72	0.44 ± 0.01	1.82	9.66
	0.80	0.84±0.02	2.31	5.54	0.85 ± 0.02	2.12	5.85	0.83 ± 0.01	1.23	3.49
HPLC-MS/MS										
	0.15	0.14 ± 0.005	3.78	8.89	0.14 ± 0.005	3.60	4.44	0.14 ± 0.011	7.71	5.33
CFZ	4.00	4.3 ± 0.26	6.08	7.58	4.06 ± 0.09	2.13	1.50	4.4 ± 0.16	3.68	10.00
	8.00	8.29 ± 0.47	5.67	3.58	8.33 ± 0.67	8.03	4.10	8.59 ± 0.32	3.71	7.35
	0.06	0.05 ± 0.005	9.68	11.11	0.06 ± 0.005	9.11	5.56	0.06 ± 0.005	9.78	6.67
CFE	0.40	0.45 ± 0.01	1.68	12.08	0.43 ± 0.03	6.78	7.92	0.45 ± 0.01	1.85	13.00
	0.80	0.89 ± 0.02	2.46	11.25	0.84 ± 0.07	8.61	4.58	0.91 ± 0.01	0.60	14.25

[a] n=6 for each level
[b] n=5 for each level

TABLE 4. CFZ and CFE stability[a] in human plasma after different storage conditions

HPLC-MS/MS	Actual Concentration (µg/ml)	Short-term stability 6 h (RT)			Short-term stability 24 h (4C)			Freeze-thaw 3 cycles			Long-term stability 1 month (−80 °C)		
Analyte		Mean ± SD	RSD (%)	RE (%)	Mean ± SD	RSD (%)	RE (%)	Mean ± SD	RSD (%)	RE (%)	Mean ± SD	RSD (%)	RE (%)
CFZ	0.15	0.14 ± 0.00	0.00	6.67	0.14 ± 0.00	0.00	6.67	0.15 ± 0.006	3.94	2.22	0.16 ± 0.006	3.69	0.00
	4.00	3.92 ± 0.27	6.93	7.75	3.95 ± 0.21	5.23	5.50	4.01 ± 0.18	4.57	0.25	3.44 ± 0.01	0.29	14.00
	8.00	6.93 ± 0.14	2.05	11.38	7.18 ± 0.17	2.38	12.50	7.53 ± 0.22	2.95	5.83	6.85 ± 0.04	0.59	13.88
CFE	0.06	0.06 ± 0.00	0.00	0.00	0.05 ± 0.00	0.00	16.67	0.06 ± 0.00	0.00	0.00	0.06 ± 0.00	0.00	0.00
	0.40	0.40 ± 0.03	6.61	5.00	0.42 ± 0.02	3.61	5.00	0.41 ± 0.03	7.10	1.67	0.34 ± 0.01	1.68	12.50
	0.80	0.70 ± 0.02	2.17	10.00	0.81 ± 0.03	3.09	1.25	0.87 ± 0.02	1.76	8.33	0.75 ± 0.02	2.05	6.25

[a] n=5 for each level

TABLE 5. CFZ, CFE and CPH stock solution stability[a]

	CFZ		CFE		CPH	
Duration / condition	Stability % (Mean ± SD)	RSD (%)	Stability % (Mean ± SD)	RSD (%)	Stability % (Mean ± SD)	RSD (%)
6h / RT	99.9 ± 1.70	1.70	101.1 ± 0.73	0.72	98.6 ± 1.11	1.13
24h / RT	87.7 ± 1.24	1.42	87.7 ± 0.71	0.80	87.2 ± 1.35	1.55
24h / 4C	93.8 ± 1.33	1.41	94.3 ± 1.54	1.63	93.7 ± 1.06	1.13
30Days/ -20°C	95.0 ± 1.82	1.92	96.3 ± 1.59	1.65	90.9 ± 1.00	1.10

[a] n=5

All calculated confidence intervals (CI) at 90% for all the geometric means values of generic/brand products for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were within the bioequivalence acceptable limits for CEF, 80–125%, Table 8, accordingly both products can be considered as bioequivalent.

