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Application of capillary zone electrophoresis to the analysis and to a stability study of cephalosporins

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Abstract

The applicability of capillary zone electrophoresis (CZE) for the determination of cephalosporin antibiotics has been studied. In the case of the separation conditions optimised for fourteen cephalosporins, the precision of migration times was smaller than 1.3% RSD, and the values of the limit of detection ranged between 0.42 and 1.62 μg/ml. The proposed CZE method was applied to study the stability of cephalosporins in water at different temperatures (+25, +4 and −18 °C). It was established that the degradation of most cephalosporins was not higher than 20% at room temperature within 4 h of dissolution of these antibiotics.

Keywords: Cephalosporins

1. Introduction

Nowadays the cephalosporins can be considered as one of the most important and most frequently used group of antibiotics in medicine. The cephalosporins are semisynthetic antibiotics having a broad spectrum for Gram-negative bacteria. The cephalosporins are derived from cephalosporin C, which is one of the fermentation products of *Cephalosporium acremonium*. This basic compound (7-aminocephalosporanic acid) consists of a β-lactam ring fused with dihydrothiazine ring. The side chains linked to carbon-7 position could improve the stability and the effectiveness of the derivatives, however the side chains linked to carbon-3 position may better the pharmacokinetic characteristics.

Capillary electrophoresis (CE) has proved to be a powerful technique for the analysis of pharmaceuticals. It is used in many industrial pharmaceutical companies and applications including the determination of related impurities, main component assay and drug residue analysis [1]. Because CE is still a rapidly developing technique, many developments and applications appear year by year. In last few years some works on the determination of cephalosporins by means of capillary zone electrophoresis (CZE) [2–4] and micellar electrokinetic capillary chromatography (MECC) [5–9] have been published. While MECC can be especially useful for the determination of drugs in samples having a high protein content (clinical samples, biofluids) reducing the disadvantageous matrix effects caused by organic materials, CZE through its simplicity and operation stability could be very advantageous for pharmaceutical determinations. So far the CZE has been

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used to analyse a single cephalosporin compound and its metabolite(s) or only a few different cephalosporins simultaneously [2–4], but the MECC was the method that was applied to separate numerous cephalosporins (six components in Ref. [8], nine components in Ref. [5]) at the same time.

In the present work, the fourteen most frequently used cephalosporins were analysed by means of CZE. A mixture of cephalosporins (more than one compound at the same time) is generally not used in medicine. The reason to study a great number of cephalosporins was to prove the applicability of CE for the analysis of cephalosporins in general. The aim of our work was also to study the solution and temperature stability of these antibiotics.

2. Experimental

2.1. Instrumentation

The CE instrument was a HP 3DCE model (Agilent, Waldbronn, Germany). In all measurements hydrodynamic sample introduction was used for injecting samples. The sample solutions were introduced at the anodic end of the capillary. Separations were performed using a polyimide-coated fused-silica capillaries of 64.5 cm (effective length: 56 cm)× 50 μm I.D. (Polymicro Technology, Phoenix, AZ, USA). The applied voltage was +25 kV. The temperature of the capillary holder was kept at 25 °C. The detection was carried out by on-column photometric measurement at 270 nm. The diode array detection system also made it possible to record the UV spectra at points (in every 0.2 s) of the electropherogram. The electropherograms were recorded and processed by CHEMSTATION computer program of 7.01 version (Agilent).

2.2. Chemicals and samples

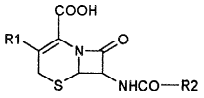
Reagents of analytical grade were obtained from various distributors. The sodium dihydrogenphosphate and sodium hydroxide for preparing buffer electrolytes were purchased from Reanal (Hungary). Benzil alcohol was used as a marker substance of electroosmosis. The sample solutions were prepared by dissolving the solid salts of cefalosporin C (Fluka), cefoxitin (MSD, The Netherlands), cefazolin, cefadroxil (Bristol-Myers, Italy), cefoperazon (Pfizer, Italy), cefamandol (Human, Hungary), cefaclor (Lilly, Italy), cefalexin (Chinoin, Hungary), cefixim (Richter, Hungary) ceftibuten (SIFI, Italy), cefuroxim, ceftazidim (Glaxo), cefotaxim (Lek, Slovenia) and ceftriaxon (Roche) in water just before the analyses. The concentration of the stock solutions of all cephalosporins were 0.15 mg/ml. The formulae and abbreviations of the analysed cephalosporins are summarized in Table 1. The capillaries were preconditioned with the buffer electrolyte for 10 min. After daily work the capillaries were flushed with 0.5 *M* NaOH (5 min), 0.3 *M* SDS (5 min) and distilled water (5 min) to remove all the components which may stick to the capillary walls. Prior to CE all the samples and buffers were filtered through a 0.45-μm syringe filter and stored in a refrigerator at +4 °C. The electrophoretic runs were performed as quickly as possible, but not later than 4 h after the solution preparation.

