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Simple HPLC method for cefazolin determination in human serum – validation and stability testing

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ABSTRACT

The paper presents an HPLC method for cefazolin determination in human serum. The preparation step was based on serum protein precipitation with acetonitrile followed by supernatant evaporation and sample reconstitution in water before injection. The separation of cefazolin and internal standard cefamandole was performed at ambient temperature under isocratic conditions on LiChrosorb RP8-5 column (250 mm × 4.6 mm) using the mixture: CH_3CN:H_2O:0.5 M KH_2PO_4 (100:894:6, v/v) as a mobile phase with a flow rate of 1.5 mL/min. UV detection was performed at 272 nm with LLOQ of 0.2 μg/mL. The precision was satisfactory in the whole range tested with RSD of 2.3–12.5% (accuracy: from −2.3% to +3.6%) and of 1.7–7.1% (accuracy: from −3.5% to +1.1%) for intra- and inter-assay, respectively. The method stability was confirmed in a series of experiments including: freeze–thaw and short- and long-term stability testing. Finally, the procedure described was found resistant to potential human errors.

1. Introduction

Cefazolin (CEF) is a first generation cephalosporin which has been administered parenterally for many years in surgical prophylaxis and treatment [1–5]. Cardiosurgery is one of the most common situations when CEF is administered in practice, very often including cardiopulmonary bypass [2,6–9]. Antibiotics are generally dosed basing on protocols and regimen only theoretically corresponding to MIC levels [5,10]. Cardiopulmonary bypass surgery by its nature creates important disturbances in pharmacokinetics of drugs. That may cause inadequate antibiotic concentrations during and after cardiac operation finally resulting in ineffective prophylaxis [2,10].

We were therefore interested in a simple but reliable HPLC analytical method which may be applied for measuring CEF serum concentrations in cardiac patients.

A number of HPLC methods have been published for cefazolin determination in human serum or plasma [11]. The application on LC–MS/MS seems very attractive [12–14]. However, because of its relatively high (in μg/mL) concentrations in serum, CEF is easily measured with most popular and inexpensive UV detection. Early methods based on inconvenient preparation procedures [6,15,16], high-volume liquid–liquid extraction [17], or on-line extraction via column switching technique [18]. Other authors published papers where CEF was isolated from biological material by protein precipitation with different reagents [19–23]. These methods were worked out on quite big volumes [19,22], presented low sensitivity [21,23] or were poorly validated [20], especially in the case of methods being modifications of papers published previously [24–26].

Our aim was to develop and validate a simple and low-cost analytical procedure suitable for pharmacokinetic and TDM studies with cefazolin, which can be utilized in hospital laboratories equipped with only basic HPLC apparatus.

2. Materials and methods

2.1. Chemicals

Cefazolin sodium salt (≥98% purity) was obtained from Fluka (Buchs, Switzerland). Cefamandole sodium salt (IS) was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from PoCh (Gliwice, Poland), HPLC-grade water was purchased from J.T. Baker (Deventer, The Netherlands), and KH_2PO_4 was from Merck (Darmstadt, Germany).

2.2. Instrumentation

The HPLC isocratic system (Spectra-Physics, San Jose, CA, USA) consisted of a pump (Model P 100), a UV detector (UV 150), an injector with 20 μL loop (Model 7125i, Rheodyne, Cotati, USA) and an integrator (Chrom Jet 4400). Universal laboratory centrifuge (5417C, Eppendorf, Hamburg, Germany) and a water bath

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(LW 502, AJL Electronic, Cracow, Poland) were used for sample preparation.

2.3. Chromatographic conditions

The separation of compounds was made on LiChrosorb RP8-5 column (250 mm × 4.6 mm) protected with LiRP8-5-10C pre-column (Hichrom, Berkshire, UK). The mobile phase was a mixture of: CH_3CN:H_2O:0.5 M KH_2PO_4 (100:894:6, v/v). Final mobile phase composition was chosen after testing the resolution of standards as well as the influence of biological matrix. Detailed information is included in Section 3.1. The flow rate was fixed at 1.5 mL/min. UV detection was set at a wavelength of 272 nm. All analyses were performed at ambient temperature.

