Acta Poloniae Pharmaceutica – Drug Research, Vol. 60 No. 6 pp. 425–433, 2003

ISSN 0001-6837
Polish Pharmaceutical Society

CAPILLARY ELECTROPHORESIS WITH A DIAMINE EOF MODIFIER AS AN APPROACH FOR GLUTAMINE DIPEPTIDE CONTAINING FORMULATIONS

MAŁGORZATA JAWORSKA*, MAŁGORZATA WILK and ZOFIA SZULIŃSKA

Department of Biochemistry and Biopharmaceuticals,
National Institite of Public Health, 30/34 Chełmska Str., 00–725 Warsaw, Poland

Abstract: Currently, glutamine is thought to be conditionally indispensable amino acid. However, the use of glutamine in clinical practice encounters serious problems related to its limited solubility and instability in water solutions. Therefore, glutamine–containing dipeptides, which have better solubility and stability in the solution, have been introduced into the medical practice. High concentration of L–alanyl–L–glutamine in the medicinal product and thermal sterilization during the manufacturing process are the reasons of many impurities formation – the products of deamidation, racemization, cyclization etc.. The purpose of the study was to develop a single step CE method that could be applied in the purity control of L–alanyl–L–glutamine containing parenteral infusions instead of three routinely used LC methods. After systematic optimization separation conditions were selected: 100 mM borate buffer at pH 8.5 with 0.4 mM diaminopropane as EOF modifier. The method developed allows the separation and determination of the main component and the related impurities. The results show that the method is suitable for quality control of glutamine dipeptide solutions offering some advantages over routinely used LC methods.

Keywords: capillary electrophoresis, dipeptides, glutamine, diamines, purity control

Although glutamine is not an essential amino acid, it plays a special role in the body. Its concentration in blood serum and muscles is the highest of all amino acids (1,2). It is a protein synthesis activator and at the same time it inhibits protein decomposition (3). It also stimulates glycogen production, and it is a precursor of nucleotide and glutathione synthesis (4). Therefore, it is one of the most important substrates for rapidly proliferous cells, such as epithelial cells – especially in the intestine, and the immune system cells (5,6).

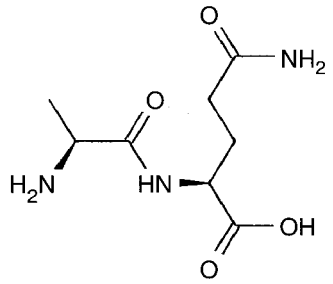
In many pathologies, high demand for glutamine in the body leads to a fast decrease of its level in the blood serum, and as a consequence, to mobilization of glutamine reserves from the skeletal muscles (5). Therefore, an adequate glutamine intake, especially in patients undergoing surgery, is essential to reduce the negative nitrogen balance, to inhibit the catabolic processes, as well as to the activation and normal function of the immune system, including the maintenance or enhancement of the integrity of the intestinal barrier (5,7,8). It has been found that glutamine administration, also as a supplement in total parenteral nutrition, leads to faster recovery and reduces the number of post–surgery complications, enhances the immune response, has a positive effect on the survival rate of patients in severe condition and reduces the convalescence time (9–12).

Currently, glutamine is thought to be conditionally indispensable, depending on the body's demand in a given condition. However, the use of glutamine in clinical practice encounters serious problems related to its limited solubility and instability in water solutions because of fast hydrolysis to glutamic acid (2). Therefore, glutamine–containing dipeptides, which have better solubility and stability in the solution, have been introduced into the medical practice.

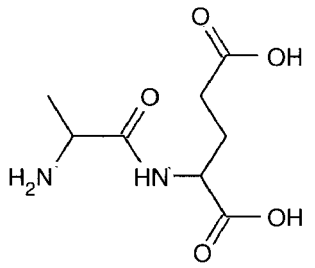
At present, there are two commercially available formulations containing glutamine dipeptides on the European market. One of them is a solution for intravenous infusions containing 20% of L–alanyl–L–glutamine (Ala–Gln), marketed in Poland. It is used as a product supplementing amino acid infusion preparations (2).