3. Results and discussion

3.1. Optimization of separation conditions

In CZE separation the resolving power is based on the difference in electric charge relative to molecular size. The electric charge depends on the number of carboxyl and amino groups of the component, but also on the pH of the electrolyte because the dissociation of these groups are controlled by pH. In a wide range of pH all investigated cephalosporin compounds have a negative net charge and thus under the experimental conditions their migration velocities are smaller than that of the electroosmotic flow. (Therefore the CZE separation could be achieved using detection at the negative polarity end of the capillary.) In Fig. 1 the influence of pH of the background electrolyte on the migration times of the cephalosporins is shown. With an increase in the pH the solutes reach the end of the capillary faster but the resolution becomes poor. The resolution would be improved by decreasing the pH, however the time of the measurement will be markedly lengthened (more than 50 min at pH 5). The optimum pH value for the separation was found to be about 7 because

Table 1
Structures of the studied cephalosporin antibiotics



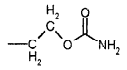
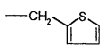
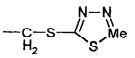
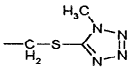
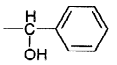
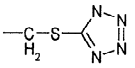
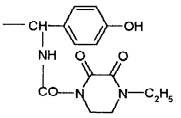
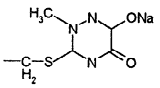
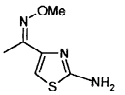
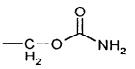
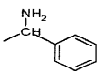
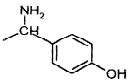
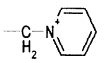
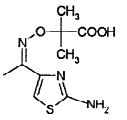
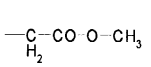
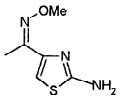
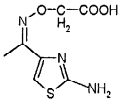
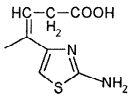
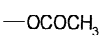
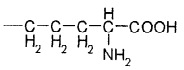
Cephalosporin	Abbreviation	R1	R2
Cefoxitin	COX		
Cefazolin	CZO		
Cefamandol	CFM		
Cefoperazon	CFM		
Ceftriaxon	CTR		
Cefuroxim	CFR		
Cefaclor	CFC		
Cefalexin	CFL		
Cefadroxil	CFD		
Ceftazidim	CZI		
Cefotaxim	CTA		

Table 1. Continued

Cephalosporin	Abbreviation	R1	R2
Cefixim	CIX		
Ceftibuten	CFB		
Cephalosporin C	CCC		

the work at this pH provides acceptable resolution of the components in a relatively short analysis time. At pH 6.8 the electrophoretic mobilities of most cephalosporins are similar and only a bit higher than that of EOF, however the CTR, CIX and CFB have higher (negative) mobilities. Studying the different buffer ionic strength (5–100 m*M* phosphate) on the resolution it was established that the buffer concentration does not have a substantial effect on the separation. When the buffer concentration increased, migration times slightly increased due to the slower EOF. As the simplest choice, 25 m*M* phosphate, pH 6.8, was applied as the buffer electrolyte. Using a 64.5-cm length of capillary all fourteen cephalosporins could be separated within 20 min. In Fig. 2 an electropherogram of a mixture of cephalosporins is shown, and for a better illustration, the peaks of 11

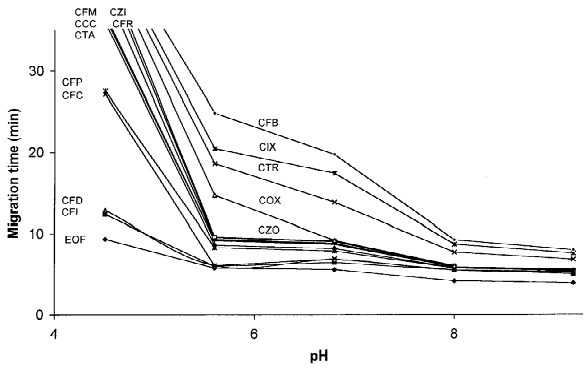


Fig. 1. Influence of pH on the separation of cephalosporins (separation conditions: capillary, 64.5 cm×50 μm I.D.; buffer electrolyte, 25 m*M* phosphate, pH 6.8; applied voltage, +25 kV; detection, UV absorption at 270 nm).

