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Modification of a micellar system for amino acid separation by MEKC—Application for amino acid profiling in formulations for parenteral use

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ABSTRACT

The paper proposes a new method for amino acid determination which can be applied for amino acid profiling in solutions for parenteral nutrition. The MEKC method based on a mixed micellar system was developed for the separation of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized amino acids. Background electrolyte was based on tris–borate buffer with high alkaline pH. Sodium dodecyl sulfate micelles were modified using 1,2-hexanediol as a co-surfactant. The effect of the modifier on amino acid migration was studied with respect to hydrophobicity of the analytes. The modifier appeared to be suitable to improve the separation of AQC-tagged amino acids without an adverse effect on buffer ionic strength or EOF velocity. The method was successfully validated and applied for amino acid profiling in medicinal preparations for parenteral nutrition. The results obtained were compared with a reference chromatographic method (amino acid analyser).

1. Introduction

Amino acids (AAs) play an important role in living organisms as building blocks for the synthesis of proteins and peptides. In case of disease, with loss of appetite and increased catabolic response, an intensive whole-body protein breakdown takes place which leads to negative nitrogen balance. Adjustment of the negative balance of nitrogen is one of the goals of clinical nutrition so that the proper concentration and composition of proteins is restored. Parenteral nutrition is used when the administration of food through the digestive tract is inappropriate or impossible. Preparations containing amino acids are important among all the infusion solutions applied in parenteral nutrition. The composition of these preparations is developed in relation to the type of primary disease (i.e. hepatic, renal or cardiovascular failure) and patient age (i.e. paediatric preparations) [1]. Usually, the solutions contain common amino acids; however, some formulations for special medical purposes require additional ones, such as taurine (Tau), ornithine (Orn), acetyltyrosine (AcTyr), acetylcysteine (NAC) or dipeptides.

Quality control of these medical products above all requires amino acid analysis. The most popular method for AA analysis is based on ion-exchange chromatography in pH-gradient elution with post-column ninhydrin derivatization [2] used for the first time by Stein and Moore almost 60 years ago (1951). Manufacturers' dossiers also describe other applications after pre-column derivatization of amino acids, followed by separation by RP-HPLC [3–5]. The separations usually require a multistep-gradient elution with long column conditioning and analysis time. Additionally, some amino acids, such as tryptophan, cysteine, acetyltyrosine and acetylcysteine, are assayed using different methods.

As amino acids are not easily detectable in their native state, the methodology generally recommends labelling of analytes with high-absorbing or fluorescent reagents. A derivatization reagent should fulfil several requirements, such as specific and rapid reaction, preferably with primary and secondary amino acids leading to sufficiently stable products and single peaks for each analyte. Considering these features, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was found to have an advantage over the others [5]. The reagent is designed for fluorescent labelling, but it can also be used for UV detection taking into account less sensitivity in that case. The labelling reaction does not only lead to improved amino acid detectability, but it also alters the physico-chemical properties of analytes, making them more hydrophobic and suitable for the micellar electrokinetic separation mode (MEKC). This approach offers advantages for the resolution of compounds with similar structure over other electrophoretic modes. CE labelling

Abbreviations: AA, amino acids; Hxd, 1,2-Hexanediol; AQC, 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate; AMQ, 6-Aminoquinoline; NHS, N-hydroxysuccinimide.

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Table 1
A list of the medicinal products tested during the study.

Medicinal product	Intended use	Manufacturer	% AA (w/v)	Nitrogen content N [g/L]	Number of AA	Amino acid composition[a]		Electrolytes
						Additional amino acids or derivatives present	Amino acids not present	
1	General	A	10	15.6	19	Orn; NAC; AcTyr	–	+
2	General	B	15	24	18	NAC; Tyr	–	+
3	General	C	10	16	13		Asp; Glu; Ser	+
4	General	C	11.4	18	18	Cys + Cst; Tyr	–	–
5	Paediatric	A	10	15	20	Cys; Tyr; Orn; Tau	–	–
6	Paediatric	C	6	9	17	NAC; AcTyr; Tau	Asp; Glu	–
7	Hepatic failure	C	8	12,9	15	NAC	Asp; Glu; Tyr	–
8	Renal failure	A	6	8,6	17	NAC; AcTyr	Asp	–

[a] A set of 16 amino acids (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Val) is treated as a basis of formulations for parenteral nutrition. The two columns give details about differences in amino acid composition.

with AQC was applied previously for enhanced detection of proteins and peptides by Le Potier [6] and Liu [7], whereas AAs were derivatized for the purpose of enantiomeric separation [8–10]. In this paper we investigate the potential use and suitability of MEKC for AQC-labelled amino acids, a novel approach in pharmaceutical analysis. The aim of the study was to develop a micellar system appropriate for the electrophoretic separation of AQC-amino acids and to verify method applicability for the quantification of amino acids in parenteral infusions. The MEKC system was modified with a class I micelle modifier that was proposed as a new approach for the separation of AQC-labelled amino acids.