Because of high concentration of active substance in the product and the manufacturing process technology (thermal sterilization), many reactions take place in the solution leading to the occurrence of impurities – the products of deamidation, racemization, cyclization etc. The most important impurities are: L–pyroglutamic acid (pyro-Glu) having an unclear effect on the nervous

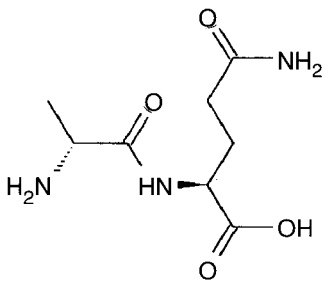
* author for correspondence: tel: (+48–22) 841–21–65, fax: (+48–22) 841–06–52
e–mail: m–jaworska@il.waw.pl



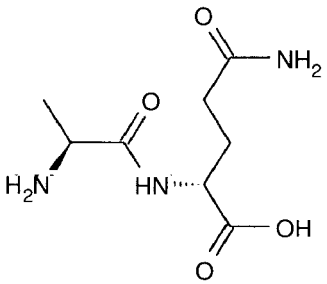
L-alanyl-L-glutamine (Ala-Gln)



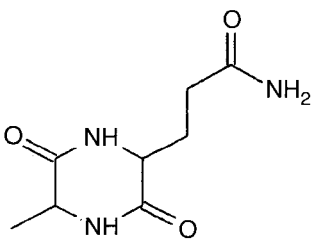
L-alanyl-L-glutamic acid (Ala-Glu)



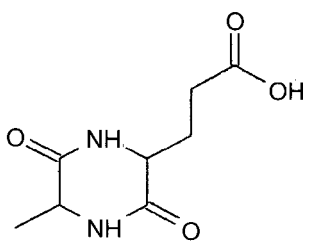
D-alanyl-Lglutamine (DL)



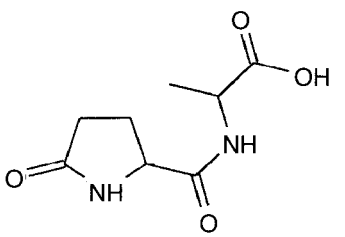
L-alanyl-D-glutamine (LD)



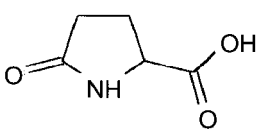
Cyclo-(L-alanyl-L-glutaminyl)
(cyclo(Ala-Gln))



Cyclo-(L-alanyl-L-glutamyl)
cyclo(Ala-Glu)



L-pyroglutamyl-L-alanine (pyroGlu-Ala)



pyroglutamic acid (pyroGlu)

Figure 1. Structures of compounds under investigation.

system (13–15) and cyclo–(L–alanyl–L–glutaminyl) (cyclo(Ala–Gln)), produced in large amounts. Other impurities present in the product are: L–alanyl–L–glutamic acid (Ala–Glu), L–pyroglutamyl–L–alanine (pyroGlu–Ala), cyclo–(L–alanyl–L–glutamyl) (cyclo(Ala–Glu)), D–alanyl–L–glutamine (DL) and L–alanyl–D–glutamine (LD) (Figure 1).

As part of the quality control of the L–alanyl–L–glutamine containing preparation, it is planned to determine, apart from the active substance, the seven mentioned impurities and other unknown, each separately and all in total. For this purpose, the following validated chromatographic methods are used:

I. determination of the active ingredient: Ala–Gln using the HPLC on a RP 18 column;

II. determination of cyclization products using the anion–exchange chromatography;

III. determination of other degradation products using the HPLC on a RP–NH_2 column.

Capillary electrophoresis has been found to be an alternative to HPLC, especially for the separation of highly charged compounds like amino acids and peptides. The main advantage of CE separation over HPLC is the fact that CE provides high speed and separation efficiency connected with low sample and chemicals consumption. In a number of cases the technique offers a single step separation of a multicomponent sample, tending to reduce the analysis cost as compared to HPLC.

Amino acids and dipeptides are a common subject of analytical research, especially by CE, mainly as far as chiral recognition is concerned (16–19). In particular, several quantitation and stability studies of Ala–Gln and related compounds using capillary isotachophoresis (cITP) (20–22) and micellar electrokinetic chromatography (MEKC) have been reported (23). Only pyroGlu and Ala–Glu were taken into consideration when purity aspects of Ala–Gln were discussed (21,22).

The purpose of the study was to develop a single step CE method that could be applied in the quality control of L–alanyl–L–glutamine containing parenteral infusions instead of three LC methods.

EXPERIMENTAL

Instrumentation

All experiments were performed on the Quanta 4000 instrument (Waters, Milford, MA, USA) with data collection by the integration software Chromax 2000 (Pollab, Warsaw, Poland) that processed the normalized peak area to compensate for minor fluctuations in the migration time. Separations were carried out at 20°C with a voltage of 350 and 500 V/cm (positive polarity). Detection wavelength was set at 185 nm.

Fused silica capillaries (75 μm ID, 60 cm total length, 53 cm to detector) were obtained from Beckman (Fullerton, CA, USA). The new capillary was treated for 30 min with 1M NaOH, for 10 min with isopropanol, and rinsed with water. Daily conditioning with 0.1M 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 was applied between the runs.

