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Capillary electrophoresis method for simultaneous determination of penicillin G, procaine and dihydrostreptomycin in veterinary drugs

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Abstract

Capillary electrophoresis method for identification and simultaneous determination of procaine, dihydrostreptomycin and penicillin G, present in multiantibiotic veterinary preparations, was elaborated. The influence of pH (5.0–9.75) and concentration of disodium tetraborate decahydrate in running buffers (0.02–0.1 M) as well as temperatures (25–40 °C) on separation efficacy were analyzed. For quantitative analysis, 0.08 M borate buffer (pH 8.0) at 35 °C and 15 kV were chosen. Method was validated, selectivity, precision, linearity, LOD, LOQ, accuracy and specificity of capillary zone electrophoresis (CZE) were evaluated.
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Keywords: Dihydrostreptomycin; Procaine; Penicillin G

1. Introduction

The combination of compounds belonging to different antibiotic groups is sometimes used in the veterinary pharmaceutical preparations. Most frequently aminoglycosides are combined with β-lactams [1] in order to obtain synergy interaction and to broaden the spectrum of antimicrobial activity. Benzylpenicillin might be applied in the drug as procaine salt to get slower absorption and therefore longer lasting activity (Fig. 1).

Dihydrostreptomycin sulfate (DHS) (Fig. 2) assay is a critical point for simultaneous determination of analyzed compounds, because lack of suitable strong UV chromophores or fluorophores in aminoglycoside antibiotics, makes assay very difficult.

Several methods for DHS determination in pharmaceutical preparations are available in the publications. Most pharmacopoeias have recommended the microbiological determination of DHS, however, existing other analytical methods involve separation techniques such as high performance liquid chromatography (HPLC) or capillary zone electrophoresis (CZE). In the HPLC method, ion-pair reversed phase with UV detection at 195 or 205 nm can be applied [2]. DHS, streptomycin and related substances can also be analyzed by CZE with direct UV detection at 205 nm, using borate complexation at pH 10.25 [3,4]. Indirect UV detection in the anionic mode with a reversed electroosmotic flow (EOF) by addition of fluorochemical surfactant FC 135 to the background electrolyte (BGE) was also applied [5,6].

The routine determination of benzylpenicillin, procaine and dihydrostreptomycin in multiantibiotic drug is performed by HPLC using ion-pair reversed phase and ultraviolet detection at 205, 210 or 224 nm [1].

The aim of this study was to adapt capillary electrophoresis system for determination of procaine benzylpenicillin and DHS, components of Veti-ps drug, simultaneously and to validate elaborated assay.

2. Experimental

2.1. Apparatus

Capillary electrophoresis experiments were carried out on QUANTA 4000E CE system (Waters, Milford, MA, USA) suitable to perform both hydrodynamic and voltage injection, equipped with a 30 kV power supply, an UV spectrophotometric detector, connected to a data collection

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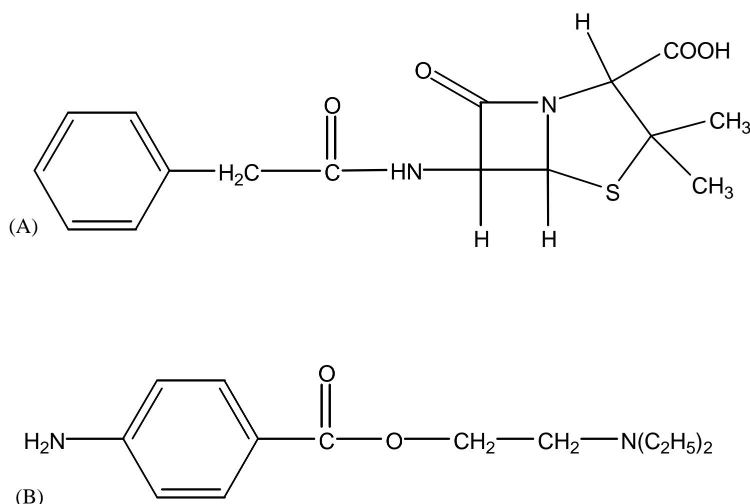

Fig. 1. Structure of benzylpenicillin (A) and procaine (B).

system. The selected detection wavelength was 185 nm using a mercury lamp with appropriate filter. Separations were performed in an uncoated fused-silica capillary Accu-Sep (75 μm i.d. × 60 cm) obtained from Waters thermoregulated at 35 °C, with applied voltage of 15 kV. The capillary was conditioned every morning by purging with 0.1 M potassium hydroxide for 5 min, water for 5 min and running buffer for 10 min. Hydrodynamic 10 s injection was performed.