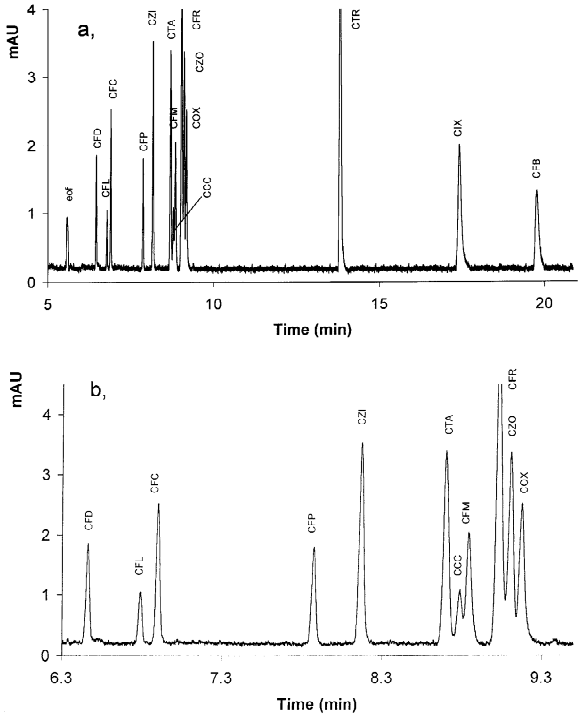


Fig. 2. Electropherograms of a mixture of 14 cephalosporins. For a better illustration two different time scales are shown. In the case of the lower figure (b) only the signals appearing between 6.3 and 9.3 min are illustrated. The concentration of each cephalosporin was 30 μg/ml; other conditions as in Fig. 1.

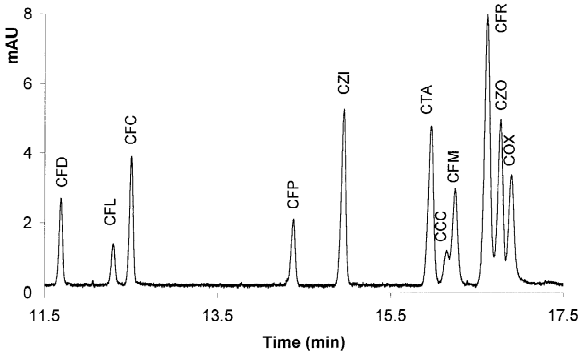


Fig. 3. Electropherogram of a mixture of cephalosporins using a 112 cm length of capillary for a comparison with Fig. 2b. The concentration of each cephalosporin was 30 μg/ml; other conditions as in Fig. 1.

cephalosporins positioned close to one another are also shown. In the case of some components (CTA, CCC, CFM, CFR, CZO, COX) baseline separation could not be achieved, but still they could be qualified and quantified with the common data collection/integration/analysis systems of the spectrophotometric detection. The use of a longer separation capillary did not bring a better resolution for the relevant components, but the migration times of the solutes were greater (Fig. 3). Each component was identified on the basis of their migration times and UV spectra. The UV spectra of the cephalosporins are relatively similar to each one another (all of them have absorption maxima at both about 200 and 270 nm), but definite, small differences can be observed in the case of all components.

