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Development of a capillary electrophoretic method for the analysis of amino acids containing tablets ☆

Małgorzata Jaworska*, Zofia Szulińska, Małgorzata Wilk
Department of Biological Preparations, Drug Institute, Chełmska 30/34 Str., 00-725 Warsaw, Poland

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Abstract

Ketosteril is an enteral medicinal product indicated for prevention and therapy in chronic renal insufficiency in connection with a low protein diet. Tablets of Ketosteril contain five essential amino acids like: Lys, His, Thr, Trp, Tyr and another five amino acids in the form of their hydroxy and keto analogues as calcium salts, that are: α-ketoleucine, α-ketoisoleucine, α-ketovaline, α-ketophenylalanine and α-hydroxymethionine. The composition of Ketosteril tablets is routinely tested with three LC methods. Capillary electrophoretic method seems to be a good alternative for amino acids and their analogues determination in multicomponent pharmaceuticals because of short analysis time and the possibility to assay all components during a single run without any pretreatment. Electrophoresis was performed in 50 μm I.D. fused-silica capillaries with 65 cm distance to the detector. Capillaries were installed in Waters Quanta 4000 electrophoretic equipment with a positive power supply and on-line UV detection at 214 nm. Separations were done in a buffer containing 40 m*M* Tris and 160 m*M* boric acid titrated with NaOH to pH 10. The method developed allows the separation of all investigated analytes with an efficiency of $n=230\,000$ and 20 min analysis time. The method was applied for determination of all components of Ketosteril in commercial tablets.

Keywords: Pharmaceutical analysis; Ketosteril; Amino acids; Keto acids

1. Introduction

Ketosteril (Fresenius, Germany) is an enteral medicinal product indicated for the prevention and therapy of chronic renal failure to be used in connection with a low protein diet [1]. Ketosteril tablets contain five essential amino acids, namely lysine (Lys), histidine (His), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) in the ratio of the potato–egg pattern and another five amino acids in the form of calcium salts of their hydroxy and keto analogues: α-ketoleucine (k-Leu), α-ketoisoleucine (k-Ile), α-ketovaline (k-Val), α-ketophenylalanine (k-Phe) and α-hydroxymethionine (OH-Met) (Table 1).

Ketoanalogues are a source of nitrogen-free carbon skeletons that, following transamination, reduce nitrogen supply without eliciting the signs of malnutrition. Consequently, Ketosteril has a positive

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* Corresponding author. Tel.: +48-228-412-165x313, +48-228-515-221x313; fax: +48-228-410-652.
E-mail address: m-jaworska@il.waw.pl (M. Jaworska).

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Table 1
Composition of Ketosteril tablet

Amino acids			Keto and hydroxy analogues (calcium salts)		
Lysine (acetate)	(Lys)	105 mg	α-ketoleucine	(k-Leu)	101 mg
Histidine	(His)	38 mg	α-ketoisoleucine	(k-Ile)	67 mg
Threonine	(Thr)	53 mg	α-ketovaline	(k-Val)	86 mg
Tryptophan	(Trp)	23 mg	α-ketophenylalanine	(k-Phe)	68 mg
Tyrosine	(Tyr)	30 mg	α-hydroxymethionine	(OH-Met)	55 mg

influence on the progression of uraemic symptoms, prevents degradation of the body's own protein and reduces proteinuria [2]. In many cases the administration of Ketosteril significantly delays the need for dialysis. Apart from the fact that calcium supplementation can improve renal osteodystrophy, it has a positive clinical effect on renal hyperphosphataemia and secondary hyperthyroidism [3].

The composition of Ketosteril tablets is routinely tested according to the manufacturer's specification (not published) with LC methods as follows:

(i) ion-exchange chromatography in a pH gradient elution with postcolumn ninhydrin derivatisation using an amino acid analyser for amino acid determination excluding tryptophan (120 min run time);
(ii) isocratic RP-HPLC for tryptophan determination (12 min run time);
(iii) anion-exchange chromatography (HPAEC) for the assay of keto and hydroxy analogues (40 min run time).

Amino acid determination using ion-exchange chromatography on an amino acid analyser is a well-established and commonly employed method [4]. Its limitations include the possibility to visualise only the amino acids with a free (not blocked) amino group, relatively long column conditioning time (up to 30 min) and analysis time (up to 2 h).