2.2. Standards and reagents

Dihydrostreptomycin sulfate (working standard of activity 741 I.E./mg), penicillin G sodium (working standard of purity 99.6%), and procaine hydrochloride (working standard of purity 99.8%) were obtained from Bela-Pharm GmbH & Co. (Vechta, Germany). Procaine benzylpenicillin CRS, containing 96.3% of benzylpenicillin and 39.7% of procaine, were obtained from European Pharmacopoeia

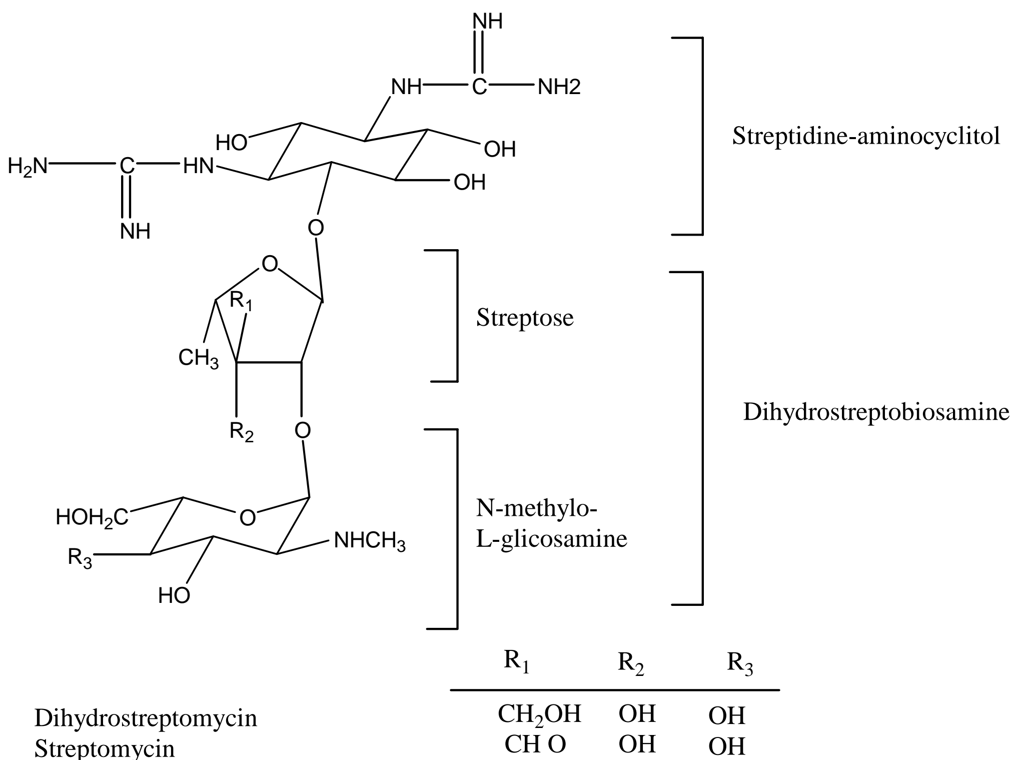

Fig. 2. Structure of dihydrostreptomycin and streptomycin.

(Strasbourg, France). Veti-ps pro-injection veterinary drug was produced by Vetimex Animal Health B.V. (AD Bladel, The Netherlands).

Monobasic sodium phosphate, disodium tetraborate decahydrate, sodium hydroxide and phosphoric acid were of reagent grade from POCh (Gliwice, Poland). Pentane-1-sulfonic acid sodium salt (PS) and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). The water used to prepare standards and samples solutions as well as running buffers, was obtained from Labconco System (Kansas City, MO, USA).