2. Materials and methods

2.1. Chemicals

The 18 individual common L-amino acid standards, and additionally taurine (Tau), L-ornithine HCl (Orn) and nor-DL-leucine (n-Leu), were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). All reagents used for buffer preparation, capillary conditioning and sample pre-treatment were of analytical grade. The materials used: tris(hydroxymethyl)-aminomethane (tris) and boric acid were from Merck (Darmstadt, Germany); 1,2-hexanediol (Hxd) and sodium dodecyl sulfate (SDS) were both supplied by Sigma–Aldrich (St. Louis, MO, USA); sodium hydroxide (NaOH) from Applichem (Darmstadt, Germany), concentrated hydrochloric acid (HCl) from POCh (Gliwice, Poland) and isopropanol (IPA) from Lab-Scan Ltd. (Dublin, Ireland). The AQC reagent was supplied by Waters as part of the AccQ Tag Reagent Kit (Waters, Milford, MA, USA) and it also contained borate buffer and acetonitrile (ACN) as an AQC diluent.

Highly purified DI water was prepared using an EASY Pure RF deionizer (Barnstead-Thermolyne, Dubuque, IA, USA). All buffers and solutions applied to the capillary were filtered through a 0.45 μm GDX MF (micro-fiber) syringe filter (Whatman, Maidstone, UK). Stock reference solutions of individual amino acids were prepared in DI water at a concentration of 1 mg/mL with a few drops of 1 M HCl for dissolution. n-Leu at a concentration of 5 μg/mL was prepared in the same manner as internal standard solution. Working reference solution containing all AAs was prepared by the appropriate mixing of stock solutions. The final concentration was adjusted to the target value based on the dilution of test samples. The test samples were taken from medicinal products for parenteral nutrition (eight formulations from three manufacturers) containing AAs at a level of 6–15% (Table 1).

Stock solutions of boric acid (200 mM), tris (200 mM) and SDS (500 mM) were prepared in DI water and filtered. MEKC background electrolyte (BGE) was prepared by mixing suitable volumes of boric acid and tris solutions to obtain the desired concentration of each component. The pH of the electrolyte was adjusted with 2 M NaOH when necessary. Finally, SDS solution and Hxd were added when required and the solution was filled up with DI water.

2.2. Equipment

BioFocus 2000 (BioRad, Hercules CA, USA) capillary electrophoresis equipment with a UV detector was used in the study. Separations were carried out in 60 cm (55.4 cm to the detector) 50 μm ID fused silica capillaries (Beckman Coulter, Fullerton, CA, USA) thermostated at 20 °C with 415 V/cm voltage (positive polarity). Samples were injected hydrodynamically at 5 psi pressure for 1 s (5 psi s). AQC-derivatives were detected at 254 nm.

A new capillary was treated with IPA, subsequently with 0.2 M NaOH solution and separation buffer for 100 s at 100 psi each. The same procedure was applied as every day pre-conditioning of the capillary. Between runs flushing with background electrolyte (40 s at 100 psi) was sufficient to achieve reproducible migration.

2.3. Determination of AQC labelled amino acids

For AA profile analysis test samples were diluted with DI water accordingly to match the quantity of AQC available for derivatization [5]. The final concentration of total AAs was set to be not more than 0.8 mM/L of total amino nitrogen. It corresponds to about 1–20 μg/mL of each component in a diluted test sample assuming over ten to twenty amino acids in the product.

Derivatization was performed according to the supplier's procedure. AQC solution (10 mM) was prepared in ACN. A specified volume of sample solution (50 μL) was mixed with 50 μL of borate buffer to achieve alkaline reaction. Subsequently, 150 μL of DI water and 50 μL of internal standard solution were added. The derivatization was accomplished by adding 20 μL of AQC solution and vortexing the mixture thoroughly. Because of rapid reaction, samples could be injected directly after preparation; however, in case of tyrosine present in a sample an additional heating step (55 °C, 10 min) was applied to convert the amino acid to a monolabelled product.

The electrophoresis of AQC-labelled AAs was performed with background electrolyte containing 40 mM tris and 100 mM boric acid adjusted to pH 10.4 with 40 mM SDS and 150 mM Hxd (0.93 mL/50 mL). The quantification of AAs was done using a calibration curve covering approximately 70–130% of the declared content of each amino acid in a test sample. The corrected peak area of each compound was subsequently normalised based on the corrected area of the internal standard peak and the resulting val-