Chromatographic methods for the determination of amino acids [5–7] and keto analogues [8–11] that have been published up to the present, are related to each group of the analytes separately and they are applied mostly to biological samples. Derivatization procedures are usually used for improving detectability of analytes. However it can require time consuming steps in sample pretreatment.

The relatively recently developed capillary electrophoresis (CE) offers several advantages over HPLC. Due to its speed, high efficiency and a low consumption of chemicals, CE is found to be an alternative of LC and may be used as a complementary method in the routine analysis of pharmaceuticals, being especially useful for multicomponent sample analysis [12–14]. While CE methods are quite often reported for amino acid analysis [15–18], CE applications for relevant keto acids have not been found. Moreover, in the case of amino acids their determination does not always require derivatisation [18–21].

Therefore CE seems to be a good alternative for amino acids and their analogues determination in multicomponent pharmaceuticals. The purpose of this study was to develop a single-step CE method that can be applied to the quality control of Ketosteril instead of three LC methods.

2. Experimental

2.1. Chemicals and samples

Amino acids (L-lysine acetate, L-threonine, L-histidine, L-tryptophan, L-tyrosine), α-keto analogues (sodium salts) were purchased from Sigma (St. Louis, MO, USA); tris(hydroxymethyl)-aminomethane (Tris), boric acid, *p*-aminobenzoic acid (PAB, used as internal standard, I.S.) sodium hydroxide (NaOH) and isopropanol (IPA) were purchased from Merck (Darmstadt, Germany). α-Hydroxymethionine calcium salt was obtained from Fresenius (Bad Homburg, Germany). Highly-purified deionised water was prepared using EASY Pure RF deioniser (Barnstead-Thermolyne, Dubuque, IA, USA).

The study was conducted on three batches of Ketosteril (Fresenius). Samples were prepared by elution of powdered tablets with water (1 tablet/100 ml), a 10-min long sonification in an ultrasound bath and filtering through a GD/X glass microfiber 0.45

μm filter (Whatman, Maidstone, UK). Aqueous solutions of standards of amino acids, α-keto and α-hydroxy analogues were prepared in concentrations analogous to expected in the sample solution.

Electrophoretic separations were performed in a borate buffer (100 m*M* boric acid titrated with 4 *M* NaOH) and a Tris–borate buffer at various concentrations and pH values. Tris–borate buffer was prepared as follows: boric acid solution was mixed with the Tris solution to achieve the final concentration of each component and then titrated with 4 *M* NaOH to reach the desired pH.

2.2. Instrumentation

Capillary electrophoresis was developed using Quanta 4000 (Waters, Milford, MA, USA). Data were collected with the integration software Chromax 2000 (Pollab, Warsaw, Poland) that processed the normalised peak area to compensate for minor fluctuations of the migration time.

Fused-silica capillaries from Bio-Rad (Hercules, CA, USA), 58 cm (51 cm to detector)×75 μm I.D. and 72 cm (65 cm to detector)×50 μm I.D.) were used. All the separations connected with the optimization of the method were performed using the 58 cm (51 cm to detector)×75 μm I.D. capillary. For final separation conditions, method validation and commercial samples assay, the 72 cm (65 cm to detector)×50 μm I.D. capillary was used. Separations were performed at 20 °C with a voltage of 415 V/cm (positive polarity). On line UV detection at 214 nm was performed.

The new capillary was treated for 30 min with 1 *M* NaOH, 10 min with IPA, and water. Daily conditioning with 0.1 *M* NaOH was continued for 20 min, then 5 min with water and 5 min with a separating buffer. A 2 min purge of background electrolyte between runs was applied. Analytes were introduced into the capillary by 10 s shiphoning using 10 cm elevation of the inlet side of capillary.

3. Results and discussion

3.1. Method development

Determining the pH value and the content and concentration of the separating buffer are crucial elements to the development of the separation method using CE. Due to the acidic properties of keto and hydroxy analogues a 100-m*M* borate buffer was selected as the electrolyte. A high concentration of the separating buffer was chosen to ensure satisfactory separation of α-ketoleucine and α-ketoisoleucine. This was followed by analysing separation courses at different pH values (8.5–10.3) (Fig. 1). In subsequent separations, as the separating buffer pH increased, which in turn resulted in increasing negative net charge of analytes, an increasingly better separation was observed coupled with a concurrent increase of the analysis time. The beneficial influence of increasing electrolyte pH was especially noticeable in the case of amino acids, which being anions in these conditions, display the largest variations in electrophoretic mobility. The best separation of the examined substances was achieved at pH 10 (Fig. 2). However, due to asymmetric and triangle-shaped peaks attempts were made to modify the buffer composition.