The samples were injected hydrodynamically by 10 sec siphoning with 10 cm elevation of the inlet side of capillary.

Chemicals and samples

L–Alanyl–L–glutamine dipeptide (Ala–Gln), L–pyroglutamic acid (pyroGlu), L–phenylalanine (used as internal standard, IS) were purchased from Sigma (St. Louis, MO, USA); boric acid, sodium hydroxide (NaOH) and isopropanol were purchased from Merck (Darmstadt, Germany); 1,3–diaminopropane (DAP) was purchased form Avocado (Karlsruhe, Germany); cyclo–(L–alanyl–L–glutaminyl) (cyclo(Ala–Gln)), L–alanyl–L–glutamic acid (Ala–Glu), L–pyroglutaminyl–L–alanine (pyroGlu–Ala), cyclo–(L–alanyl–L–glutamyl) (cyclo(Ala–Glu)), D–alanyl–L–glutamine (DL) and L–alanyl–D–glutamine (LD) were obtained from Fresenius AG (Bad Homburg, Germany). Highly–purified deionised water was prepared using EASY Pure RF deioniser (Barnstead–Thermolyne, Dubuque, IA, USA).

The study was conducted on five batches of Dipeptiven (Fresenius AG, Bad Homburg, Germany) containing 20% solution of Ala–Gln for parenteral infusions. The samples were prepared by dilution with deionized water: 200–fold for main component assay and 40–fold for purity determination. Aqueous solutions of standards were prepared in concentrations analogous to those expected in the sample solution.

Electrophoretic separations were performed in a borate buffer (100 mM boric acid titrated with 4 M NaOH) at various pH values. When the electrolytes with diamine modifier were prepared, 20 mM aqueous solution of DAP was added to the borate buffer to achieve the required concentration of the modifier.

RESULTS AND DISCUSSION

Method development

The lack of chromophores in amino acid molecules causes low detectability, especially in

capillary electrophoresis when the sensitivity is limited by short optical path length. In order to improve the detectability of compounds under investigation, 185 nm as detection wavelength and 75 μm ID capillary were selected. To assure a satisfactory signal to the noise ratio, the borate buffer was used as a low UV background electrolyte.

The optimum pH of the electrolyte was studied by varying the pH value from 7.8 to 10.2. Based on data from collected electrophoregrams, effective mobilities (μ_{ef}) of the solutes were calculated from the equation (**1**):

$$\mu_{ef} = \frac{L \cdot l}{V \cdot t_m} - \frac{L \cdot l}{V \cdot t_{EOF}}$$

where:
L – total lenght of capillary [cm]
l – effective lenght of capillary (to detector) [cm]
V – voltage applied [V]
t_m – solute migration time [s]
t_{EOF} – electroosmotic flow (EOF) „migration time" [s]

The influence of increasing pH of the separating buffer on the effective mobility of the analytes is presented in Figure 2. The higher the electrolyte pH was, the lower effective mobilities corresponding to protraced migration times were found for all analytes, especially for pyroGlu and Ala–Glu that are the most acidic compounds. For these substances, an inversion of migration order was observed between pH = 8.5 and 8.6.

As DL and LD are enantiomers, they showed the same mobility in the form of a single peak on the electrophoregrams (DL/LD peak). However, DL and LD are diastereoisomers in relation to Ala–Gln and a separation of DL/LD from the main peak in the achiral conditions was expected.

During the study, two pairs of peaks were found to be critical to achieve good separation of all compounds under investigation. The Ala–Gln and DL/LD pair and the pyroGlu–Ala and cyclo(Ala–Glu) pair presented similar mobilities to each other which was shown as closely situated lines on the mobility plot (Figure 2). Considering the DL/LD peak migrating close to the main component peak, it is important to achieve a sufficient resolution for good peak recognition and for the determination of the impurity at a level of 0.2% in the presence of a large amount of Ala–Gln. For the Ala–Gln and DL/LD peaks, the best resolution occurred at pH = 8.4 (R_s = 2.58). For the pyroGlu–Ala and cyclo(Ala–Glu) peaks, the best resolution was found at pH = 9.5 (R_s = 0.78), while at pH = 8.4 the resolution was 0.41.

In the following study the optimization of electrophoretic conditions has been tried to achieve by using 1,3–diaminopropane (DAP) as buffer additive. Diamines and polyamines are frequently used as electroosmotic flow (EOF) modifiers or dynamic coating agents preventing from wall adsorption of proteins (24–27).