2.4. Sample preparation

A 200 μL of serum was transferred to a 1.5 mL standard Eppendorf tube, then mixed with 20 μL of internal standard (cefamandole) working solution (10 μg/mL) and vortexed for 30 s. Next, 400 μL of acetonitrile was added to precipitate proteins and the sample was again vortexed for 30 s. After centrifugation (10,000 rpm, 10 min), a 200 μL volume of supernatant was transferred to a 10 mL Pyrex conical glass tube and evaporated to dryness in a water bath at 30 °C under a stream of argon. Then the dried extract was reconstituted in 200 μL of water and a 20 μL aliquot was injected onto the column. The presented procedure was experimentally recognized as optimal and robust. Its stability was then evaluated and is presented in Section 3.3.

2.5. Calibration and solutions

Stock solutions of cefazolin (1 mg/mL) and cefamandole (0.5 mg/mL) were prepared by dissolving appropriate amounts of chemically pure substances (as sodium salt) in water. The analytes were weighted with correction for sodium salt. A stock solution of CEF was stable for 2 weeks when stored in the dark at 4 °C, while a stock solution of IS was prepared in water fresh daily. The CEF working solutions for calibration and controls were prepared from the stock solution by adequately diluting in water. Consequently, for calibration seven levels of working solution were prepared containing CEF concentrations of 2, 10, 50, 100, 250, 400, and 700 μg/mL; and for HLOQ – undiluted stock solution (1000 μg/mL) was used. Working solutions (20 μL) were added to drug-free serum (200 μL) to obtain the CEF concentration levels of 0.2, 1, 5, 10, 25, 40, 70, and 100 μg/mL. Similarly, for QC three levels of working solution were prepared containing CEF concentrations of 6, 200, and 800 μg/mL; these working solutions (20 μL) were added to drug-free serum (200 μL) to obtain the CEF concentration levels of 0.6, 20, 80 μg/mL. The following procedure was as described above for sample preparation. For the purpose of evaluating the response from the detection system – CEF aqueous solutions with concentrations of 0.2, 1, 5, 10, 25, 40, 70, and 100 μg/mL were made from stock solution corresponding to drug concentration in serum in the range of 0.56–280 μg/mL.

3. Results

3.1. Method optimization

3.1.1. Extraction

The use of acetonitrile for deproteinization of serum samples is a simple and rapid stage of preparing the samples for HPLC. That is especially attractive for drugs whose serum concentration during routine administration is relatively high (in μg/mL) thus no further sample volume concentration before HPLC is necessary in contrast to those drugs having lower (in ng/mL) serum concentration. Consequently, we could use such a procedure for CEF determination. The absolute recovery was analysed by comparing the peak areas for extracted calibration standards with those obtained from direct injection of equivalent quantities of standards. The analytical procedure based on serum protein precipitation resulted in satisfactory recoveries yielding: 74.8 ± 3.37% (n = 8) for CEF and 77.5 ± 3.03% (n = 16) for IS. The results were stable (72.9–83.0%) for CEF concentrations covering the calibration range. The choice of final conditions for extracting analytes from serum sample was extensively and carefully evaluated. Accordingly to stability requirements, the deviation of mean measured value should be within ±15% of the nominal concentration [27]. In the following experiments, the results were obtained for four repeated measurements (n = 4). In the first experiment, the same amount (corresponding to ~20 μg/mL for 0.2 mL of serum) of CEF standard was added to different volumes (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL) of drug-free serum. The peak area of IS decreased with sample volume increasing. Only a slight effect of sample volume on the finally calculated CEF concentrations (deviation from −2.48 to +7.48%) with reference to value for target volume (0.2 mL) was observed. In a similar experiment, we checked the influence of acetonitrile volume in sample precipitation step using 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL of acetonitrile and following the procedure. Generally, no significant effect was observed (deviation from −7.85 to +3.49%) with reference to value for target volume (0.4 mL) except +16.08% for the smallest volume tested (0.1 mL) for which elevated interference & sample contamination were also noted. The influence of extraction time was also examined proving that no significant difference occurred for vortexing in the range from 15 to 60 s (deviation from −0.01 to +1.84%). Satisfactory stability [deviation from −0.75 to +4.88% with reference to value for target volume (0.2 mL)] was noted when we evaporated different volumes of supernatant (from 0.1 mL till maximal volume of ca. 0.58 mL). Reaching a compromise between method LLOQ and evaporation time, we finally set supernatant volume at 0.2 mL. A separate experiment was performed for testing evaporation temperature at a range of 25–50 °C. No difference was observed for CEF concentration obtained [deviation from −0.35 to +1.97% with reference to value for chosen temperature (30 °C)], however, a rise of temperature above 35 °C, apart from shortening the evaporation time, caused the reduction of peak areas both for CEF and IS.