3.2. Analytical performance

The proposed separation method was evaluated on the basis of precision (migration time and peak area), linearity, linear range, limit of detection (LOD) and limit of quantification (LOQ). For all these measurements the optimised separation parameters were used. The LOD values at 270 nm are summarized in Table 2. The calculation of the LODs of the compounds were based on a signal-to-noise ratio of 3. Very similar migration times and peak areas were obtained for ten repeated measurements of the fourteen compounds, the RSD values of the migration times were generally smaller than 1% RSD indicating that the separation system was stable during the measurements. The precision of the response was slightly worse (smaller than 3% RSD), because the cephalosporins slowly decompose in aqueous medium, thus the peak areas are gradually decreased over a long time. To achieve these suitable

Table 2
Analytical parameters of determination of cephalosporins

	Migration time (min)	Mobility μ_{ep} (cm^2/kV min)	LOD[a] (μg/ml)	RSD (%)[b]	
				Peak area	Migration time
CFD	6.465	−3.49	0.99	1.51	0.51
CFL	6.791	−4.56	1.62	1.50	0.30
CFC	6.901	−4.90	0.70	1.31	0.37
CFP	7.876	−7.49	0.98	2.13	0.85
CTA	8.174	−8.16	0.50	1.86	0.87
CCC	8.701	−9.23	0.53	1.72	0.89
CFM	8.787	−9.39	1.61	2.86	0.98
CZI	8.844	−9.50	0.84	1.67	0.90
CFR	9.041	−9.85	0.42	1.26	0.90
CZO	9.107	−9.97	0.53	2.08	0.91
COX	9.178	−10.09	0.71	1.93	0.92
CTR	13.844	−15.40	0.42	1.56	0.98
CIX	17.441	−17.55	0.89	1.72	1.16
CFB	19.893	−18.57	1.36	1.63	1.26

[a] $S/N=3$.
[b] $c=100$ μg/ml, $n=10$.

Table 3
Linearity regression data for cephalosporins

Cephalosporin	Regression equation	Correlation coefficient	Range (μg/ml)
CFD	$y = 0.054x - 0.011$	0.9989	2–150
CFL	$y = 0.041x - 0.061$	0.9955	5–200
CFC	$y = 0.072x - 0.0466$	0.9979	2–150
CFP	$y = 0.041x + 0.028$	0.9987	5–200
CTA	$y = 0.097x + 0.043$	0.9979	2–150
CCC	$y = 0.036x - 0.052$	0.9992	5–200
CFM	$y = 0.0514x - 0.0106$	0.9944	5–200
CZI	$y = 0.0647x - 0.022$	0.9974	2–150
CFR	$y = 0.347x - 0.115$	0.9989	1–100
CZO	$y = 0.170x - 0.143$	0.9986	2–150
COX	$y = 0.056x - 0.010$	0.9995	2–150
CTR	$y = 0.289x - 0.012$	0.9990	1–100
CIX	$y = 0.187x - 0.462$	0.9971	5–200
CFB	$y = 0.101x - 0.205$	0.9972	5–200

precision data the use of the (automatic) buffer replenishment system (refreshment of the buffer) after each measurements was applied. The individual linear regression equations (response–concentration) for each cephalosporins were calculated according to five concentrations and three replicates per concentrations (Table 3). The calibration graphs of the 14 components are shown in Fig. 4. The peak areas were found to be linear ($R^2 > 0.994$) in a concentration range specified in Table 3 in each case with a precision better than 4%. Thorough accuracy (recovery tests) studies in matrix of biological/clinical materials were performed elsewhere [10].

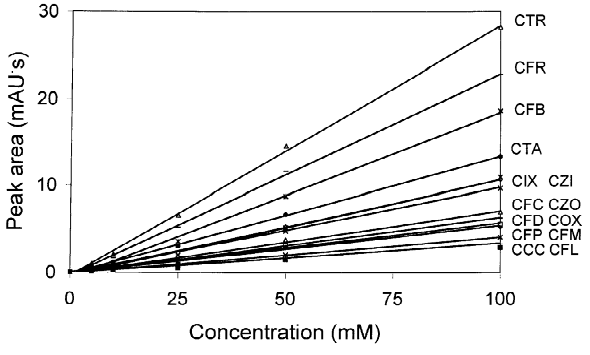


Fig. 4. Calibration graphs of 14 cephalosporins (conditions were as in Fig. 1).

3.3. Solution stability of cephalosporins

Although the stability of cephalosporin antibiotics in solid state are generally satisfying, cephalosporins dissolved in water gradually convert to different degradants by hydrolysis. After dissolution of each antibiotic in water (at room temperature) the sample solution was reinjected several times over a duration of 13 h (Fig. 5). The rate of the decrease of the concentration was similar for all cephalosporins, however, some differences can be established in hydrolytic stability of certain compounds.