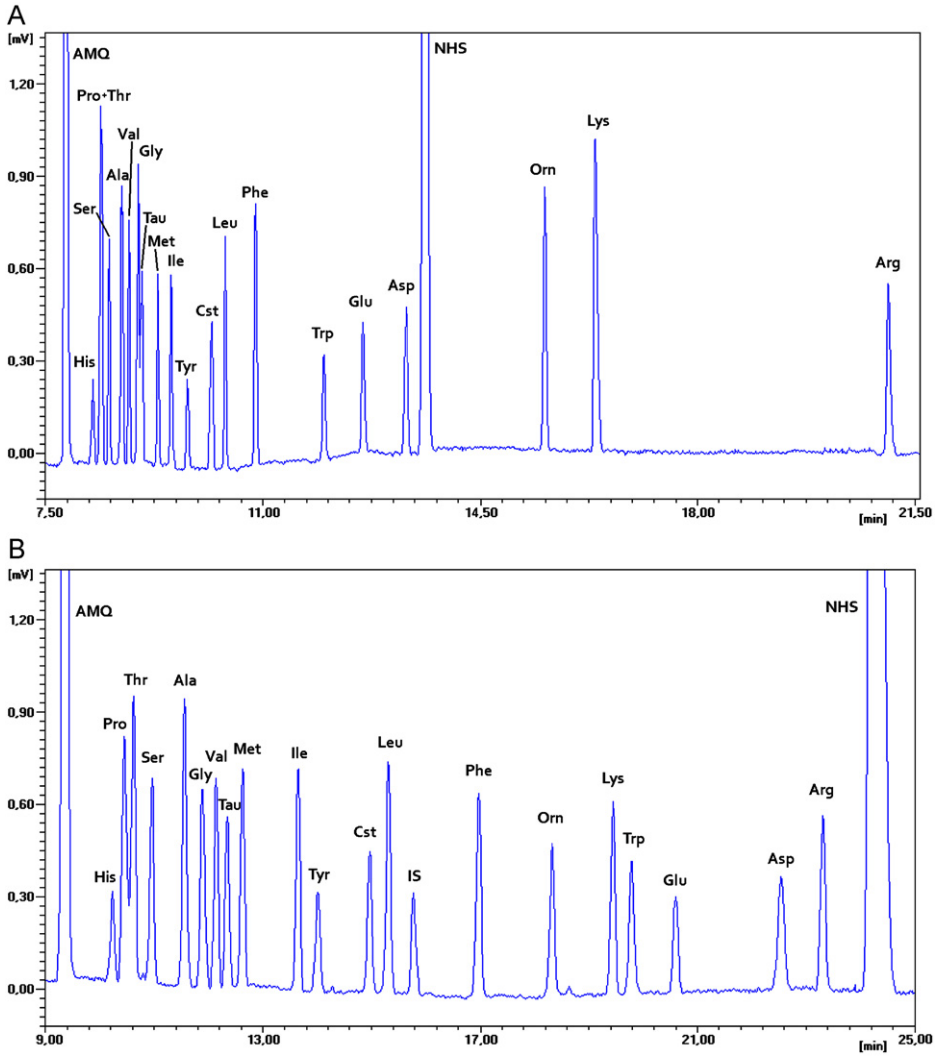

Fig. 1. AQC-AA separation in optimised MEKC conditions: (A) without modifier: background electrolyte 40 mM Tris, 100 mM boric acid adjusted to pH 10.4 with 20 mM SDS; (B) with modifier: background electrolyte 40 mM Tris, 100 mM boric acid adjusted to pH 10.4 with 40 mM SDS and 150 mM Hxd; AMQ – 6-aminoquinoline; NHS – N-hydroxysuccinimide; IS – internal standard n-Leu.

ues were used to plot a curve in relation to the concentration of the amino acid. Each result was obtained in triplicate.

2.4. Reference method

Ion-exchange chromatography with post-column ninhydrin derivatization was selected as the reference method [2]. Amino acids were analysed using a Biotronik LC 6001 automated amino acid analyser (Biotronik, München, Germany) with a cation-exchange resin (type BTC 2710, 30 mm × 4 mm column size, Biotronik, München, Germany), a gradient of lithium citrate elution buffers and post-column derivatization with ninhydrin, all according to working recipes and the manufacturer's instruction. Quantification was performed against a commercial amino acid standard (Sigma–Aldrich, St. Louis, MO, USA) using single point calibration. n-Leu was used as the internal standard.

3. Results and discussion

3.1. Method development

In MEKC a number of factors has to be considered when optimal conditions are sought. The most important are: buffer composition and concentration, pH and surfactant concentration. After some experiments (data not shown) tris–borate buffer was found to be better then borate buffer for the separation of solutes tested. Owing to the use of tris as a buffer additive buffer ion mobility was matched to solutes mobility which is important for minimising peak shape distortions and improving peak shape. Considering the concentration of the buffer added to samples during the derivatization process, high concentration of background electrolyte was chosen to prevent an anti-stacking effect. Therefore, the electrolyte containing 40 mM tris and 100 mM boric acid was selected to perform next optimisation steps.