3.1.2. Mobile phase

The influence of acetonitrile content in mobile phase on retention times (RT) was examined experimentally. The results are shown in Fig. 1A. A rise of acetonitrile percentage resulted in shortening retention times of CEF and IS. Reasonable RTs occurred when exceeding 9% of CH_3CN, however when the percentage rose more than 11% the interference from the biological matrix made chromatography unacceptable.

Similarly, the influence of KH_2PO_4 content reflecting ionic strength on retention times was checked in a series of experiments. A rise of KH_2PO_4 content in mobile phase resulted in prolongation of CEF and IS retention times, presented in Fig. 1B. Acceptable elution (RT for IS up to 10 min) was found with phosphate below 1%. Unfortunately, disturbing interference was observed from 0 up to 0.4% and also from 0.7 to 5% of phosphate content. That finding forced us to set an analytical "window" for KH_2PO_4 percentage between 0.5 and 0.6%. It should be noticed that the interference mentioned above was significant only for CEF concentrations at low levels not exceeding 2 μg/mL.

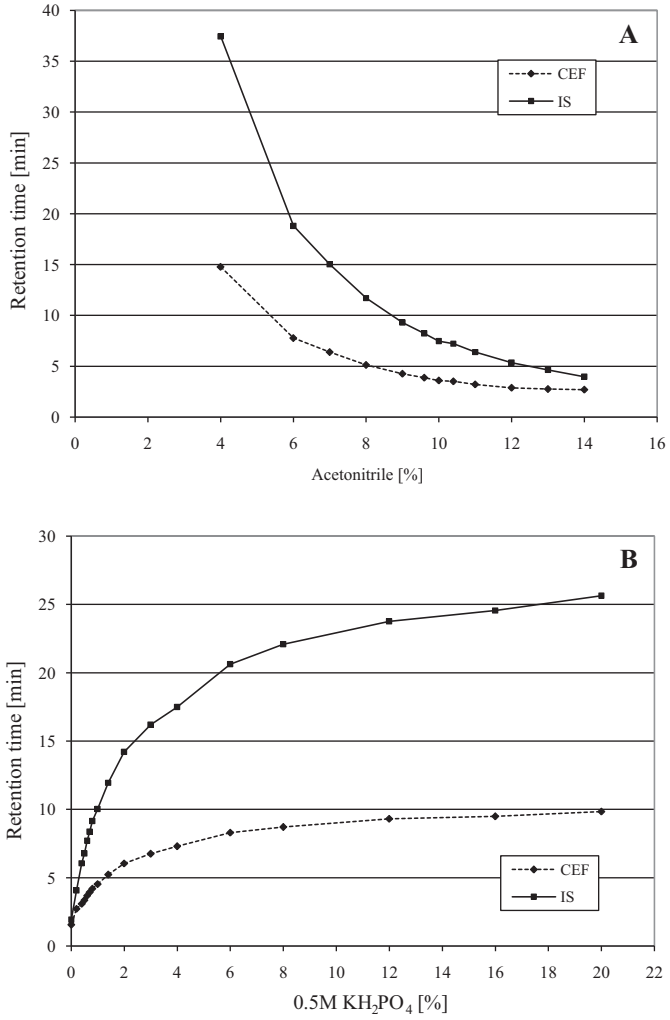

Fig. 1. The influence of acetonitrile (A) and KH_2PO_4 (B) content in mobile phase on retention of analytes.

3.1.3. Analytical wavelength

CEF and IS ultraviolet absorption profiles were drawn from a series of injections ($n = 2$) at different wavelengths. The results are shown in Fig. 2. Going down from 260 nm an interference with biological background arose in the cefazolin retention time, therefore the optimal λ was set at 272 nm due to absorption profiles and clarity of sample after extraction.

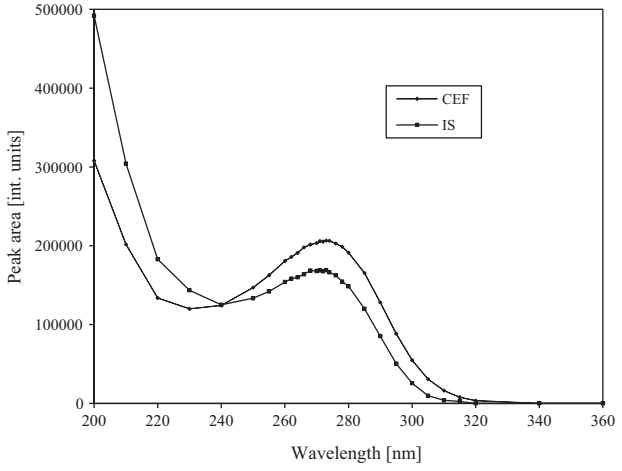

Fig. 2. Ultraviolet absorption profiles of cefazolin (CEF) and cefamandole (IS).

3.2. Method validation

The method was validated according to the incoming (into effect from 1 February 2012) European Medicines Agency (EMA) guideline (Guideline on bioanalytical method validation, EMEA/CHMP/EWP/192217/2009 of 21 July 2011) [27]. The following parameters: specificity, linearity, precision and accuracy of intra- and inter-assay, limit of detection, range and stability were established in a course of validation.