A 100 m*M* solution of Tris was used as the modifier. Electrophoretic separations of the investigated substances were analysed in buffers containing 100 m*M* boric acid and 100 m*M* Tris in various proportions (titrated to pH 10 with NaOH) (Fig. 3).

In the separation buffers with a high content of 100 m*M* Tris (60–90%) analytes displayed a similar, very short migration time, the resulting peaks were strongly tailing, which rendered their identification very difficult. Decreasing the content of Tris in the separating buffer (from 50 down to 20%) resulted in the elongation of migration times of the investigated substances and improved peak symmetry, therefore increasing the resolution and efficiency of the system. The use of an electrolyte containing 20% of 100 m*M* Tris solution and 80% of 100 m*M* boric acid solution proved optimal for the course of the separation (Fig. 4).

Due to the fact that in these conditions baseline separation of ketoleucine and ketoisoleucine peaks was not achieved, the separating buffer concentration was doubled (200 m*M* Tris+200 m*M* boric acid, 20:80, titrated to pH 10 with NaOH). However, due to the high value of the current it was necessary to employ a capillary with a lower diameter (50 μm). In order to improve the resolution the capillary was

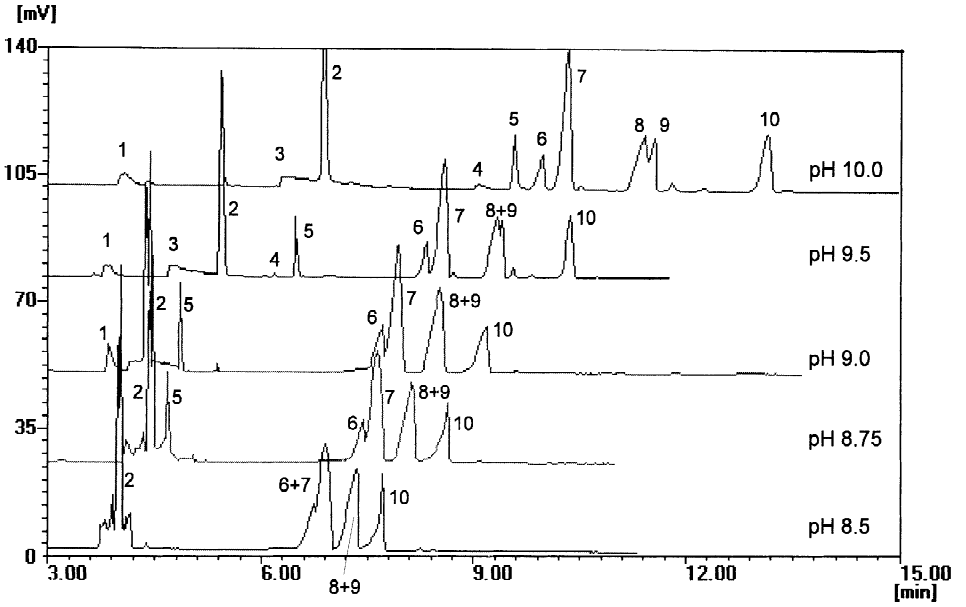

Fig. 1. Effect of pH of buffer on migration of peaks. Conditions: fused-silica capillary 58 cm (51 cm to detector)×75 μm I.D., 100 m*M* borate buffer, 24 kV, detection at 214 nm. Analytes concentration as in sample solution (see Experimental). 1–Lys; 2–Trp; 3–His; 4–Thr; 5–Tyr; 6–OH-Met; 7–k-Phe; 8–k-Ile; 9–k-Leu; 10–k-Val.

