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Quantitative high-performance liquid chromatography–tandem mass spectrometry impurity profiling methods for the analysis of parenteral infusion solutions for amino acid supplementation containing L-alanyl-L-glutamine

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ABSTRACT

Potential impurities in a parenteral infusion solution for amino acid supplementation containing alanyl-glutamine (AlaGln) and glycyltyrosine (GlyTyr) as peptide constituents have been determined. Such complex multicomponent pharmaceutical formulations with reactive ingredients may yield a multitude of impurities in stress testing samples. Thus, three stability indicating LC–ESI-MS/MS methods were developed for the establishment of quantitative impurity profiles employing a Chiralpak QN-AX and a Polysulfoethyl A stationary phase in HILIC mode as well as a Gemini C18 stationary phase in gradient RPLC mode. The primary goal was to separate isobaric compounds (stereoisomers, constitutional isomers, retro-peptides) and to provide quantitative data of impurities identified in stressed nutritional infusion solutions. The optimized methods were calibrated by standard addition in the samples and validated according to ICH guidelines. The methods were then applied for the analysis of stressed sample solutions stored under different conditions. Major peptide impurities found in concentrations above the qualification threshold in stressed solutions stored at 40 °C for 6 months comprised cyclo(AlaGln) 808 μg/mL, pyroGluAla 122 μg/mL, AlaGlu 117 μg/mL, cycloGlyTyr 60 μg/mL, AlaGln epimers (DL + LD) 38 μg/mL, and TyrGly 27 μg/mL. A number of impurities above the reporting threshold were also detected including AlaAlaGln 18 μg/mL, cyclo(AlaGlu) 16 μg/mL, AlaGlu(AlaGln) 17 μg/mL, and AlaGlu(His) 12 μg/mL. The study showed that bioactive peptides may be formed in amino acid infusion solutions by condensation of amino acids and a careful control of these impurities is mandatory.

1. Introduction

The establishment of qualitative and quantitative impurity profiles of drug substances and drug products is a crucial part in the course of the development of new pharmaceutical formulations. Thereby, stability testing with forced degradation is considered to be an important tool to uncover degradation processes and unwanted side reactions [1]. The *International Conference on Harmonization of Clinical Requirements for Registration of Pharmaceuticals for Human Use* (ICH) has published a set of quality documents that provide guidelines on various aspects of impurity profiling and stability testing. Among others these guidelines state that drug developers should summarize the degradation products that can be observed during manufacture and/or stability studies of new products. Further, these impurities have to be quantified reliably by validated assays to enable classification into those that need to be reported (<0.05% relative to the precursor compound at daily dosage >1 g), identified (0.2% above 10 mg daily dose and 0.1% above 2 g) and qualified (i.e. by assessment of their biological safety) (0.2% and 0.15% above 100 mg and 2 g, respectively) [2–4]. Such strict demands should help to preserve product safety. Hence, analytical assays that allow for the accurate and reliable quantification of all detected impurities are required. The present report deals with such methods that have been developed for identified impurities in a parenteral infusion solution for amino acid supplementation. Due to limited stability, glutamine (Gln) [5] is sometimes substituted by AlaGln dipeptide. Several clinical studies revealed that AlaGln is quickly hydrolyzed in the extracellular space and thus the free amino acids Ala and Gln are set free and can be absorbed quickly [6,7].

The object of the present study was the examination of the impurity profile of a pharmaceutical nutritional infusion solution that contained not only AlaGln as main component, but also GlyTyr and various amino acids as well as other constituents. It was of special interest to uncover degradation pathways and side products of AlaGln. The impurity pattern in a stressed infusion solution (40 °C