3.2.1. Specificity

CEF and IS were well separated at retention times of 3.6 and 7.3 min, respectively. The peaks were of good shape, completely resolved from one another. Twenty serum samples randomly taken from individual but not treated with CEF cardiac patients were analysed and evaluated for interference. No significant interference neither with serum matrix constituents nor with co-administered drugs was noted under finally established chromatographic conditions. The mobile phase guaranteed satisfactory repeatability of retention times. The chromatograms are presented in Fig. 3.

3.2.2. Calibration and linearity

The linearity of the detection system response was examined using specially intended CEF working solutions covering the concentrations of 0.56–280 μg/mL, as described in Section 2.5. The detector response for CEF was linear in the whole range tested ($y = 41{,}018x + 565$, $r^2 = 0.9999$).

Having confirmed the response from the UV detector, the method was calibrated and found linear up to 100 μg/mL. The calibration curves were obtained by analysing two serum samples for each of eight concentrations tested, i.e. 0.2, 1, 5, 10, 25, 40, 70 and 100 μg/mL in a single analytical run. Due to observed heteroscedasticity, the curves were calculated by a weighted linear regression analysis with $w = 1/x$ implemented for improving adjustment at low concentrations. The five calibration curves obtained were linear and described by following equations:

$$\text{CEF} = 44.387 \times F + 0.0009, \quad r^2 = 0.9996. \tag{I}$$

$$\text{CEF} = 43.235 \times F - 0.0428, \quad r^2 = 0.9984. \tag{II}$$

$$\text{CEF} = 44.222 \times F - 0.0301, \quad r^2 = 0.9992. \tag{III}$$

$$\text{CEF} = 42.257 \times F + 0.0167, \quad r^2 = 0.9990. \tag{IV}$$

$$\text{CEF} = 44.585 \times F + 0.0377, \quad r^2 = 0.9979. \tag{V}$$

where CEF stands for cefazolin concentration in μg/mL, and F is a factor obtained from peak areas: CEF/IS.