DAP was added to the background electrolyte at concentrations 0.2 and 0.4 mM. As expected, the EOF mobility decreased with higher DAP concentrations in the buffer (Figure 3). The EOF suppression efficiency went down with increasing pH of the electrolyte due to deprotonation of the amino groups. In order to compensate for the protraced analysis time because of lower EOF, separations in

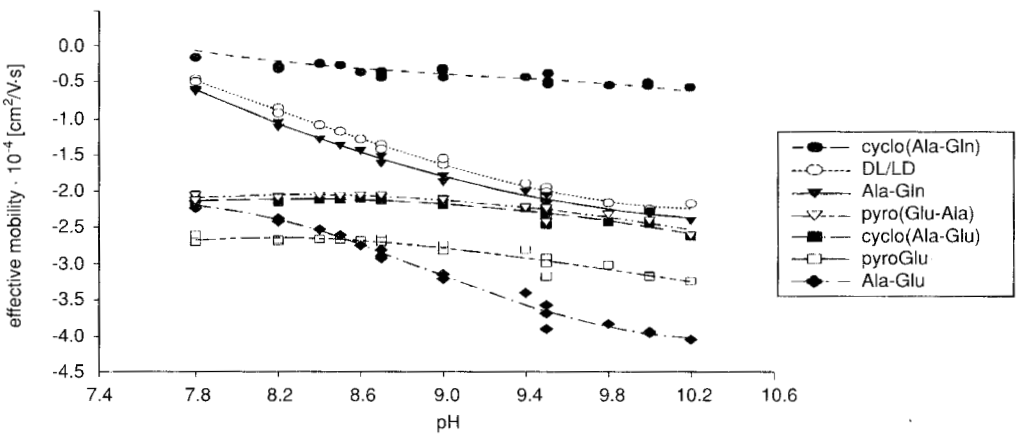


Figure 2. Effect of pH of 100 mM borate buffer on effective mobility of analytes.

buffers containing DAP were conducted with voltage increased from 350 to 500 V/cm.

Applying higher voltage caused an increase of a current in the capillary and for that reason the study of optimal separating conditions was limited to the pH range of 7.8 – 9.4 because of excessive heat generation at higher pH.

Calculating the resolution of close migrating peaks after electrophoresis in buffers containing various amounts of the modifier (from 0 to 0.4 mM), the shift of the pH value was observed where the maximum resolution of pyroGlu–Ala and cyclo(Ala–Glu) peaks occurred (Figure 4B). For the electrolyte containing 0.4 mM DAP, the best resolution was found at pH = 8.2 (R_s = 1.48). Additionally, the plot of effective mobility of the solutes in borate–DAP buffers of various pH values (Figure 5) showed a lower pH value (8.4) of migration order inversion for pyroGlu and Ala–Glu compared to buffers without DAP. Those results are probably connected with the ion–pairing effect of DAP. The pH of the borate–DAP buffer with the best resolution between DL/LD and Ala–Gln peaks was found unchanged (R_s = 3.39 at pH = 8.4).

After the experiments described above, 100 mM borate 0.4 mM DAP buffers at pH = 8.2 or 8.5 were expected to be optimal for separation of Ala–Gln and related impurities. Thus the suitability of selected buffers was evaluated by application for real samples analysis.

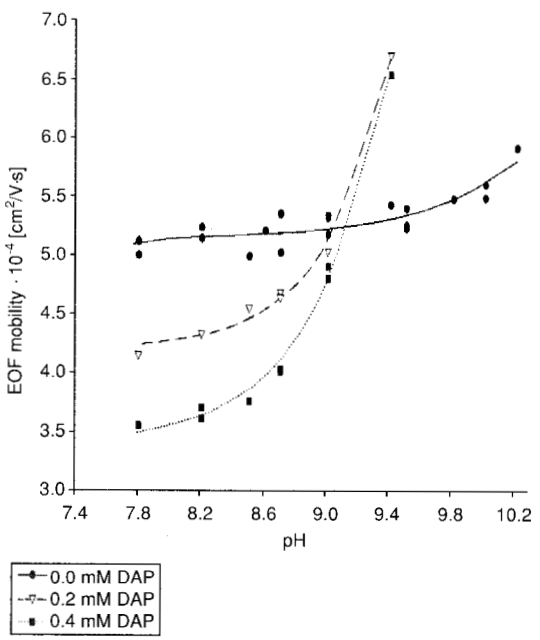


Figure 3. Effect of DAP concentration on EOF mobility. Background electrolyte: 100 mM borate.

Figure 6 presents electrophoregrams of Dipeptiven samples in borate–DAP buffers pH = 8.2 (A) and pH = 8.5 (B) where all defined impurities are indicated and a few unknown secondary peaks can be distinguished. Parameters taken into consideration for the evaluation of buffer suitability are listed in Table 1. Based on these data, 100 mM borate 0.4 mM DAP at pH = 8.5 electrolyte was selected for the analysis of commercial samples.