The optimisation of electrolyte pH and SDS concentration was carried out simultaneously in a number of experiments. The best conditions were found at pH 10.4 and 20 mM SDS allowing the separation of 18 of 20 amino acids under investigation – threonine (Thr) and proline (Pro) were recorded as a single peak and glycine (Gly) and taurine (Tau) were partially resolved (Rs = 0.7) (Fig. 1A). Further increase of SDS concentration set back the resolution between polar AAs. Furthermore, the higher retention of 6-aminoquinoline (AMQ, a product of AQC hydrolysis) resulted in co-migration with other AA peaks.

The modification of MEKC buffer selectivity was performed using 1,2-hexanediol (Hxd), a class I modifier (co-surfactant) for

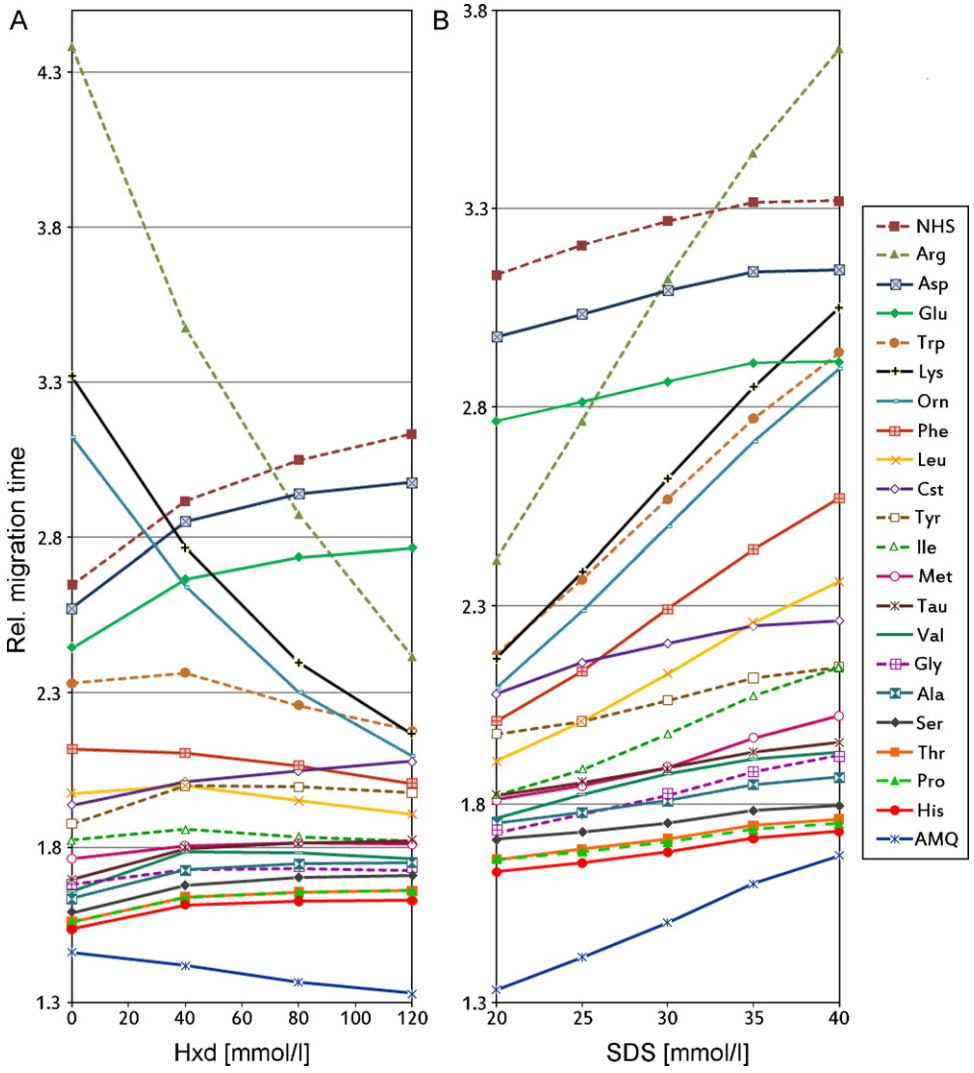

Fig. 2. Relative migration time changes dependent on background electrolyte composition: (A) increase of Hxd concentration with constant SDS 20 mM; (B) increase of SDS concentration with constant Hxd 120 mM. AMQ – 6-aminoquinoline; NHS – N-hydroxysuccinimide.

micellar systems [11,12]. A number of buffers containing various amounts of SDS and Hxd were tested to find the best proportion for the separation. As reported previously [12], the modifier had little impact on EOF. The observed decrease in EOF mobility compared to unmodified BGE was less than 7% throughout the optimised range of SDS and modifier concentrations. The changes in analyte migration depending on the composition of BGE are shown in Fig. 2. The addition of Hxd to the separation buffer resulted in increased distance between AMQ and the first migrated His peak (Fig. 2A) and thus made it possible, as a next step, to increase SDS concentration without unwanted overlapping of AMQ with AA (Fig. 2B).