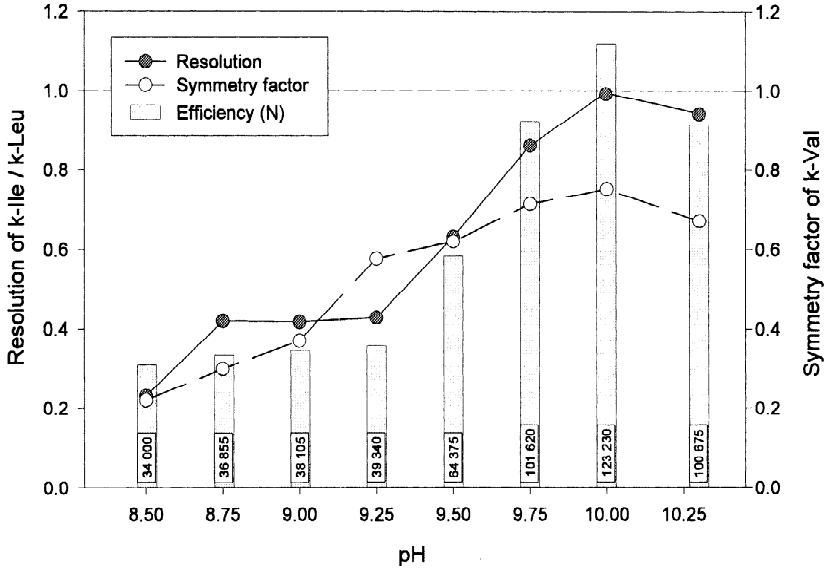

Fig. 2. Resolution of α-ketoisoleucine (k-Ile) and α-ketoleucine (k-Leu) (left axis) and symmetry factor of k-Val (right axis) as a function of pH of buffer. Plot shows optimal pH of buffer at 10 where the resolution attained maximum. Peak shape determined by symmetry factor (for the last migrating peak—k-Val) reaches the highest value at pH 10 indicating the highest efficiency of system. Bars represent efficiency as a number of theoretical plates (*N*) calculated for k-Val. Separation conditions as in Fig. 1.

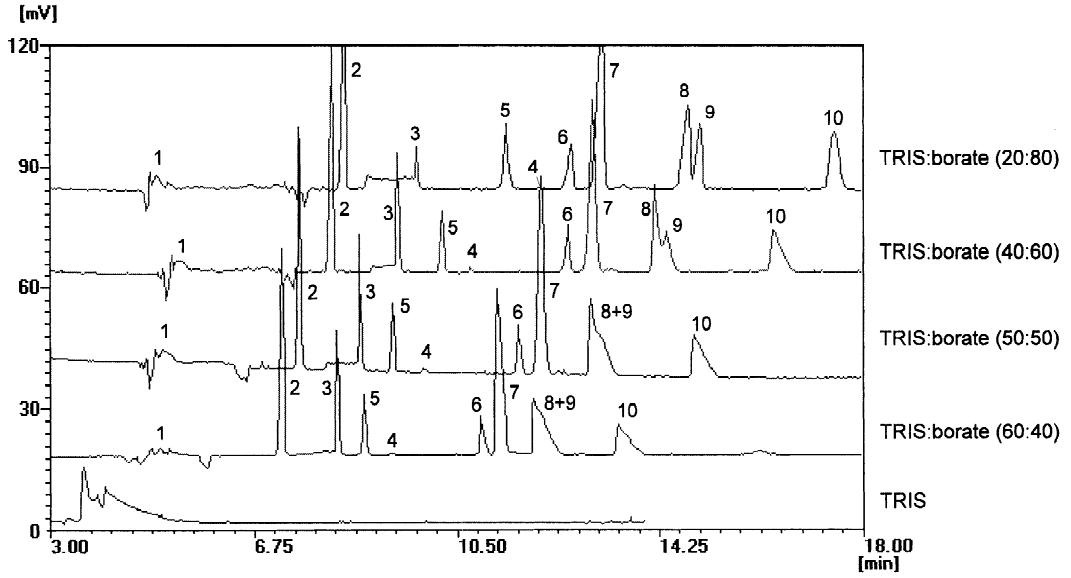

Fig. 3. Effect of buffer composition on migration of peaks. Conditions: fused-silica capillary 58 cm (51 cm to detector)×75 μm I.D., 100 m*M* boric acid+100 m*M* Tris (mixtures in various proportions, titrated to pH 10 with NaOH), 24 kV, detection at 214 nm. Analytes concentration as in sample solution (see Experimental). 1–Lys; 2–Trp; 3–His; 4–Thr; 5–Tyr; 6–OH-Met; 7–k-Phe; 8–k-Ile; 9–k-Leu; 10–k-Val.

elongated to 72 cm, which enabled us to achieve separation of ketoleucine and ketoisoleucine peaks down to the baseline with the resulting resolution being 1.25.