Application

Optimized separation conditions were applied for the analysis of Dipeptiven samples. Figure 7

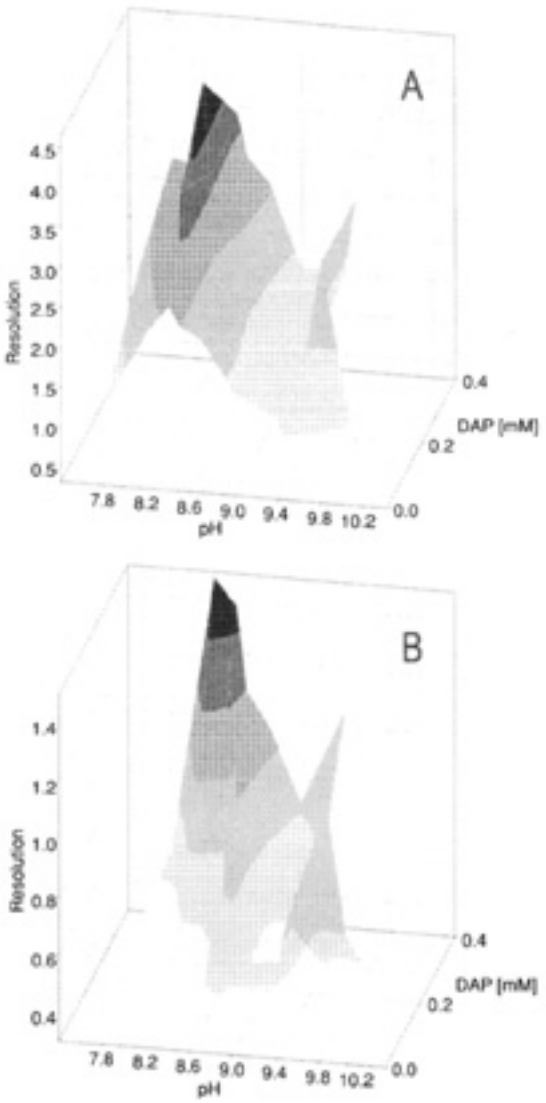


Figure 4. Resolution between two pairs of close migrating peaks: [**A**] – DL/LD and Ala–Gln, [**B**] – pyroGlu–Ala and cyclo(Ala–Glu) as a function of pH of electrolyte and DAP concentration in background electrolyte.

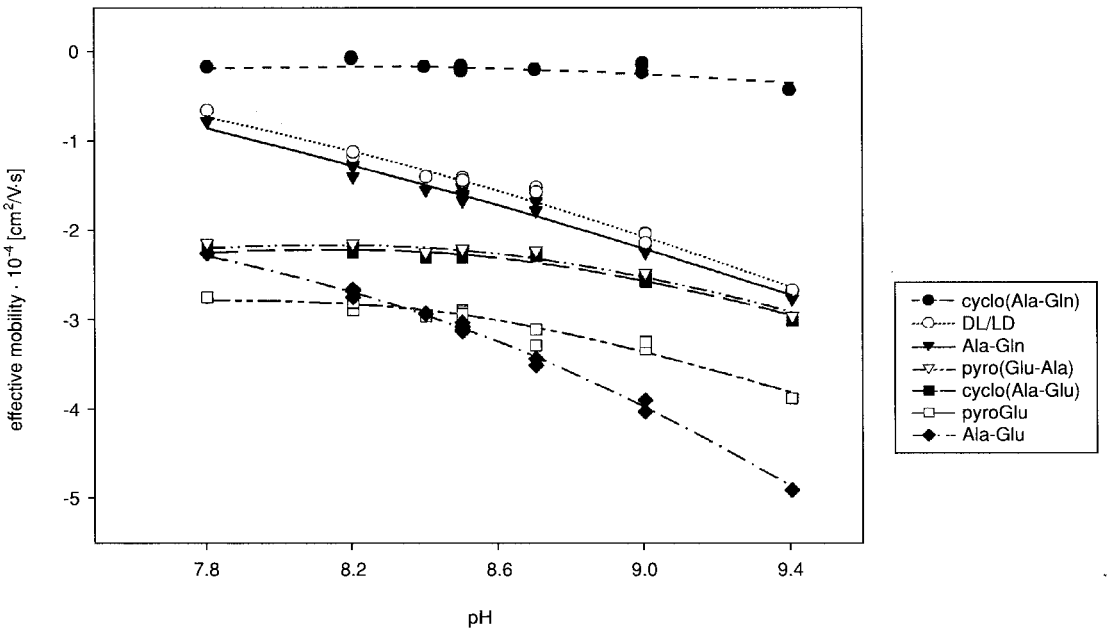


Figure 5. Effect of pH of 100 mM borate 0.4 mM DAP electrolyte on effective mobility of analytes.