In the finally selected conditions (40 mM tris and 100 mM boric acid adjusted to pH 10.4 with 40 mM SDS and 150 mM Hxd) amino acids were separated within 25 min with the average efficiency of 300,000 theoretical plates. The adjacent Pro/Thr pair was separated with Rs = 0.88 and the remaining AAs were fully resolved (Fig. 1B).

3.2. Effect of 1,2-hexanediol on MEKC separation of amino acids

The effect of alkyl polyols, including Hxd, on MEKC separation was studied by Allen [11] and Wall [12]. They found a decrease in critical micelle concentration (CMC) for SDS in the presence of the modifier. Interaction of the modifier with the micellar phase and embedding in the micelle structure were indicated as factors altering the partition coefficient of solutes and changing system selectivity. The advantage of this class of modifiers was demonstrated in the separation of phenols, dansyl-AA and alkylphenylketones. No further application of alkyl polyols in MEKC was described.

The effect of Hxd on the separation of AQC-AAs has been currently studied based on the data obtained during method development. With modified buffer analytes distribution was more uniform along the retention window, whereas with unmodified BGE almost all the amino acids were located at the front part of the electrophoregram (Fig. 1). Some of AQC-AAs altered the migration order with changed Hxd content in BGE (Fig. 2A). The most significant change occurred for charged AAs (Glu, Asp, Orn, Lys, Arg). With increased Hxd concentration, the migration time of acidic AAs increased much more compared to neutral AAs. On the contrary, basic amino acids tended to migrate considerably faster. An opposite phenomenon was observed with the increasing surfactant/co-surfactant ratio (Fig. 2B). It was expected that the effect was related to the log P value of the solutes [13]. Treating derivatized AAs as homologues differing in amino acid side chains (except for double derivatized Cst, Lys, Orn) the effects could be analysed with respect to the hydrophobic properties of the amino acid itself. Since there is no hydrophobicity data available for Cst