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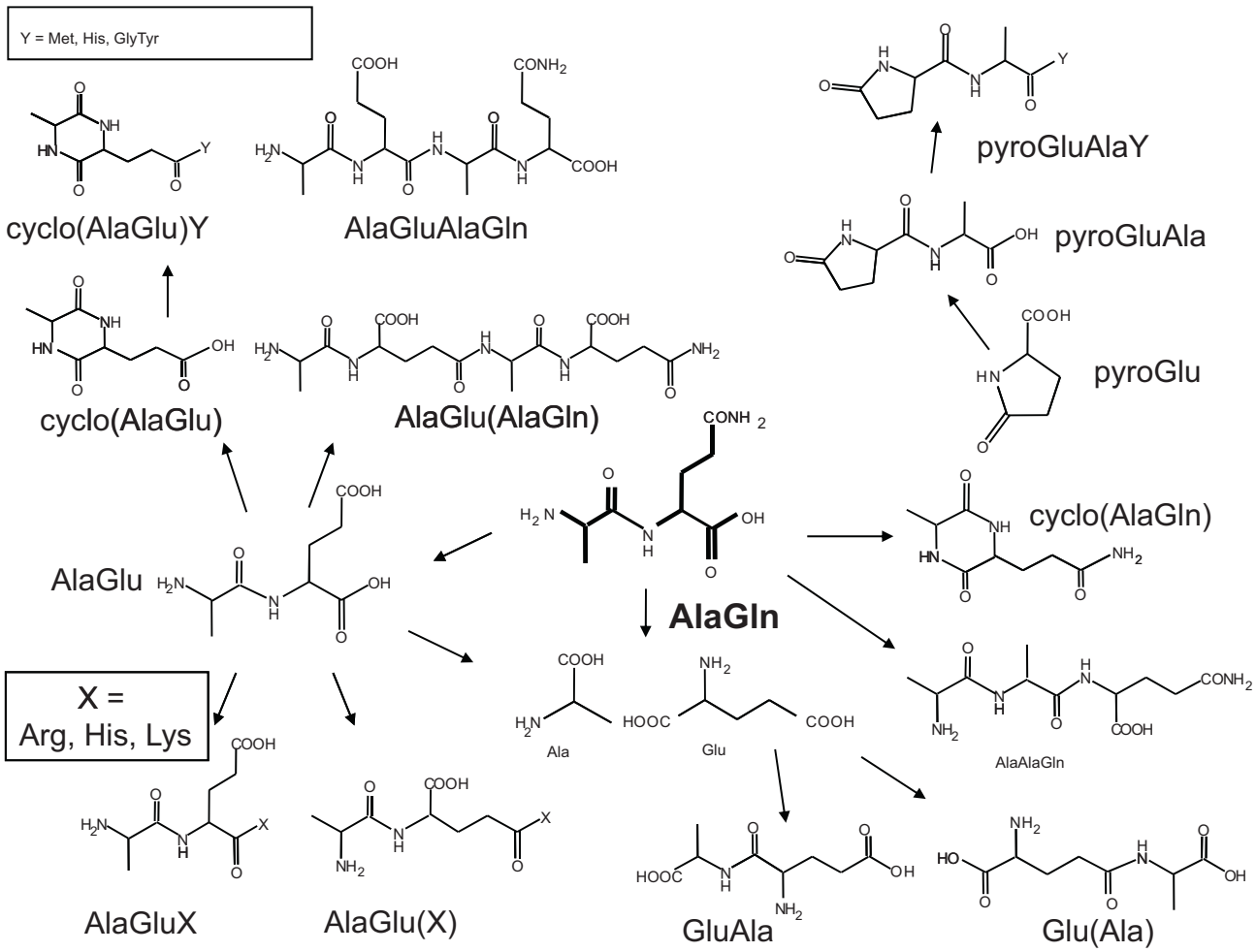

Fig. 1. Scheme of the degradation pathways of AlaGln and its follow-up reactions.

for 12 months) was quite complex and was analyzed by a multidimensional analysis approach consisting of offline two-dimensional HPLC combined with ion-trap (IT) MS and charged aerosol detector (CAD) [8]. This study revealed an impurity profile as illustrated in Fig. 1. Degradation products of AlaGln formed by side chain and main chain hydrolysis leading to AlaGlu as well as to the free amino acids Ala and Glu were reported previously [16]. The reporting threshold for impurities proposed by the ICH for maximal daily doses of the active agent exceeding 2 g is 0.05% relative to the parent compound. Hence, the LOQ of the employed analysis method should reach at least a concentration level of 0.05% of the impurity related to the parent compound. Due to its relatively universal and consistent detector response (for non-volatile compounds) the offline multidimensional HPLC method with CAD allowed a preliminary quantification of the impurities by use of a universal calibration function as reported in the first part of this study [8]. Herein, we present accurate quantitative LC–MS/MS assays for impurities identified as relevant in this prior study employing calibration functions established with authentic standards to confirm the results obtained with HPLC-CAD.

Thus, three LC–MS/MS methods in selected reaction monitoring (SRM) mode were utilized for specific detection of target impurities [9–13]. The hydrophilicity of the identified impurities constituted a major challenge and a number of isomeric or isobaric impurities that cannot be distinguished by MS required selective chromatographic separation. Moreover, in order to be able to accurately and reliably analyze low abundant (structurally similar) degradation-related impurities in the sub-percentage range, it is usually necessary to fully or at least partly separate them from their parent compounds to avoid errors from interferences/cross-talk and ion suppression due to coelution of highly abundant ingredients [17,18]. Matrix-matched calibration by standard addition was carried out in each of the three methods by spiking distinct amounts of standard to the sample solutions. This assured similar conditions during calibration and measurement of the samples alleviating the problem of errors from matrix effects. This method was expected to produce more accurate results because several calibrants are utilized in a narrow relevant concentration range besides matrix. Validation was performed according to the ICH guidelines [14,15]. Unknown new degradation products of AlaGln formed in nutritional infusion solutions have been identified and quantified.