Table 1. Parameters taken into consideration for selecting the most suitable electrolyte

Parameter	pH = 8.2	pH = 8.5
DL/LD and Ala–Gln resolution (R_s)	2.07	3.21
PyroGlu and Ala–Glu resolution (R_s)	1.48	1.15
Number of unknown peaks visible	8	9
Number of unknown peaks well separated, available for quantification or not interfering others	5	9
Average efficiency (N)	75 000	115 000
Run time [min]	18	15

shows an electrophoregram of a sample prepared for the L–alanyl–L–glutamine assay. Phenylalanine was used as internal standard to compensate for injection volume fluctuations which is a routine procedure in capillary electrophoresis. An external standard method for the Ala–Gln determination was applied. The linearity of the compound was tested in a range of 0.1 – 1.0 mg/ml and correlation coefficient 0.9977 was found. Detection limit (LOD) of Ala–Gln was defined as 2 μg/ml.

Purity of Ala–Gln in the final formulation was determined as percentage of the normalized peak area of each impurity in relation to the Ala–Gln peak. LODs estimated for all defined impurities are presented in Table 2. Based on the data, relative LODs connected with the Ala–Gln solution at concentration used for purity tests (5 mg/ml) were calculated. Impurity concentrations at detection limits are lower than required in the product specification.

The electrophoresis of Dipeptiven samples has shown a few unknown peaks that we tried to identify. The presence of L–alanine (Ala), L–glutamine (Gln) and L–glutamic acid (Glu) was found (Figure 6B). The identity was established by either comparing the migration times or by spiking with individual compounds. No quantitative evaluations were made for the amino acids detected. The peaks along with other undefined impurities were calculated as a total of unknown in order to ensure uniform result presentation with LC methods.