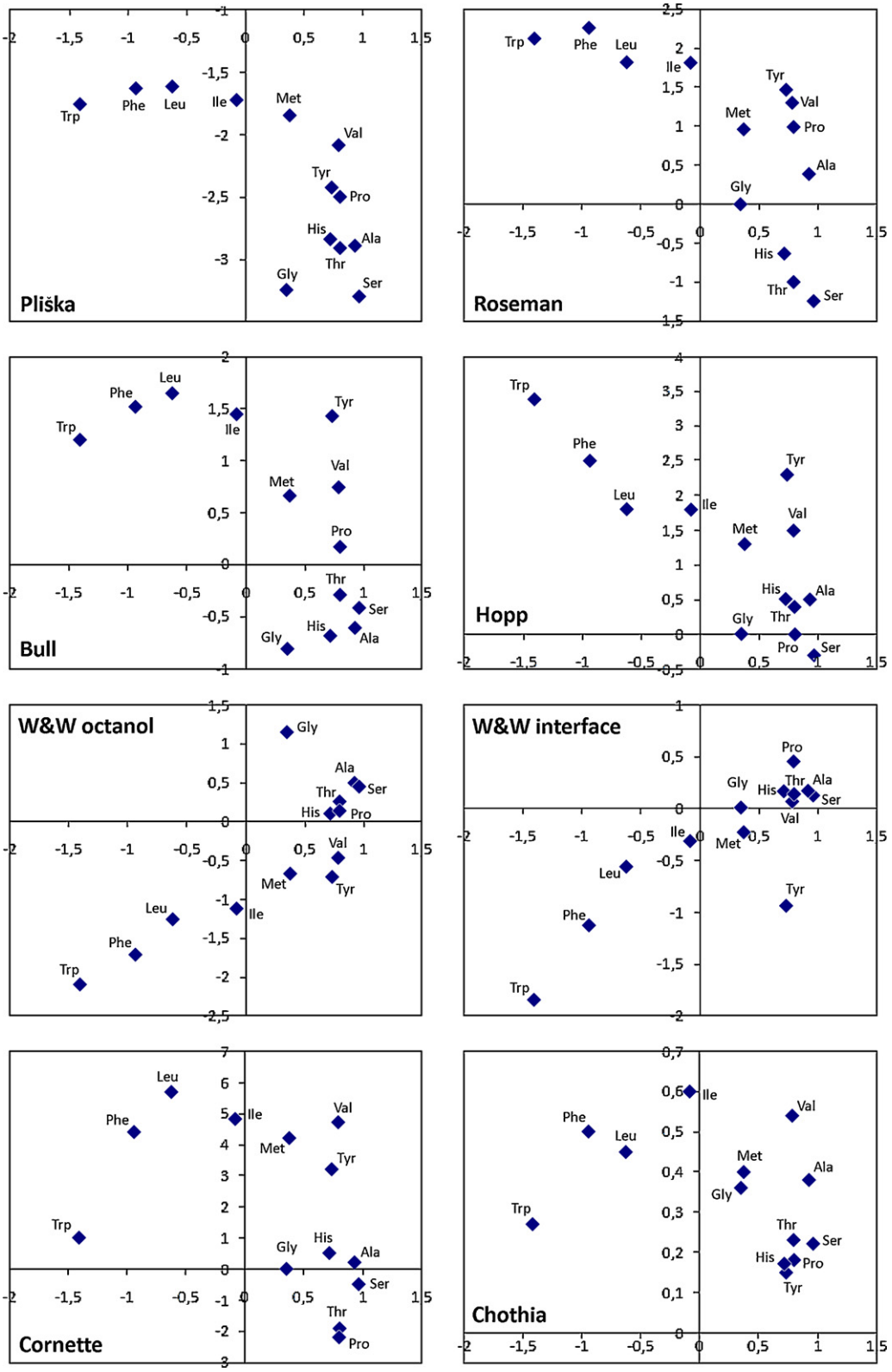

Fig. 3. The dynamics of changes of AQC-AA relative migration time (abscissa*) plotted versus various hydrophobicity scales (ordinate). The following scales are shown as an example: Pliška [14] – based on chromatographic data; Roseman [19] – based on partitioning methods; Bull [22] – based on physical property methods; Hopp [23] – based on prediction of antigenic determinants of proteins; Wimley and White [24] – based on partitioning to octanol phase on the left and interaction with lipid bilayer (interfacial partitioning) on the right; Cornette [26] – based on statistical evaluation; Chothia [27] – based on accessible surface area method. Charged AA (Glu, Asp, Lys, Arg) were excluded from the plots. *The values were multiplied by factor 100 for better presentation.

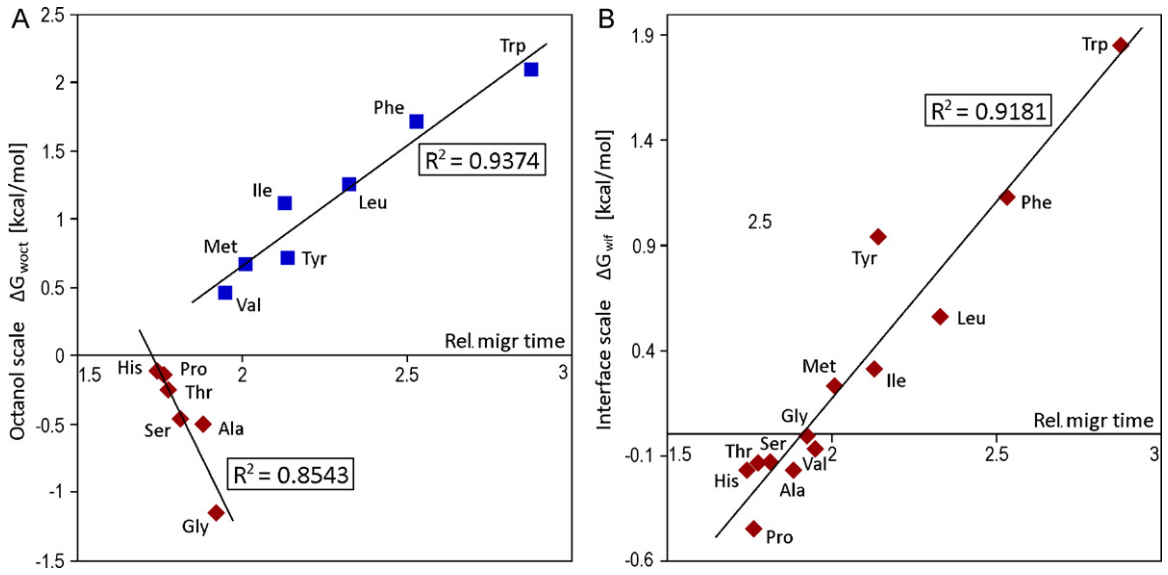

Fig. 4. Relative migration time of AQC-AA in the modified micellar system plotted versus "octanol scale" (A) and "interface scale" (B) [24]. Fitting lines added as an overview.

and for non-protein Tau and Orn, they were excluded from the analysis.

The changes in relative migration of AQC-AAs depending on the variation in BGE composition were transformed into single values represented as a slope of the lines from Fig. 2 approximated by linear regression. The addition of Hxd to the separation buffer made some of neutral AAs (Trp, Phe, Leu, Ile) show a negative tendency in changes of relative migration indicating weaker interactions with modified micelles compared to the unmodified ones. At the same time remaining neutral AAs showed a positive tendency of migration changes which could be interpreted as a stronger partition to the mixed micelles. Based on these observations it is noted that in the presence of Hxd the micellar system became "less hydrophobic" (or "more hydrophilic") and thus more attractive to solutes of lower hydrophobicity.