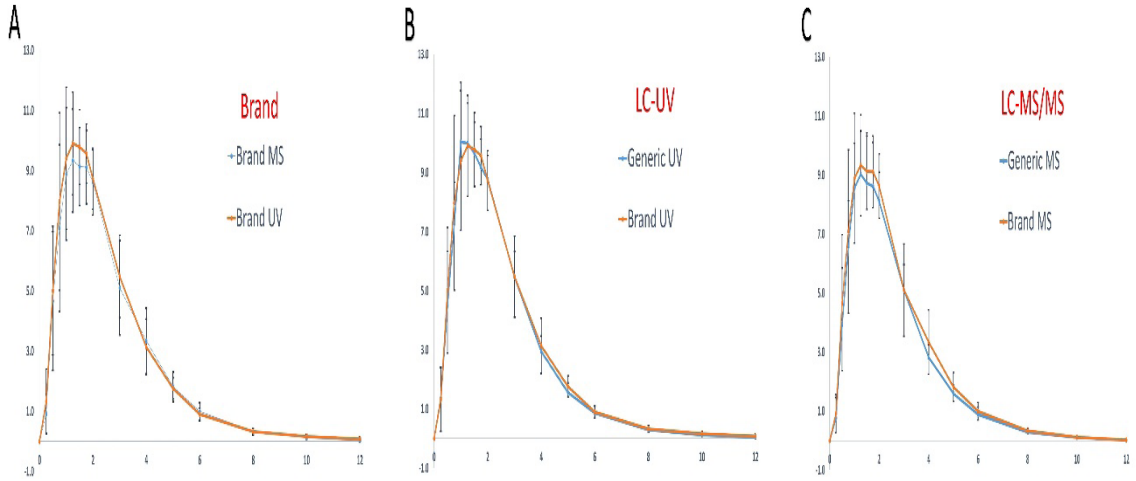

Fig. 5. Mean plasma concentration curves for the ten volunteers' vs sampling time points; A) for brand product determined using both methods, B) for generic vs brand products determined using HPLC-UV method and C) for generic vs brand products determined using HPLC-MS/MS method.

TABLE 6. CEF mean plasma concentrations[a] (mean ± SD) of over time.

Time (h)	HPLC-UV		HPLC-MS/MS	
	Brand	Generic	Brand	Generic
0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.25	1.32 ± 1.08	1.36 ± 1.05	0.91 ± 0.64	0.77 ± 0.69
0.50	5.01 ± 2.13	4.52 ± 1.82	4.67 ± 2.30	3.93 ± 1.92
0.75	7.97 ± 2.95	7.27 ± 1.41	7.09 ± 2.77	6.58 ± 1.55
1.00	9.41 ± 2.36	10.03 ± 2.02	8.89 ± 2.21	8.56 ± 1.51
1.25	9.90 ± 1.70	9.99 ± 1.36	9.33 ± 1.71	9.02 ± 1.47
1.50	9.78 ± 1.25	9.66 ± 1.05	9.13 ± 1.29	8.73 ± 1.60
1.75	9.57 ± 0.99	9.18 ± 0.95	9.11 ± 1.22	8.62 ± 1.49
2.00	8.73 ± 1.01	8.76 ± 0.80	8.62 ± 1.10	8.10 ± 0.99
3.00	5.48 ± 1.37	5.47 ± 0.85	5.10 ± 1.57	5.09 ± 0.88
4.00	3.14 ± 0.93	2.95 ± 0.53	3.34 ± 1.09	2.82 ± 0.43
5.00	1.76 ± 0.36	1.55 ± 0.33	1.81 ± 0.49	1.59 ± 0.28
6.00	0.90 ± 0.21	0.86 ± 0.21	0.99 ± 0.30	0.89 ± 0.25
8.00	0.32 ± 0.12	0.30 ± 0.08	0.33 ± 0.10	0.28 ± 0.08
10.00	0.16 ± 0.07	0.12 ± 0.04	0.11 ± 0.04	0.10 ± 0.03
12.00	0.07 ± 0.08	0.04 ± 0.04	0.02 ± 0.03	0.02 ± 0.03

[a] n=10

TABLE 7. CEF pharmacokinetics[a] (mean ± SD) and confidence intervals %.