Systematic optimisation of electrophoretic conditions for substances under investigation resulted in the determination of the separating buffer composition: a buffer containing 40 m*M* Tris and 160 m*M* boric acid titrated with NaOH to pH 10 (Fig. 5). Using Tris as a constituent of the separating buffer reduced wall effects that very frequently hinder amino acid separation. Selection of buffer composition also caused the matching buffer ion mobility to solutes mobility which was important for minimizing peak shape distortions. Due to stacking phenomena, the high electrolyte concentration ensured narrow peaks of analytes and enabled baseline separation of ketoleucine and ketoisoleucine peaks. The efficiency of the system was estimated by counting the average number of theoretical plates, which amounted to 230 000.

The method was applied for identification components of Ketosteril by either comparing relative migration time or by spiking with the individual compounds.

3.2. Quantification and assay of commercial tablets

In order to compensate for injection errors the internal standard method was used to carry out quantifications of compounds under investigation. Signal dependence linearity range relative to the concentration and the detection limit (LOD) for the individual analytes were determined. Regression analysis of calibration curves showed good linearity in a wide range of concentrations. Results are presented in Table 2.

Validation of method was completed by checking precision and repeatability. Results are listed in Table 3.

Determination of investigated components in selected batches of Ketosteril was performed. The content of individual substances was determined for each compound by comparing the normalised peak area with the standard curve. Results of CE assays

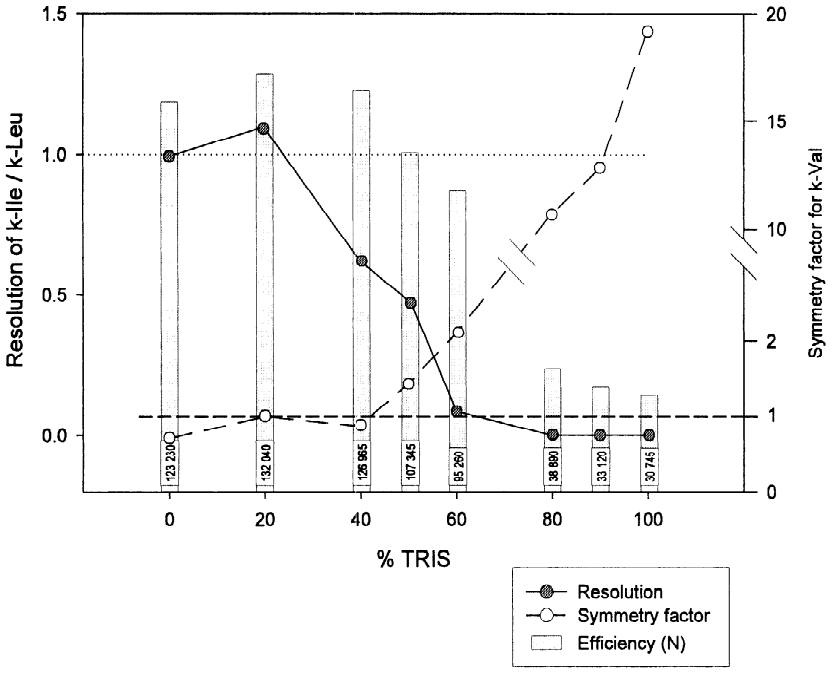

Fig. 4. Resolution of α-ketoisoleucine (k-Ile) and α-ketoleucine (k-Leu) (left axis) and symmetry factor of k-Val (right axis) as a function of buffer composition. Plot shows optimal buffer content as 20% Tris and 80% boric acid (pH 10) where the resolution attained maximum. Symmetry factor of k-Val is 1.008 for the same buffer composition. Bars represent efficiency as a number of theoretical plates (*N*) calculated for the last migrating peak—k-Val. Separation conditions as in Fig. 3.