Charged amino acids (Asp, Glu, Lys and Arg) behaved in a different way because of a different ionisation state of the molecule in highly alkaline BGE compared to neutral AAs. AQC-labelled Glu and Asp had net charge −2 and thus were strongly attracted to the anode. AQC-Lys (and similarly Orn) was of net charge −1 but double derivatized which decreased the charge to mass ratio and made it more hydrophobic compared to others. AQC-Arg remained a zwitterion (net charge 0) which could affect the highest outcome hydrophobicity resulting in elution as the last peak in unmodified micellar BGE. Even though similar interpretation concerning solute-micelle interaction could be drawn – stronger when the slope value was positive and weaker in case of a negative one – it seemed that changes in migration of this AA group were also affected by additional factors.

The dynamics of changes in relative migration time was analysed against various amino acid hydrophobicity scales [14–28]. A number of hydrophobicity scales were checked while looking for the best correspondence to the experimental data. Depending on a principle the scale is developed on, various patterns were obtained (Fig. 3). The Wimley–White hydrophobicity scales [24] based on partition to the octanol phase and interaction with a lipid bilayer (interfacial partitioning) were found in the best agreement with the experimental observations for neutral AAs. Remarkably, the "interface scale" fits much better the current data than the "octanol scale" does (Fig. 4), which was also observed by Burns et al. in regard to uncharged benzene derivatives [29]. Relative migration time of AQC-AAs in the modified micellar system plotted versus the "octanol scale" resulted in the appearance of two groups with very different slopes of the regression lines. On the contrary the correlation with the "interface scale" gave a clear linear fit within all the neutral AAs. It seemed that it could indicate the similarity of partition mechanism in both Wimley–White lipid bilayer vesicles and Hxd-SDS mixed micelles.

To conclude, Hxd was able to modify the properties of SDS micelles making them more selective especially for less hydrophobic small and polar amino acids. The modifier appeared suitable to improve the separation of AQC-derived amino acids without any adverse effect on buffer ionic strength or EOF velocity.

3.3. Method validation

With the optimised MEKC method amino acid relative migration times as well as resolution were found to be stable and repeatable except for Tyr. The ionisation of the Tyr side chain (pKa 10.07) depending on small pH variation in BGE resulted in a significant migration shift. Thus the resolution between Ile and Tyr was set as the system suitability criterion with the limit of 1.5.

The method was successfully validated. Linearity and the working range of the method was established with the upper level limited to the amount of AQC available for derivatization and the lower level based on LOQ. The results allow the quantification of analytes down to 5 μM for aromatic (Phe, Tyr, Trp) and double derivatized (Lys, Orn, Cst) amino acids and to 10 μM for the remaining ones. Sensitivity (LOD) of the method was evaluated as 2 and 5 μM, respectively. Precision and intermediate precision were checked based on repetitive analysis of a commercial medicinal product which contained all the amino acids under investigation (product no. 5, see Table 1). Method precision was expressed as relative standard deviation (RSD) of six consecutive assays performed the same day. Intermediate precision was calculated as RSD of assays performed within four successive days (one assay per day). RSD values of precision and intermediate precision are given in Table 2. The accuracy of the method was verified by comparison to results obtained with the reference LC method [2]. The results of AA assay were in accordance with chromatographic analysis (Table 2). A statistical comparison of the results using a paired *t*-test indicated no significant differences between these methods ($p = 0.318$).

Table 2
MEKC method for AQC-AA assay – the results of precision (intra-day repeatability), intermediate precision (day-to-day repeatability) and comparison to the reference LC method.

Amino acid	Declaration [g/L]	LC method	MEKC method		
		Result, $n=2$ [% declaration]	Result, $n=4$ [% declaration]	Precision, $n=6$ RSD [%]	Intermediate precision, $n=4$ RSD [%]
Ala	8	102.13	100.94	1.31	2.34
Arg	8.4	98.40	96.97	1.76	2.91
Asp	6	103.22	101.72	1.14	2.26
Cys[a]	1.89	99.45	98.50	2.22	3.21
Glu	10	98.24	99.79	1.28	2.42
Gly	4	96.11	98.43	1.20	2.83
His	3.8	99.22	101.83	1.12	2.26
Ile	6.7	101.78	102.01	0.63	1.59
Leu	10	101.13	99.62	2.04	2.30
Lys	11	98.16	97.51	0.52	0.76
Met	2.4	100.73	101.49	0.67	1.03
Orn	2.49	102.17	99.92	0.70	1.54
Phe	4.2	99.37	97.72	0.81	1.85
Pro	3	102.20	101.72	1.19	2.68
Ser	4	98.74	99.45	1.05	1.55
Tau	0.6	102.06	100.76	1.62	2.84
Thr	3.7	97.16	98.61	1.31	2.78
Trp	2	103.87	104.06	0.91	1.84
Tyr	0.45	105.27	102.50	2.36	3.58
Val	7.6	99.15	98.09	0.96	2.30

[a] As the formulation of the medicinal product was built based on cysteine HCl, the results obtained were recalculated appropriately.