2.3. Preparation of running buffer

The running buffers for CE experiments were prepared as follows: sodium tetraborate decahydrate (0.08 M) was dissolved in about 80 ml of water. The pH was adjusted to value 8.0 with concentrated phosphoric acid and finally diluted to 100 ml with water.

2.4. Sample preparation

All reference and sample solutions were prepared in water and stored at refrigerator. Veti-ps sample (0.4 ml) was thoroughly mixed with water and diluted up to 100 with the same solvent. The following concentrations of samples were prepared: 1.0 mg/ml for DHS, 0.48 mg/ml for penicillin G and 0.32 mg/ml for procaine.

3. Results and discussion

Great efforts were undertaken to establish and optimize simultaneous separation conditions of multiantibiotic

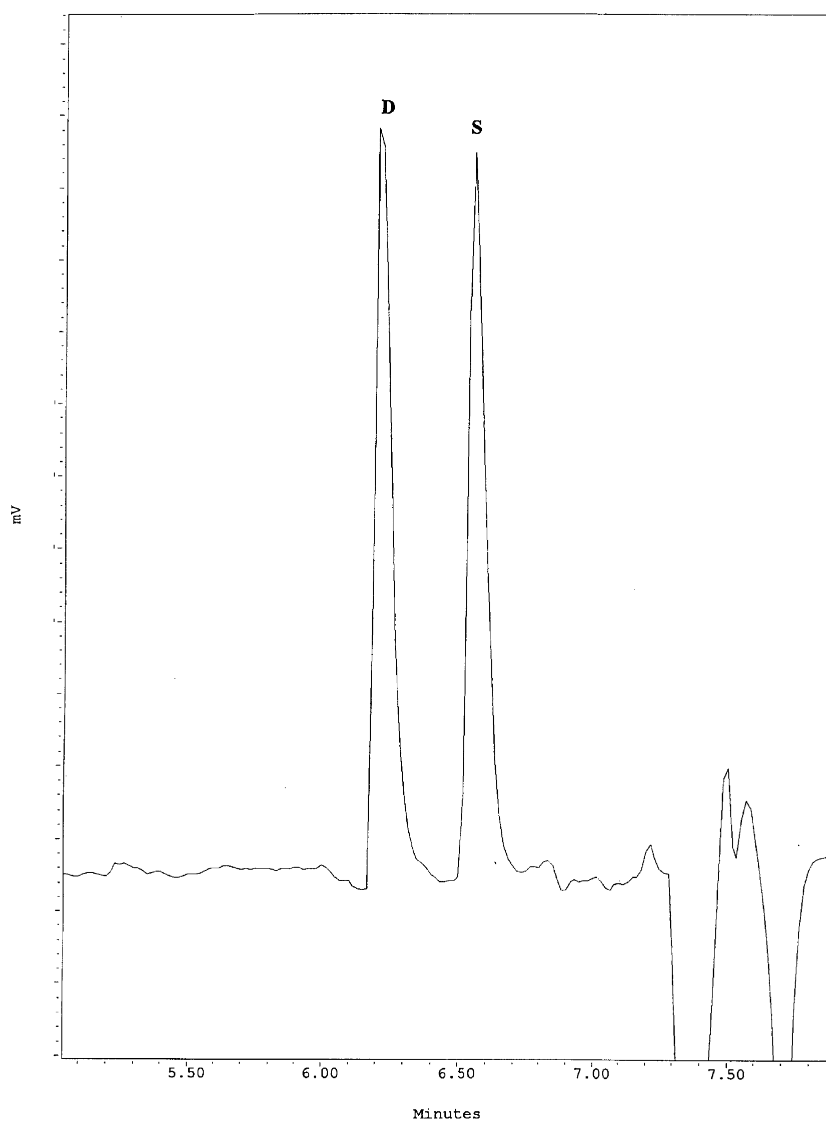

Fig. 3. Electrophoretic separation of dihydrostreptomycin (D) and streptomycin (S), obtained under the selected conditions: 0.02 M phosphate–borate buffer supplemented with pentane-1-sulfonic acid sodium salt (1.74 g/100 ml), pH 9.75, 18 kV, 25 °C.

drug active compounds. Evaluated capillary electrophoresis method could be further validated.