The back calculated individual concentrations of the calibration standards differed by less than 9.69% from the nominal value (±15% accepted), except for the LLOQ for which the concentrations differed by less than 15.68% from the nominal value (±20% accepted) [27]. Only for one curve (IV) the accuracy at LLOQ slightly exceeded accepted limit yielding 21.91%.

3.2.3. Precision and accuracy

The precision of the method was examined using three levels of control samples (L, M, H) as well as lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) standard samples for intra-assay ($n = 6$) and inter-assay (different days, $n = 6$). The precision was satisfactory in the whole range tested with relative standard deviation (RSD) of 2.3–12.5% for intra-assay and of 1.7–7.1% for inter-assay.

The accuracy of the method was calculated using the data from precision testing. The within-run accuracy was found between

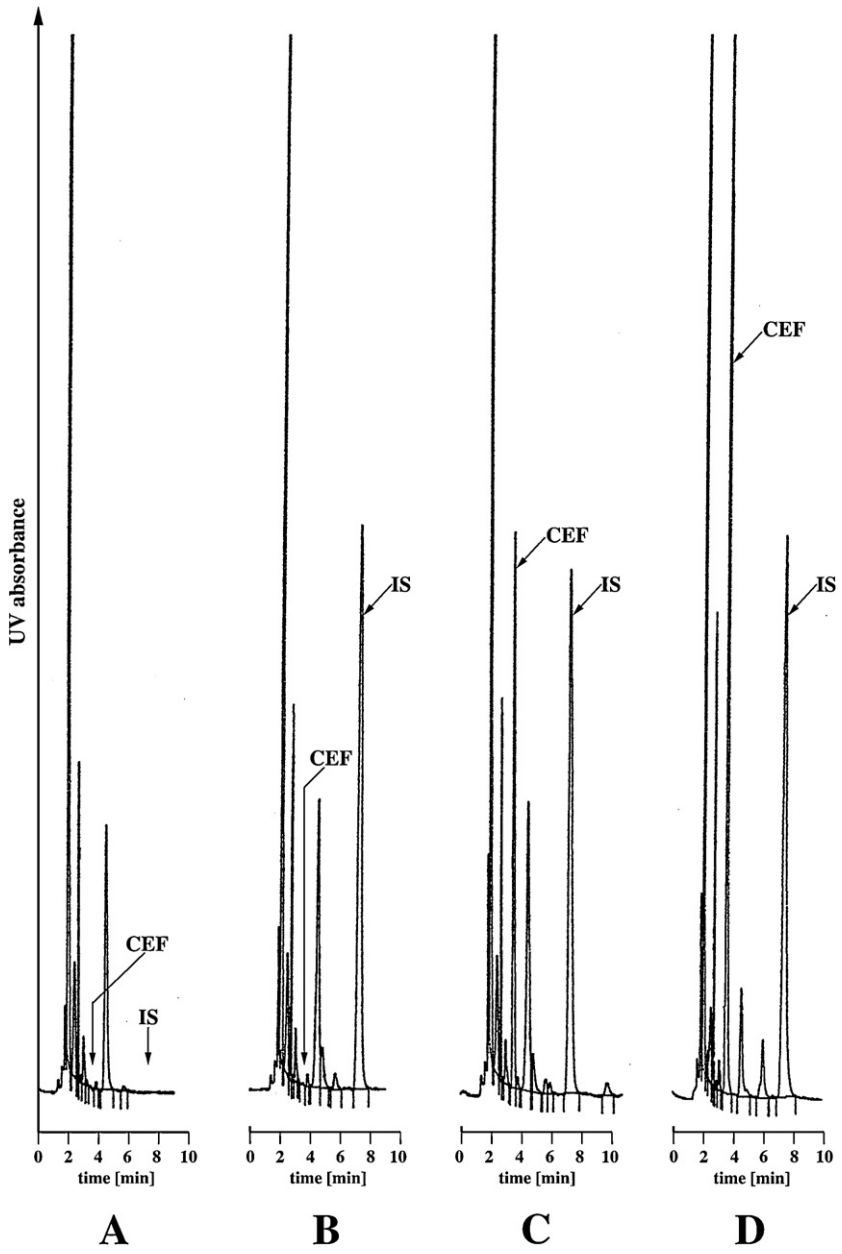

Fig. 3. Chromatograms of serum samples analysed as described in Material and methods (signal attenuation 32): (A) drug-free serum analysed without IS, (B) drug-free serum, (C) drug-free serum spiked with CEF to obtain the concentration of 20 μg/mL, (D) serum sample taken from the patient treated with cefazolin containing 41.41 μg/mL of CEF. Peaks: CEF, ~3.6 min; IS, ~7.3 min.

Table 1
Precision and accuracy of the method ($n = 6$).

Concentration added [μg/mL]	Intra-assay			Inter-assay		
	Concentration determined (mean ± SD) [μg/mL]	Precision [%]	Accuracy [%]	Concentration determined (mean ± SD) [μg/mL]	Precision [%]	Accuracy [%]