Precision and intermediate precision were below 2.4% and 3.6%, respectively, for all the amino acids. The method occurred to be slightly less precise for Tau and Trp, although it came from the low level of the amino acid in the medicinal product tested. Not fully resolved Pro and Thr presented acceptable repeatability indicating that integration with a perpendicular dropped to the baseline is a good assumption for the quantification. Cysteine (Cys) present in the test sample was determined as cystine (Cst) due to oxidation occurring in alkaline medium. The oxidation seemed to be efficient enough that no Cys peak was recorded, although this could make the peak area of higher variability. Based on the validation results it can be considered that the MEKC method is appropriate for the intended purpose.

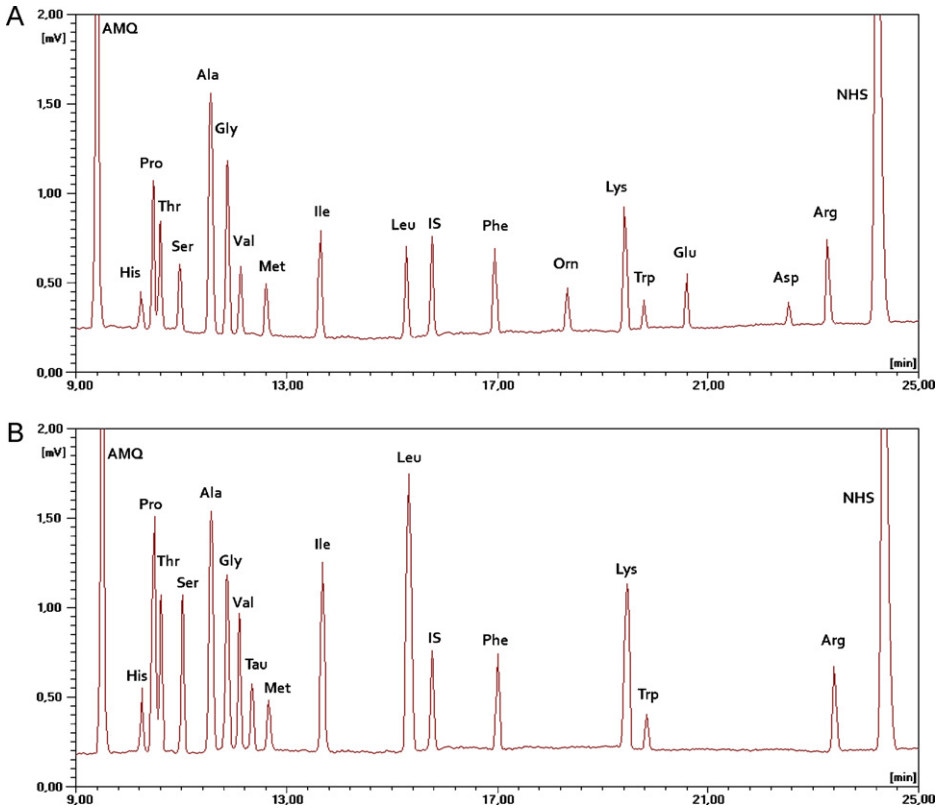

Fig. 5. Amino acids profiling in commercial medicinal products for parenteral nutrition: (A) product no. 11000-fold dilution in water and (B) product no. 6600-fold dilution in water. See Table 1 for detailed product information. CE conditions – as in Fig. 1B.

3.4. Application of the method to commercial preparations

The method was applied in amino acid identification and assay of commercial medicinal products for parenteral nutrition. The method demonstrated good reproducibility between tests. Fig. 5 presents electrophoregrams of amino acid profiles in two of the products listed in Table 1, as an example. Practical application of the developed CE method revealed a number of advantages compared to the reference amino acid analyser method. The most significant is short analysis time: 25 min by CE vs. more than 100 min that is usually required for AA separation with an amino acid analyser. The CE method also has improved sensitivity. An amino acid analyser is able to detect 50–100 pmol of AA per injection [30], while the mass detection limit for AQC-labelled AAs is one magnitude lower (20–50 attomoles with an injection volume of about 9 nL). In addition, the CE method requires much less reagents and consumables and shorter system equilibration. Consequently, the implementation of the method in laboratory practice can reduce the time and cost of analysis.

4. Concluding remarks

A new, rapid and sensitive method was developed for the analysis of amino acids in pharmaceuticals. The method allows the separation and identification of a mixture of 20 amino acids derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The electrophoretic system used (40 mM Tris and 100 mM boric acid adjusted to pH 10.4 containing 40 mM SDS and 150 mM Hxd) is able to separate the analytes in less than 25 min with a system efficiency of 300,000 theoretical plates. 1,2-hexanediol used as a co-surfactant increased the resolution between co-migrated and adjacent amino acid peaks. The investigated effect of the modifier on analyte migration showed good correlation with the interface hydrophobicity scale based on partition to lipid bilayer vesicles. Hexanediol can be an efficient modifier of micellar systems affecting selectivity and improving separation for close related polar compounds, such as amino acids. Based on the validation carried out and comparison with a reference LC method, suitability of the present method was confirmed for the quantitative testing of amino acids in medicinal products.

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