Several screening experiments were performed to study the influence of some electrophoretic parameters on separation of basic compounds: procaine, DHS and streptomycin, which were chosen as critical peaks. The relationship between the concentration of tetraborate, pentane-1-sulfonic acid sodium salt, the pH value and temperature for electrophoretic separation was also analyzed.

Based on our previous experiments applying CE for assay of β-lactam antibiotics [7], 0.02 M phosphate-borate buffer (pH range 5.0–9.75) was used. Satisfactory separation of procaine, DHS and streptomycin in buffer pH 8.0, only after PS (1.74 g/100 ml) addition was achieved. The best resolution for DHS and streptomycin was obtained in buffer pH 9.75. During our further studies, basing on the information, that addition of borate to the solutions of mono and oligosaccharides increases absorbance [8] and taking into consideration presence of sugar residues in DHS moiety, higher tetraborate concentrations were introduced. The separations were carried out in tetraborate buffer, since addition of the tetraborate generally improved peaks shape.

The tetraborate concentration (range from 0.02 to 0.1 M) in BGE had a significant influence on the DHS and streptomycin separation. The effect was negative for low ionic strength of BGE (DHS and streptomycin co-migrated while DHS peak was tailing) and positive for the high (0.08–0.1 M) concentration of tetraborate. The best resolution was obtained in 0.1 M tetraborate, but repeatability of peaks area was worse than in 0.08 M tetraborate buffer, because of the high generated current flow (270 μA). Therefore, 0.08 M tetraborate electrolyte, as a compromise between resolution

