Cefuroxime, (6R,7R)-3-(carbamoyloxymethyl)-7-[(2Z)-2-(furan-2-yl)-2-methoxyiminoacetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (CEF, Fig. 1, R = H), is a second-generation cephalosporin used against different kinds of bacterial infections. Cefuroxime axetil (CEFA) is its 1-acetyloxyethyl ester. After oral administration, CEFA is absorbed from the gastrointestinal tract and rapidly hydrolyzed by nonspecific esterases in the intestinal mucosa and blood to CEF, which is subsequently distributed throughout the extracellular fluids. Following oral administration of CEFA tablets, maximum CEF concentration in plasma occurs at 1–4 hours. The elimination half-life is 1–2 hours (1, 2).

The aim of the presented study was to adapt and validate the existing high performance liquid chromatography (HPLC) method [1] for the determination of CEF in human plasma to allow pharmacokinetic studies in humans after oral administration of CEFA tablets.

**EXPERIMENTAL**

**Standard substances**

Cefuroxime sodium salt (reference standard) was supplied by Sigma-Aldrich, St. Louis, MO, USA and cefalexin monohydrate (the internal standard, I.S.), (6R,7R)-7-{[(2R)-2-amino-2-phenylacetamino]amino}-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid hydrate (Fig. 2), was supplied by Tarchomińskie Zakłady Farmaceutyczne „Polfa”, Warszawa, Poland.

Methanol, acetonitrile (both POCH, Gliwice, Poland) were of HPLC grade, glacial acetic acid, potassium dihydrogen phosphate and ortho-phosphoric acid were supplied by Merck KGaA, Darmstadt, Germany. Other chemicals were of analytical grade.

The highest calibration standard (12.0 µg/mL) was prepared by spiking drug free human plasma with the stock solution of CEF in methanol at 1.0 mg/mL. The other calibration standards and quality

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**Figure 1. Chemical structures of cefuroxime axetil (1) and cefuroxime (2)**

1. R = CH₃CHOCOCH₃
2. R = H

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control samples (QC – at concentrations of 0.5, 6.0 and 10.0 µg/mL) were prepared by appropriate dilution with blank plasma.

**Instrument and chromatographic conditions**

The liquid chromatography Shimadzu system consisting of a controller SCL-10AVP, a pump LC-10ADVP, an autosampler SIL-10A, a column oven of detector Decade II and an UV detector SPD 10-AVP was used. The data were processed using Shimadzu Class-VP version 6.0 software.

The chromatographic separation from endogenous compounds was performed on Supelcosil column LC-18-DB 250 × 4.6 mm, 5 µm (Supelco, Bellefonte, PA, USA). HPLC column was preceded by a guard column C18, 4.0 × 2 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 8.5% of acetonitrile in 0.07 M potassium dihydrogen phosphate solution (pH = 3.0) and the flow rate 1.7 mL/min was selected. The oven temperature was set at 40 ± 2°C, while the autosampler temperature was set at 20 ± 5°C. The retention times of cefuroxime and I.S. were around 13.9 min and 9.6 min, respectively, while the analyses run time was set at 17.0 min. The UV wave length \( \lambda = 275 \text{ nm} \) was selected (Fig. 3).

**Sample preparation**

The frozen plasma samples were thawed at a room temperature and centrifuged for 5 min at 3500

![Chemical structure of cefalexin (I.S.)](image1)

Figure 2. Chemical structure of cefalexin (I.S.)

![UV spectrum of cefuroxime sodium](image2)

Figure 3. UV spectrum of cefuroxime sodium

![HPLC chromatogram of blank human plasma](image3)

Figure 4. HPLC chromatogram of blank human plasma
Validated HPCL method for determination of cefuroxime in human plasma

rpm prior to protein precipitation procedure. A 500 mL aliquot of plasma was transferred to Eppendorf vial and mixed with 50 mL of the I.S. solution (cefalexin at 200 µg/mL). Then, 100 mL of 5% acetic acid was added and mixed. Afterwards, 1000 mL of cooled acetonitrile was added and mixed well. The mixture was centrifuged for 15 min at 12000 rpm at 4 ± 2°C. After that, the supernatant was transferred to a glass tube and evaporated to dryness under the stream of nitrogen in a water bath at 40 ± 2°C. The residue was reconstituted in 250 µL of 10% acetonitrile aqueous solution (v/v), shaken for 1 min on a vibra mixer at 2000 rpm and centrifuged for 5 min at 3500 rpm at 4 ± 2°C. After centrifugation, the solution was transferred to an autosampler vial and 50 µL aliquot was injected into the HPLC system.

RESULTS

Method validation

The validation parameters were defined according to respective European Medicines Agency (EMA) (3, 4) and U.S. Food and Drug Administration (FDA) (5) guidances.

The limit of detection (LOD) was determined at 0.025 µg/mL, whereas the lower limit of quantification (LLOQ) was determined at 0.2 µg/mL. The accuracy for LLOQ was 101.1% (RSD = 3.75%, n = 6) within one day and 99.1% (RSD = 5.91%, n = 6) within three days.