In the case of four antibiotics (CZI, CFR, CTR and CTA) the reactions were followed for a longer time after their dissolution in water. In Fig. 6 it can be seen that while <2% of the original compounds of CFR, CTR and CTA remained after 260 h, ceftazidim was still present at ~20%. From the curves of Figs. 5 and 6 it is obvious that within 4 h after dissolving these antibiotics in water at room temperature their degradation (hydrolysis) is generally not higher than 20%.

The degradation of the compounds depends on the temperature of the medium, with more degradation (hydrolysis reaction) at higher temperatures. In our experiments, three temperatures (+25 °C as room temperature, +4 °C as temperature in refrigerator, −18 °C as temperature in a freezer) were tested. In Fig. 7 the solution stability diagrams of four cephalo-

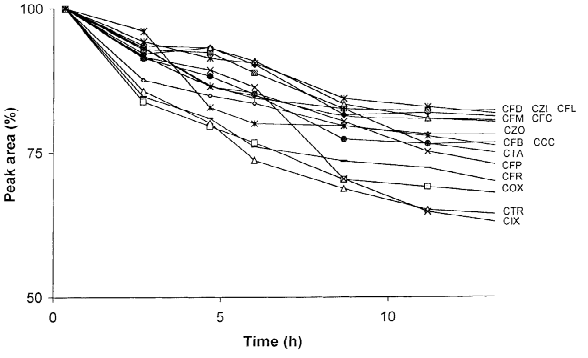


Fig. 5. Monitoring the amount of the 14 cephalosporins (main components) for up to 15 h after their dissolution in water. The initial concentration of each cephalosporin was 150 μg/ml. Peak areas obtained for the first analysis (injection of the sample in 20 min after dissolution) was regarded as 100%.

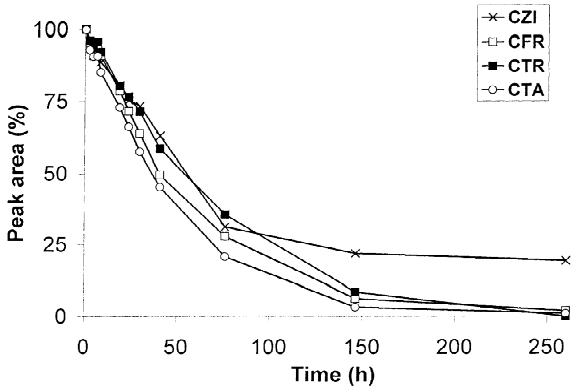


Fig. 6. Monitoring the amount of four cephalosporins (main components) for up to 260 h after their dissolution in water. The initial concentration of each cephalosporin was 150 μg/ml. Peak areas obtained for the first analysis (injection of the sample in 20 min after dissolution) was regarded as 100%.

sporins at +25 °C, +4 °C and −18 °C are shown. At +4 °C the degradation of the compounds is considerably decreased, and the stability of the components is similar as in the frozen condition. The degradation probably depends also on the pH and the matrix of the dissolution solvent, but further investigations are necessary.

The antibiotics convert to different (mostly unknown) hydrolysis products. Fig. 8 shows the time dependent diagram of the degradation of cefuroxime and the formation of its four unknown hydrolysis products (D1, D2, D3 and D4). The D1 (9.209 min) degradant is an unstable compound and it has totally decomposed by the time the original compound has disappeared in the solution. The D2 (14.916 min), D3 (11.949 min) and D4 (10.665) degradants start to form only after about 50 h after dissolution of the antibiotics. This stability investigation was facilitated by the automatic measurement repetition/time programming mode of the CE instrument.