0.2 (LLOQ)	0.21 ± 0.03	12.52	+3.17	0.20 ± 0.01	7.06	+1.05
0.6 (L)	0.62 ± 0.02	3.25	+3.63	0.58 ± 0.04	6.69	−3.51
20 (M)	19.54 ± 0.47	2.39	−2.28	20.18 ± 0.66	3.25	+0.92
80 (H)	78.90 ± 1.85	2.34	−1.38	79.83 ± 1.39	1.74	−0.21
100 (ULOQ)	98.93 ± 2.74	2.77	−1.07	99.19 ± 3.26	3.29	−0.81

Table 2
Freeze–thaw stability ($n = 4$).

Cycle/storage time	Low concentration tested (mean ± SD) [μg/mL]	Stability [%]	High concentration tested (mean ± SD) [μg/mL]	Stability [%]
Initial–0 h	3.40 ± 0.06	100.00	70.77 ± 1.84	100.00
I–72 h	3.54 ± 0.12	104.09	69.61 ± 1.14	98.36
II–120 h	3.49 ± 0.04	102.64	67.31 ± 0.62	95.11
III–168 h	3.49 ± 0.26	102.68	66.27 ± 1.35	93.64

−2.3% and +3.6% and the between-run accuracy was between −3.5% and +1.1%. Detailed information is given in Table 1.

3.2.4. Limit of detection, limit of quantification, range and dilution

The limit of detection (LOD, signal-to-noise ratio = 3:1) was experimentally set at 50 ng of CEF per mL of serum. LLOQ parameter was the lowest calibration standard with acceptable accuracy and precision (Table 1). LLOQ was set at 0.2 μg/mL. The calibration covered CEF concentrations up to 100 μg/mL. For checking applicability of the method for CEF concentrations exceeding ULOQ – the dilution protocol was examined. Serum sample with CEF at a level of 500 μg/mL was 10-fold diluted with drug-free serum to obtain CEF concentration of 50 μg/mL. Eight diluted samples were then analysed, and the mean CEF concentration determined and recalculated was 448.34 ± 6.86 μg/mL yielding 89.67% of initial value. The results fulfilled EMA requirements [27].