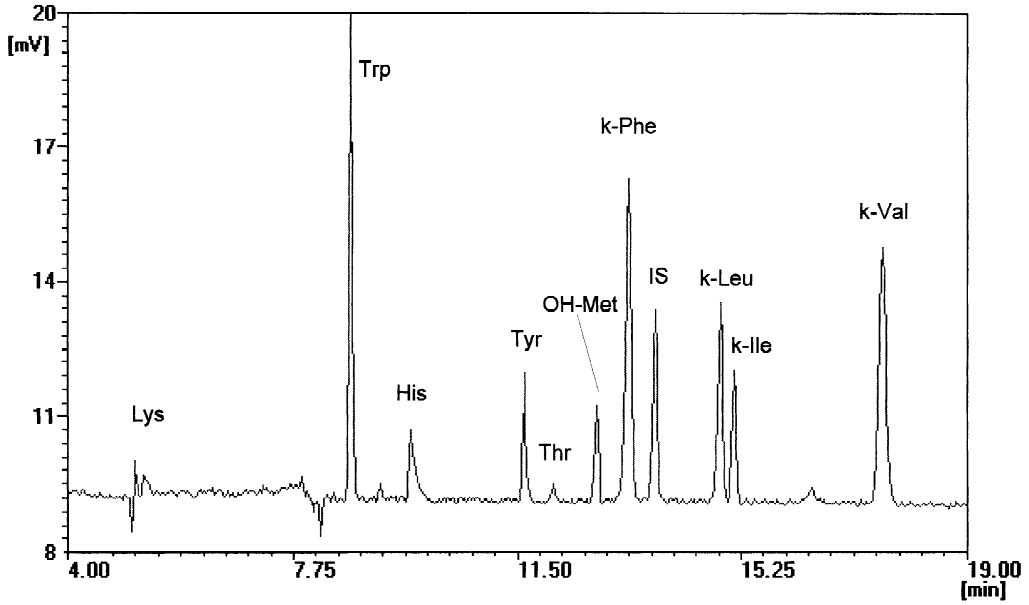

Fig. 5. Final separation conditions: fused-silica capillary 72 cm (65 cm to detector)×50 μm I.D., buffer: 40 m*M* Tris and 160 m*M* boric acid, titrated to pH 10.0 with NaOH, 30 kV, 95 μA, detection at 214 nm. Analytes concentration as in sample solution (see Experimental). I.S. *p*-Aminobenzoic acid.

Table 2
Detection limits and linearity for all components under investigation

Component	LOD (μg/ml)	Linearity range (μg/ml)	Correlation coefficient (R^2)	N
Lys	50	100–1100	0.9823	5
His	15	30–4000	0.9956	9
Thr	80	150–5 800	0.9969	7
Trp	5	10–3600	0.9978	10
Tyr	2	5–2100	0.9995	10
k-Leu	20	40–3400	0.9995	6
k-Ile	20	40–3300	0.9996	6
k-Val	20	40–3800	0.9994	6
k-Phe	15	30–4100	0.9993	7
OH-Met	20	40–3600	0.9994	6

N = number of points for calibration curves.

Table 3
Precision and repeatability of the method ($n = 5$)

Component	Precision, RSD (%)	Repeatability, RSD (%)
Lys	0.56	2.19
His	0.48	1.64
Thr	0.71	3.49
Trp	0.57	1.88
Tyr	0.71	1.58
k-Leu	0.97	2.50
k-Ile	0.91	1.84
k-Val	1.16	2.36
k-Phe	0.72	1.57
OH-Met	0.97	2.48

were compared to ones obtained with routine analytical methods (amino acid analyser, RP-HPLC, HPAEC) which were found in the manufacturer's batch release certificates. Table 4 shows the results of CE assays as an average of two independent tests for each batch, presented as a percentage of certificate data. It was found that the results obtained using CE are comparable to ones obtained using LC methods.

4. Conclusions

The method described in this study enables a simultaneous analysis of amino acids and their keto and hydroxy analogues in Ketosteril tablets. Separations were performed in a buffer containing 40 m*M* Tris and 160 m*M* boric acid titrated with NaOH to pH 10. The resolution of the closest peaks, α-ketoleucine and α-ketoisoleucine is 1.25. All components of Ketosteril are well separated with the efficiency of $N = 230\,000$ and 20 min analysis time.

Optimised separation conditions were applied for identification and also for quantification of all active ingredients. The method can be routinely used for quality control of Ketosteril.

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Table 4
The results of CE assays as an average of two independent tests for each batch, presented as a percentage of data originated from manufacturer's batch release certificate

Batch tested	Lys	His	Thr	Trp	Tyr	k-Leu	k-Ile	k-Val	k-Phe	OH-Met
1	101.4	94.9	96.2	93.6	95.5	107.0	109.0	106.5	89.7	88.7
2	97.2	93.9	98.1	97.9	96.8	103.0	102.6	103.7	98.9	104.4
3	103.6	100.0	106.7	105.8	102.8	100.1	107.9	103.9	105.2	98.4

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