Time (h)	HPLC-UV		HPLC-MS/MS	
	Brand	Generic	Brand	Generic
C_{max} (µg/L)	10.55 ± 1.38	10.56 ± 1.71	9.97 ± 1.29	9.90 ± 1.45
$t_{1/2}$ (h)	1.60 ± 0.62	1.32 ± 0.10	1.27 ± 0.12	1.17 ± 0.23
T_{max} (h)	1.18 ± 0.33	1.20 ± 0.16	1.35 ± 0.36	1.38 ± 0.24
AUC_{0-t} (µg /L·h)	30.89 ± 3.34	30.04 ± 2.54	29.71 ± 3.93	28.38 ± 2.38
$AUC_{0-\infty}$ (µg /L·h)	31.10 ± 3.30	30.16 ± 2.52	29.86 ± 3.93	28.52 ± 2.37
MRT_{0-t} (h)	2.61 ± 0.27	2.55 ± 0.23	2.63 ± 0.32	2.75 ± 0.49
$MRT_{0-\infty}$ (h)	2.69 ± 0.29	2.59 ± 0.23	2.68 ± 0.31	2.80 ± 0.48
CL (L/h/kg)	8.11 ± 0.79	8.34 ± 0.73	8.5 ± 1.11	8.82 ± 0.73

[a] n=10

TABLE 8. Confidence intervals % and p-values.

	Confidence intervals %		P-values	
	HPLC-UV	HPLC-MS/MS	Brand-MS : Brand-UV	B-MS/G-MS : B-UV/ G-UV
C_{max} (µg/L)	88.9 - 113.4	101.4- 116.8	0.12	0.05
AUC_{0-t} (µg /L·h)	94.6 - 111.4	97.2 - 111.7	0.08	0.51
$AUC_{0-\infty}$ (µg /L·h)	95.1 - 111.5	97.2 - 111.8	0.07	0.61

B-MS; Brand-MS
G-MS; Generic-MS
B-UV; Brand-UV
G-UV; Generic-UV

Conclusions

The developed and validated methods for CEF diastereoisomers determination in human plasma are simple, cheap, highly reproducible and reliable methods compared to other previously published methods. Both methods were successfully applied to the study of CEF pharmacokinetics and bioequivalence for two pharmaceutical products and they were bioequivalent.

The comparison applied for the obtained pharmacokinetics values from both methods for the same product or even for the ratio of the products demonstrates that; the pharmacokinetics values obtained from different validated determination methods had non-significant differences.

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دراسة عملية للتحقق من كفاءة طرق جديدة لقياس مركب السيفبروزيل في البلازما باستخدام جهاز الكروماتوجرافيا السائلة والمزود بوحدة قياس كتلي و الاخري باستخدام جهاز كروماتوجرافيا مزود بوحدة قياس طيفي: تم التطبيق في دراسة تكافؤ حيوي مصغرة

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يقدم هذا البحث طريقتان جديدان لتحديد و قياس المكونان الرئيسيان لمركب السفبروزيل باستخدام جهاز الفصل الكروماتوجرافي السائل. الطريقة الاولي تستخدم الاسعة فوق البنفسجية للقياس و الطريقة الثانية تستخدم مقياس الكتلة كوحدة للقياس. تلك الطريقتان اثبتا فاعليتاهما و دقتهتمها العالية لقياس هذا المركب في بلازما الانسان. تم استخدام تلك الطريقتان في تحديد تركيو مركب السيفبروزيل في دراسة تكافؤ حيوي. تلك الطريقتان اعطت نتائج متقاربة جدا وهو ما يثبت امكانية استخدامهما في تلك الدراسات.