3.2.5. Carry-over

The carry-over effect was detected by injecting blank samples (drug-free) after the highest calibration standard sample. No carry-over effect was observed.

3.3. Stability

The stability tests for newly developed and introduced analytical methods are necessary for proving its applicability especially for long-term usage. The method stability indicates how changes in an analytical procedure can influence the final results. The stability of working solutions, short- and long-term stability and freeze–thaw stability tests were performed. An analytical procedure should be stable at each stage, however the manual preparation step seems to be the most "fragile" stage. During optimization phase we also checked how significant might be potential errors in pipetting of serum, acetonitrile and supernatant, in evaporating and vortexing (data discussed in Section 3.1); finally, the ruggedness of the assay was proved in determinations made by different analysts (see Section 3.3.5).

3.3.1. Freeze–thaw stability

Freeze–thaw stability was done both with low (~3.5 μg/mL) and high (~70 μg/mL) CEF serum samples prepared from drug-free serum. The samples were determined as described, placed in the freezer (at −20 °C) and subsequently analysed after 72, 120 and 168 h. After thawing, the samples were frozen again in the same conditions. The data given in Table 2 proved CEF stability during the test.

3.3.2. Short-term stability

Serum samples with CEF added to reach the concentration ~20 μg/mL were analysed with the standard analytical procedure ($n = 4$) for testing short-term CEF stability in serum. The determination of another 4 simultaneously prepared samples was preceded by samples resting for 4 h at room temperature. The stability of dried extract in a glass tube left for 4 h after evaporation at room temperature was tested in 4 samples prepared similarly. After storage at that stage, the procedure was resumed, the samples reconstituted and injected onto the column. The standard analytical course was also interrupted just before injection for another 4 samples, which were injected onto HPLC after 4 h of resting reconstituted at ambient temperature. The results are included in Table 3. No significant changes were noted.

3.3.3. Long-term stability

The long-term stability was tested in 6-week period using CEF samples at low and high concentration. Samples were prepared using drug-free serum similarly to those for freeze–thaw tests. Before storage, the samples were analysed for recording initial CEF values. Next, the samples were transferred into plastic tubes and stored in a freezer at −20 °C. In pre-defined times (1, 2, 3, 4 and 6 weeks) the samples in the number of 4, were thawed, brought to room temperature and analysed. The stability of the analytes in serum after long-term storage (6 weeks) at −20 °C was found satisfactory. The results of long-term stability are presented in Table 4.

3.3.4. Working solution stability

The stability of CEF in aqueous solutions was established at three levels: 6, 25 and 100 μg/mL (working solutions are described in Section 2.5) corresponding to CEF concentration levels of 0.6, 2.5 and 10 μg/mL of serum, respectively.

The stability of working solutions was evaluated for 2 weeks. Aqueous solutions containing CEF were injected (20 μL) onto column when prepared. Next, the solutions were stored at 4 °C for 14 days and injected on 10 occasions during the observation period. The working solutions were stable for 7 days after being prepared (99.08, 99.49 and 99.28% of initial value for 6, 25 and 100 μg/mL, respectively). Further CEF decomposition was more evident, especially for the lowest concentration tested (90.54% at day 14). The stability is presented in Fig. 4. A stock solution (being at the same time the working solution) of IS (0.5 mg/mL) in water was freshly prepared every analytical day as cefamandole was found unstable

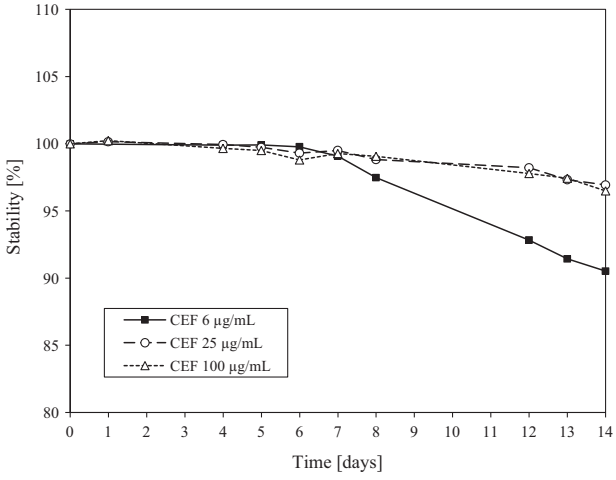

Fig. 4. Working solution stability ($n = 4$).