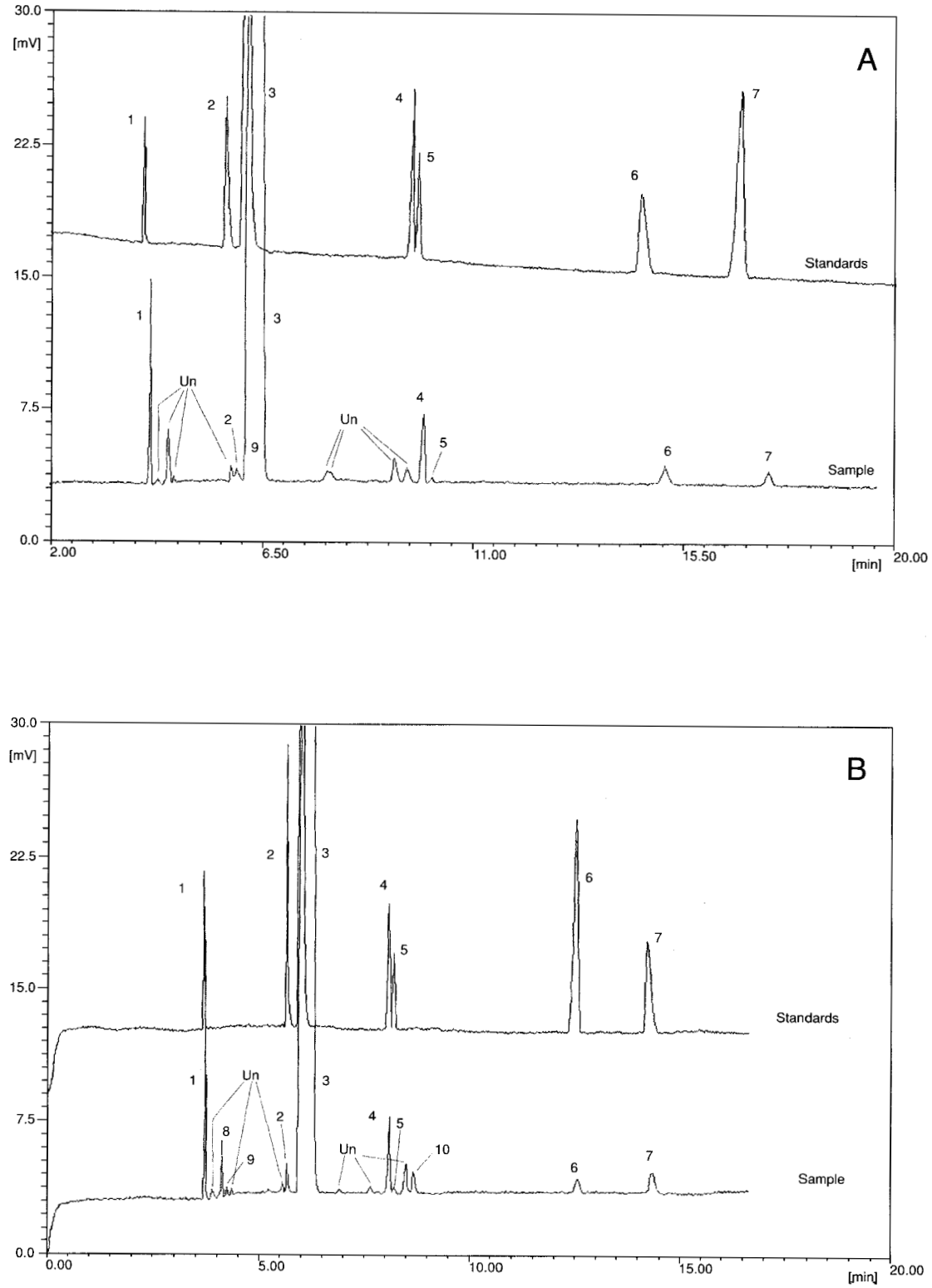


Figure 6. Separation of Ala–Gln and relative species: 60/53 cm, 75 m ID fused–silica capillary, 100 mM borate buffer 0.4 mM DAP, 30 kV, 20°C, 185 nm; **[A]** – pH = 8.2; **[B]** – pH = 8.5. Dipeptiven sample 40–fold dilution in water. 1–cyclo(Ala–Gln), 2–DL/LD, 3–Ala–Gln, 4–pyroGlu–Ala, 5–cyclo(Ala–Glu), 6–pyroGlu, 7–Ala–Glu, 8–Ala, 9–Gln, 10–Glu, Un–unknown.

Table 2. Detection limits for all defined Ala–Gln impurities

Component	LOD [μg/ml]	relative LOD in relation to 5 mg/ml Ala–Gln solution [%]	maximum permissible impurity level [% of Ala–Gln]
cyclo(Ala–Gln)	1	0.02	6.0
DL/LD (sum)	2	0.04	0.50
pyroGlu–Ala	1	0.02	0.25
cyclo(Ala–Glu)	0.5	0.01	0.05
pyroGlu	1.5	0.03	0.25
Ala–Glu	1	0.02	1.0

Table 3. The results of CE assays as an average of two independent tests for each batch, presented as percentage of data originated from manufacturer's batch release certificates

Batch tested	Ala–Gln	cyclo(Ala–Gln)	DL/LD	pyroGlu–Ala	cyclo(Ala–Glu)	pyroGlu	Ala–Glu	sum of unknown
1	97.9	90.0	190.0	105.0	100.0	120.0	114.8	108.0
2	98.8	102.6	105.0	122.5	100.0	113.3	94.3	112.0
3	101.6	107.7	100.0	132.5	100.0	140.0	115.0	112.9
4	98.8	111.5	160.0	127.5	50.0	130.0	120.0	120.0
5	99.0	89.2	87.5	90.0	100.0	100.0	93.3	96.7

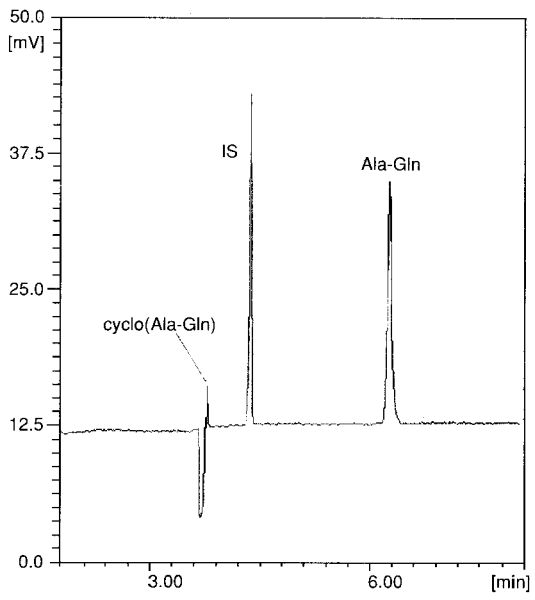


Figure 7. Electrophoregram of Dipeptiven sample prepared for main component assay. 100 mM borate buffer 0.4 mM DAP pH = 8.5, 30 kV, 20°C, 185 nm; sample: 200–fold dillution in water. IS – internal standard.

The results of the Ala–Gln assay and purity determination were compared to those obtained with LC methods (given above) which were found in the manufacturer's batch release certificates. Table 3 shows the results of CE assays as an average of two independent tests for each batch, presented as a percentage of the certificate data. It was found that the results obtained with CE are similar to those obtained using LC methods. A few CE results, in particular for DL/LD and pyroGlu, are distinctly higher than the certificate data, suggesting that the impurity formation process occurs during sample storage. No CE results for Dipeptiven purity level were out of the specification.