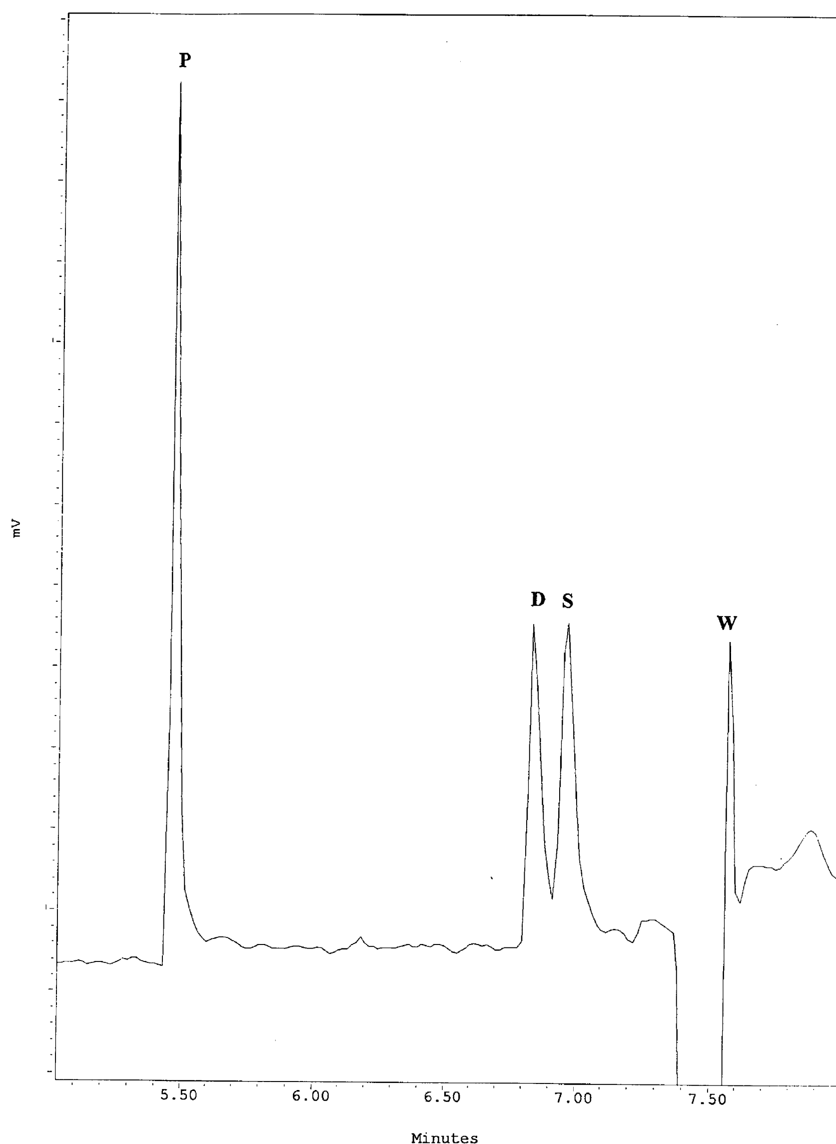

Fig. 4. Separation of procaine (P), dihydrostreptomycin (D) and streptomycin (S), obtained under the selected conditions: 0.08 M borate buffer, pH 8.0, 15 kV, 35 °C, peak from water (W).

and repeatability of peaks area, was chosen. Under these conditions more stable baseline was obtained.

The investigations were performed in the pH range 5.0–9.75. No resolution was observed between DHS and streptomycin, when lower pH buffer (5.0–7.5) was used, however, in higher pH (8.0–9.75), good separation of both compounds was achieved.

In strongly alkaline BGE buffer (pH > 9.5), procaine underwent transformation from cationic to neutral form and had the same migration time as uncharged solute (DMSO EOF marker). Therefore, further experiments were undertaken in tetraborate buffer pH 8.0.

The influence of temperature (range 25–40 °C) on the electrophoretic separation also was examined. It has proved to affect the resolution between DHS and streptomycin greatly, however, the temperature higher than 35 °C had no further influence.

Finally, two different experimental modes were chosen as the best for separations of streptomycin and DHS: 0.02 M phosphate–borate buffer with 1.74 g/100 ml pentane-1-sulfonic acid sodium salt, pH 9.75, 18 kV, 25 °C and 0.08 M borate buffer (pH 8.0), 15 kV, 35 °C (Figs. 3 and 4). For quantitative analysis 0.08 M borate buffer (pH 8.0) at 35 °C and 15 kV were used (Fig. 5).

As a part of validation of CZE method according to European Pharmacopoeia, following parameters were evaluated: selectivity, precision, linearity, LOD, LOQ, accuracy and specificity.

The linearity of the method was estimated by analyzing series of DHS and procaine benzylpenicillin standards. In this study, five agents concentrations were chosen, ranging from 0.025 to 2.5 mg/ml for procaine benzylpenicillin and from 0.05 to 1.25 mg/ml for DHS. Each sample concentration was injected twice. The assay was repeated three times.