Table 3
Short-term stability ($n = 4$).

Procedure description	Concentration tested (mean ± SD) [μg/mL]	Stability [%]
Standard analytical procedure	19.79 ± 0.24	100.00
Sample stability before analysis	19.63 ± 0.11	99.19
Dried extract stability	20.14 ± 1.00	101.77
Reconstituted sample stability	21.52 ± 0.58	108.74

Table 4
Long-term stability ($n = 4$).

Storage time at −20 °C [weeks]	Low concentration tested (mean ± SD) [μg/mL]	Stability [%]	High concentration tested (mean ± SD) [μg/mL]	Stability [%]
0 (initial)	3.51 ± 0.07	100.00	71.76 ± 1.43	100.00
1	3.20 ± 0.09	91.20	66.64 ± 2.03	92.86
2	3.30 ± 0.08	94.07	72.16 ± 0.94	100.56
3	3.21 ± 0.06	91.47	67.70 ± 2.58	94.34
4	3.57 ± 0.05	101.75	68.18 ± 0.98	95.01
6	3.27 ± 0.09	93.29	64.70 ± 5.52	90.16

Table 5
The influence of analyst ($n = 4$).

Analyst	1	2	3	4
Concentration determined (mean ± SD) [μg/mL]	18.07 ± 0.49	18.23 ± 0.10	18.61 ± 0.29	18.78 ± 0.24
Deviation from the mean [%]	−1.90	−1.03	+1.03	+1.95

during storage at 4 °C. This solution was approved for analytical run if deviated by less than 2%.

3.3.5. Ruggedness

Finally, four analysts were asked to perform the whole procedure simultaneously but independently. Mean values differed by less than 2%. Detailed information is given in Table 5.

4. Discussion

The presented method for CEF in serum was developed and validated as an economic analytical tool for pharmacokinetic studies and for antibiotic monitoring. Necessary HPLC equipment is truly not sophisticated and the manual preparation is logical and simple. Robustness may be further improved by the use of autosampler. Classic liquid–liquid extraction is not efficient for CEF [23], SPE or column extraction looks more rational [18,28]; yet, protein precipitation is, in our opinion, the method of choice due to its efficiency, repeatability and overall simplicity. Sample pre-treatment and mobile phase preparation consume mostly water and demand only relatively low amounts of acetonitrile.

The parameters of the method obtained during validation verified the assay positively. The range is optimal for low dosage of CEF applied in clinical practice. However, the range may be inadequate for some samples taken early after iv. CEF administration, but it may be safely resolved using dilution protocol or by setting higher ULOQ standard for calibration. The precision and accuracy are satisfactory both with regard to EMA regulations [27] and to pharmacokineticists' requirements.

It is also important if the method defends successfully against factors that may disturb it during clinical usage. Stability tests proved excellent repeatability and performance of the procedure presented. It was applied to measure CEF in more than 400 serum samples from a pharmacokinetic trial examining the influence of cardiopulmonary bypass on CEF disposition. From the developmental stage we noticed that the procedure may be easily adapted for other cephalosporines – only minor shifts in mobile phase composition allow a satisfactory resolution and uninterrupted by interfering compounds to be obtained.

5. Conclusion

The HPLC–UV method described for cefazolin determination in human serum was established in course of validation as precise, accurate and stable. This simple procedure may be recommended for basically equipped laboratories for pharmacokinetic studies and for therapeutic drug monitoring.

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