CONCLUSIONS

In this study, systematic investigations were reported for CE analysis of L–alanyl–L–glutamine containing formulations. The method developed allows the separation and determination of the main component and the related impurities. The separations were performed in 100 mM borate buffer at pH = 8.5 with 0.4 mM diaminopropane as]EOF modifier. In these electrophoretic conditions, the separation achieved is good enough for peak recognition and quantification. The results show that the method is suitable for quality control of glutamine dipeptide solutions offering some advantages over routinely used LC methods. Optimized separation conditions were applied for the identification and assay of all compounds under investigation in a single step analysis owing to the test procedure simplification and the analysis cost reduction.

REFERENCES

1. Cardona Pera D.: Nutr. Hosp. 13, 8 (1998).
2. Fürst P., Pogan K., Stehle P.: Nutrition. 13, 731 (1997).
3. Holecek M., Skopec F., Sprongl L., Mraz J., Skalska H., Pecka M.: Amino Acids 22, 95 (2002).
4. Yu J., Jiang Z., Li D., Yang N., Bai M.: Zhongguo Yi Xue Ke Xue Yuan Xue Bao. 20, 103 (1998).
5. Calder P.C., Yaqoob P.: Amino Acids. 17, 227 (1999).
6. Lin M.T., Kung S.P., Yeh S.L., Lin C., Lin T.H., Chen K.H., Liaw K.Y., Lee P.H., Chang K.J., Chen W.J.: Clin. Nutr. 21, 213 (2002).
7. Kircher B., Eibl G., Enrich B., Latzer K., Herold M., Niederwieser D.: Wien Klin Wochenschr. 114, 702 (2002).
8. Tazuke Y., Wasa M., Shimizu Y., Wang H.S., Okada A.: J. Parenter. Enteral. Nutr. 27, 110 (2003) .
9. van Acker B.A., Hulsewe K.W., Wagenmakers A.J., von Meyenfeldt M.F., Soeters P.B.: Am. J. Clin. Nutr. 72, 790 (2000).
10. Lin M.T., Saito H., Furukawa S., Fukushima R., Kazuhiko F., Lee P.H., Chang K.J., Chen W.J.: J. Parenter. Enteral. Nutr. 25, 346 (2001).
11. Zhou Y., Sun Y., Jiang Z., He G., Yang N.: Zhonghua Shao Shang Za Zhi 18, 343 (2002).
12. Goeters C., Wenn A., Mertes N., Wempe C., Van Aken H., Stehle P., Bone H.G.: Crit. Care. Med. 30, 2032 (2002).
13. Caccia S., Ghezzi P., Garattini S., Salmona M., Takasaki Y., Torii K.: Toxicol. Lett. 16, 225 (1983).
14. de Mello C.F., De La Vega D.D., Pizutti L.T., Lopes F.P., Rubin M.A., Homerich J.G., Melo C.R., Somer J.E., Souza D.O., Wajner M.: Neurochem. Res. 20, 1437 (1995).
15. Silva A.R., Silva C.G., Ruschel C., Helegda C., Wyse A.T., Wannmacher C.M., Wajner M., Dutra-Filho C.S.: Neurochem. Res. 26, 1277 (2001).
16. Zhao S., Liu Y.M.: Electrophoresis 22, 2769 (2001).
17. Verleysen K, Van den Bosch T, Sandra P.: Electrophoresis 20, 2650 (1999).
18. Kuhn R., Riester D., Fleckenstein B., Wiesmuller K.H.: J. Chromatogr. A. 716, 371 (1995).
19. Sanger-van de Griend C.E.: Electrophoresis 21, 2397 (2000).
20. Stehle P., Kühne B., Pfander P., Fürst P.: J. Chromatogr. 249, 408 (1982).
21. Stehle P., Pfander P., Fürst P.: J. Chromatogr. 294, 507 (1984).
22. Stehle P., Fürst P.: Clin. Chim. Acta 169, 323 (1987).
23. Jones D., Scarborough A., Tier C.M.: J. Chromatogr. A 661, 1 (1994).
24. Che F.Y., Song J.F., Shao X.X., Wang K.Y., Xia Q.C.: J. Chromatogr. A 849, 599 (1999).
25. Verzola B., Gelfi C., Righetti P.G.: J. Chromatogr. A 868, 85 (2000).
26. Pacáková V., Hubená S., Tichá M., Maděra M., Štulik K.: Electrophoresis 22, 459 (2001).
27. Kubo K., Hattori A.: Electrophoresis 22, 3389 (2001).

Received: 9.10.2003