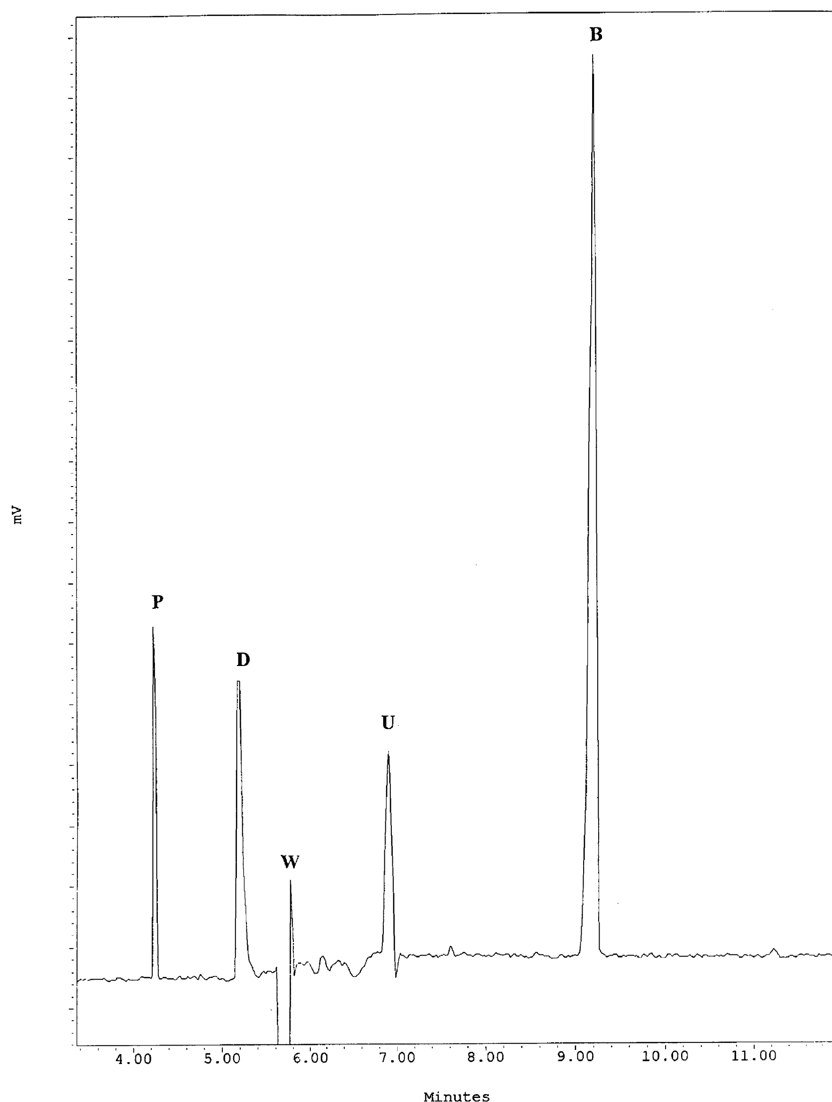

Fig. 5. Typical electropherogram obtained under the finally chosen electrophoretic conditions: 0.08 M borate buffer, pH 8.0, 15 kV, 35 °C, dihydrostreptomycin (D), procaine (P), penicillin G (B), peak from water (W), unknown (U).

Table 1
Results of procaine, dihydrostreptomycin sulfate (DHS) and benzylpenicillin analyses

Parameter	Procaine	DHS	Penicillin G
Repeatability of migration time[a] (R.S.D., %)	0.18, 0.27, 0.71	0.10, 0.32, 0.87	0.85, 0.39, 0.67
Repeatability of correct area[a] (R.S.D., %)	1.73, 1.71, 1.44	1.13, 2.88, 1.23	0.50, 0.23, 0.39
Migration time[b] (min)	About 4.5	About 5.5	About 10.5
Correlation coefficient (*R*)	0.997	0.998	0.998
Quantification limit (mg/ml)	0.01	0.05	0.012
Detection limit (mg/ml)	From 0.003	From 0.020	From 0.0035

[a] Three cycles of five repetitions were performed.
[b] During investigations, some deviations of migration times for all substances were noticed, because of the capillary exchange. Analyzed substances migrated faster in the new capillary.

Table 2
Robustness of migration times and peaks areas upon the pH change of 0.08 M borate buffer

Analyzed agent	pH 7.5	pH 8.0	pH 8.5
Procaine			
Time	4.25 (0.51)	4.25 (0.18)	4.45 (0.90)
Peak area	112833 (1.13)	115135 (1.73)	113867 (1.00)
DHS			
Time	5.36 (0.48)	5.21 (0.10)	5.20 (1.28)
Peak area	240400 (1.64)	234123 (1.13)	211924 (0.95)
Penicillin G			
Time	9.99 (0.08)	9.33 (0.85)	9.42 (0.96)
Peak area	868726 (0.64)	787854 (0.50)	678480 (0.84)

Each sample injected five times. R.S.D. (%) are listed in parentheses.

Table 3
Robustness of migration times and peaks areas upon the change of borate concentration at pH 8.0.