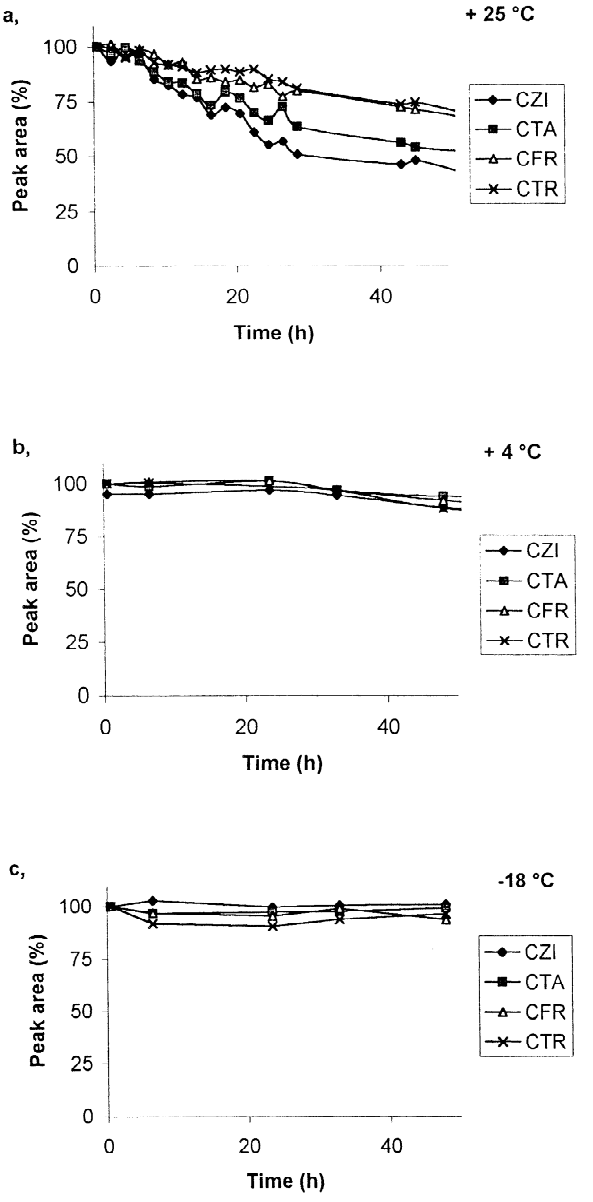


Fig. 7. Solution stability diagrams of four cephalosporins stored at different temperatures (a) +25 °C; (b) +4 °C and (c) −18 °C). Peak areas obtained for the first analysis (injection of the sample in 20 min after dissolution) was regarded as 100%.

4. Conclusion

CE has proven to be a significant and versatile technique for the analysis of the numerous cephalosporins investigated. Using the described optimized conditions the technique can be used for the analysis and identity confirmation of drugs in formulated products and also for resolving complex mixtures of drugs; other cephalosporins (not investigated in this work) can probably be likewise analyzed.

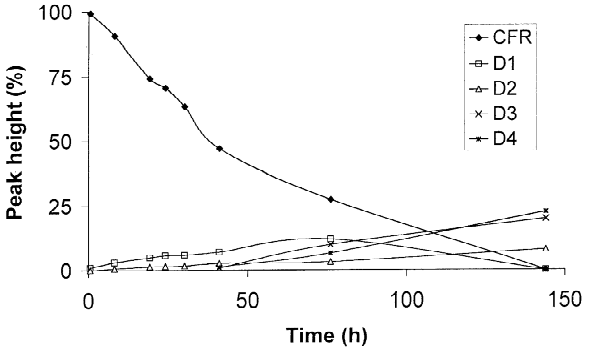


Fig. 8. Changes of the amount of the cefuroxim (CFR) and its four different degradants (D1, D2, D3, D4). The initial concentration of the cefuroxim was 150 μg/ml. Peak areas obtained for the first analysis (injection of the sample in 20 min after dissolution) was regarded as 100%.

The stability studies showed that the extent of the hydrolysis of cephalosporins in water is highly dependant on the time and the temperature. Therefore the concentration value of cephalosporin determined in a certain sample is valid just for the injection time into the CE instrument, but does not provide information about the amount of the cephalosporin at the time of sampling step (e.g. in serum of a patient at the time of taking the blood sample). In the case of the investigated 14 cephalosporins their degradation at room temperature within 4 h of dissolving these antibiotics is not higher than 20%, and smaller than 10% for most of cephalosporins. But these values are much smaller in the case of samples stored at lower temperatures. Therefore it is highly advisable that sample solutions be stored under refrigeration, especially if the sample can not be analyzed shortly after sampling or dissolution of the cephalosporin antibiotic. It was established that the the solution obtained by solving the cephalosporin compound in water (or taking a blood sample) should be stored at refrigerator (at +4 °C) and analysed within 24 h of dissolution. This is especially important for the developed method being applied for direct determination of cephalosporins in clinical samples [10].

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