The selectivity test, in which blank plasma samples from six different sources were analyzed, did not show any significant interferences in the places of cefuroxime and I.S. (Figs. 4 and 5).
The carry-over experiment in which blank human plasma samples were analyzed after the highest concentration calibration standards did not show any signals influencing quantification.

The calibration curve was linear in the range 0.2–12.0 µg/mL regarding the peak area ratio of cefuroxime and I.S. versus the nominal concentration of cefuroxime. The curve was obtained by a weighted linear regression analysis with \( w = 1/y^2 \) chosen according to the minimum sum of percentage relative errors (RE%) (6). The values of regression parameters for the curve, described by the equation: 
\[ y = ax + b \]
were calculated as: \( a = 0.155 \), \( b = 0.001 \) and \( r^2 = 0.991 \) (n = 6). All regression parameters were statistically significant (\( \alpha = 0.05 \), df = n – 2). The percentage relative error plotted against cefuroxime nominal concentration is presented in Figure 6.

The precision and accuracy of the method were determined within one day and within three days using three QC concentrations of cefuroxime (0.5, 6.0 and 10.0 µg/mL), each in six replicates. The accuracy results within one day ranged between 94.5 and 97.2% (RSD 0.46–1.71%), while within three days they ranged from 99.1 to 100.0% (RSD 3.53–4.52%). The extraction recovery for cefuroxime and the I.S. was in the range of 57.6–64.8% and 41.6–45.5%, respectively. The extraction recovery of cefuroxime was stable across the concentration range and also the recovery of I.S. was not influenced by the concentration of cefuroxime.

The confidence intervals were applied for the calculation of cefuroxime stability in plasma samples and during analysis (7, 8). The experiments confirmed the stability of cefuroxime in plasma samples during long-term storage (at least 72 days at \( = -14^\circ C \), n = 5), short-term storage at room temperature (at least 4 h, n = 6) and after 3 freeze-thaw cycles (at = -14°C, n = 6). Both cefuroxime and the I.S. were stable in reconstituted samples in an autosampler (at least 48 h at room temperature, n = 6). The stability of cefuroxime and the I.S. in stock and working solutions was confirmed for the desired storage period in the appropriate conditions.

The possibility of plasma samples dilution, e.g., in the case of concentration exceeding the upper limit of quantification of 12.0 µg/mL, was studied for the quality control samples at 24.00 µg/mL, which were diluted 1:1 (v/v) with blank human plasma prior to sample processing and analysis. The obtained results were multiplied by the dilution factor of 2. The result for accuracy 98.5% (RSD = 0.70%, n = 6) confirmed the dilution integrity.

The validation parameters are summarized in Table 1.

### DISCUSSION AND CONCLUSIONS

The cefalexine, which is a first-generation cephalosporin, was used as the I.S. It was previously applied as the I.S. for CEF determination by Al-
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Said et al. (1). The results obtained during the described study confirmed proper selection of the I.S.

The methods for the determination of CEF in human plasma described in the literature used an HPLC with an UV detection (1, 2) and a liquid chromatography coupled to mass spectrometry (9, 10). As expected, the LC-MS-MS methods were substantially more sensitive and allowed for cefuroxime quantification in the range 0.025–50.0 µg/mL, using only 100 µL of plasma (10). Still, much less expensive HPLC-UV methods enable sufficient sensitivity, i.e., LLOQ = 0.2 µg/mL for the pharmacokinetic studies of cefuroxime administered at standard doses.

The previously reported HPLC-UV method, assuring linearity in the range 0.25–10.0 µg/mL, used protein precipitation followed by the direct injection of supernatant into an HPLC system (1). The method described in this paper allows to determine CEF within a broader range of concentrations, i.e., 0.2–12.0 µg/mL, which may be further extended to 24.0 µg/mL after sample dilution. The proposed sample preparation procedure is slightly more complicated than in the method proposed by Al-Said et al. (1), but significantly simpler than in the method published by C. Pistos et al. (2), where protein precipitation followed by liquid-liquid extraction was used. Moreover, trichloroacetic acid used in (1) was replaced by a more user and environmentally friendly acetic acid.

The detailed study of CEF stability in plasma samples and in solutions, based on the application of confidence intervals was performed. The obtained results confirmed the stability of CEF in the conditions applied during the bioanalytical part of pharmacokinetic studies.

The full validation of the bioanalytical method of CEF determination in human plasma in the range of 0.2 – 12.0 µg/mL was performed according to EMA (3, 4) and FDA (5) guidances. The study was performed in compliance with the OECD Principles of Good Laboratory Practice (GLP) (11). All validation parameters met the acceptance criteria, that proves that the method for the determination of CEF in human plasma leads to reliable results. The method described above may be applied to pharmacokinetic studies in humans, e.g., after oral administration of CEFA tablets.

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