Analyzed agent	Borate concentration		
	0.07 M	0.08 M	0.09 M
Procaine			
Time	4.49 (0.33)	4.45 (0.71)	4.56 (0.81)
Peak area	121253 (0.90)	117412 (1.44)	113089 (1.31)
DHS			
Time	5.51 (0.27)	5.50 (0.87)	5.56 (0.97)
Peak area	240670 (0.90)	224996 (1.23)	216282 (3.94)
Penicillin G			
Time	10.34 (1.73)	10.36 (0.67)	10.61 (0.74)
Peak area	885873 (0.75)	824050 (0.39)	78946 (0.97)

Each sample injected five times. R.S.D. (%) are listed in parentheses.

The following, very high correlation coefficients of 0.997, 0.998, and 0.998 for procaine, DHS and benzylpenicillin, respectively, were calculated.

Detection limit defined as signal to noise ratio of 3:1, was 0.003 mg/ml for procaine, 0.02 mg/ml for DHS and 0.0035 mg/ml for benzylpenicillin. The quantitation limit defined as signal to noise ratio of 10:1, was 0.010 mg/ml for procaine, 0.012 mg/ml for benzylpenicillin as well as 0.05 mg/ml for DHS. Determined parameters are enclosed in Table 1. Instrumental precision was calculated from five consecutive Veti-ps solution injections. Veti-ps analysis showed good migration times and peak areas repeatability for each of analyzed substance.

During further experiments, robustness—influence of deliberate small changes of buffer pH value and tetraborate concentration on the method performed—was tested. Introduced changes varied from pH 7.5 to 8.5 (Table 2) and concentration of tetraborate from 0.07 to 0.09 M (Table 3).

During these investigations, some deviations of migration times for all substances were noticed. The highest deviations, concerning peaks area were observed for benzylpenicillin.

The specificity of the method was confirmed by analysis of additive substances, which are present in the Veti-ps preparation. Absence of interfering peaks on electropherograms confirmed the specificity of the elaborated

Table 4
Determination of active compounds of Veti-ps preparation by CE, performed independently by two analysts in two assays

	Procaine		DHS		Penicillin G	
	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
Mean[a]	73.78	73.00	163.33	161.02	103.33	103.03
S.D.	0.72	1.41	3.28	3.25	0.48	0.73
R.S.D. (%)	0.97	1.93	2.01	2.02	0.46	0.71
Mean from two assays	73.39		162.18		103.18	
S.D.	1.13		3.31		0.59	
R.S.D. (%)	1.54		2.04		0.58	

[a] Number of samples is five.

Table 5
Comparison of CE and HPLC methods for determination of active compounds of Veti-ps preparation

	Procaine		DHS		Penicillin G	
	CE	HPLC	CE	HPLC	CE	HPLC
Mean[a]	73.78	76.80	163.33	159.39	103.22	97.89
S.D.	0.72	0.56	3.28	1.67	0.48	0.77
R.S.D. (%)	0.97	0.63	2.01	1.05	0.46	0.68

[a] Number of samples is five.

method. Additionally, the specificity was confirmed by electrophoresis of the degradation compounds of procaine benzylpenicillin, which were created during: acid and alkaline hydrolysis, high temperature (60 °C) treatment, addition of H_2O_2, sonication and UV radiation. We observed that procaine benzylpenicillin was well separated from impurities under the established conditions. DHS possess different degradation substances, however, only those containing guanidine groups like: streptidine, mannosidodihydrostreptomycin (DHS B) and streptomycin, could be detected by UV methods [2]. According to the European and US Pharmacopoeias, only streptomycin content should be estimated. Under the established electrophoresis conditions, DHS and streptomycin peaks were well separated.

Independent assays performed by two analysts on different days proved good repeatability and intermediate precision (Table 4). As a final step, comparable assays of examined drugs by CE and HPLC methods were performed (Table 5). Contents determined by CE and HPLC in Veti-ps showed differences. The HPLC method (manufacture specification) was not validated during this study, which may explain why this value differs significantly. Nevertheless, all results obtained either by CZE or by HPLC were situated within the specification range.

4. Conclusions

The elaborated and validated CZE method can be used for identification and determination of procaine, DHS and penicillin G simultaneously. The results obtained in this study as well as the validation performed, showed CZE as suitable method for the pharmaceutical analysis of Veti-ps pro-injection preparations.

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