2. Experimental

2.1. Chemicals

The investigated drug formulation was a parenteral solution for supplementation of amino acids. It contained *N*-acetyl-L-cysteine, L-alanine, L-alanyl-L-glutamine, L-arginine, glycine, glycyl-L-tyrosine, L-histidine, L-isoleucine, L-leucine, L-lysine acetate, L-methionine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, L-tryptophan and L-valine as active metabolites.

Preliminary experiments and method development were carried out with three preparations of nutritional infusion solutions which were subjected to different treatments. One was kept under optimal storage conditions (<−20 °C) whereas the two other

solutions were stressed at increased temperatures of 40 °C for 12 months and at 60 °C for 9 months, respectively (see ICH guidelines on stability testing of new drug substances and products). In the course of stress testing further preparations of infusion solutions were kept under various stress conditions and finally analyzed employing the developed methods.

L-Ala-L-Gln and 2,4-dinitrofluorobenzene were purchased from Sigma–Aldrich (Vienna, Austria). D-Ala-L-Gln, L-Tyr-L-Gly, N,N′-diacetylcystine ($(AcCys)_2$), AlaAlaGln, AlaGlu, cyclo(AlaGlu), cyclo(AlaGln) and pyroGluAla were supplied by Bachem (Bubendorf, Switzerland). cyclo(GlyTyr) and the two structural isomeric tetrapeptides AlaGluAlaGln and AlaGlu(AlaGln) were provided from various suppliers. The standards of cyclo(AlaGlu)His (TFA salt), pyroGluAlaHis (TFA salt), AlaGlu(His) (TFA salt), AlaGlu(Arg) (TFA salt), AlaGlu(Lys) (TFA salt), AlaGluLys (TFA salt), cyclo(AlaGlu)Met, pyroGluAlaMet, cyclo(AlaGlu)GlyTyr were custom synthesized by piChem (Graz, Austria). Standards of pyroGluAlaGlyTyr, AlaGluHis and AlaGluArg were obtained from GenScript Corporation (New Jersey, USA). Aqueous ammonium hydroxide (NH_4OH) 25%, glacial acetic acid (AcOH), trifluoroacetic acid (TFA), ammonium acetate and sodium carbonate anhydrous were purchased from Fluka, formic acid (FA) and sodium bicarbonate were from Riedel-de Haën (Seelze, Germany). Acetonitrile (ACN) of HPLC grade was from VWR (Vienna, Austria). The employed water was purified with a Millipore (Elze, Germany) water filtration system.

2.2. Instrumentation

The instrumental set-up consisted of an Agilent 1200 HPLC system (Waldbronn, Germany) composed of a thermostatted autosampler (adjusted to 4 °C), a binary pump and a column thermostat (25 °C), hyphenated to a Q-Trap 4000 from ABSciex (Thornhill, Canada). For the quantification of degradation products of AlaGln and other identified impurities measurements were performed in the selected reaction monitoring (SRM) mode. Compound specific parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were determined using the "Quantitative Optimization" tool of the Analyst software (version 1.4.2). For this purpose standard solutions of the compounds were prepared at concentrations of 250 μg/L using solvents with a composition similar to the mobile phase conditions during detection. The standard solutions were infused with a syringe pump at a flow rate of 30 μL/min. For each analyte two specific transitions were monitored, one of which served for quantification (quantifier) and the other as identifier (qualifier) minimizing the risk of false peak assignment. Optimized values for MS parameters of all impurities investigated in the study can be found in Table 1.

A turbo ion spray (TIS) was employed as ion source. TIS voltage was adjusted to +4500 V in the positive mode and to −4300 V in the negative mode. Source temperature was set to 600 °C and the flow of curtain, nebulizer and heater gas were kept at 10, 50 and 60 psi, respectively. Pressure of the collision gas was adjusted to medium and a dwell time of 100 ms was utilized. The chromatograms were separated into periods and only the transitions of compounds eluting within this time frame were measured.

2.3. Chromatography

2.3.1. Stereoselective analysis of L-Ala-L-Gln stereoisomeric impurities

20 μL of nutritional infusion solutions or standard solution was combined with 500 μL of carbonate buffer, which was prepared by mixing 0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3 to yield pH 9.5. After addition of 200 μL of Sanger's reagent (5% 2,4-dinitrofluorobenzene in ACN, w/v) the reaction mixture was incubated on a shaker at room temperature for 60 min. To remove apolar side products of the derivatization, reaction solutions were extracted twice with 500 μL diethylether. In the next step, mobile phase (vide infra) was added to the derivatized samples to yield a volume of 1 mL and further 1:10 dilution was prepared.

The samples were analyzed on the LC–MS/MS (Q-Trap) system in the negative ionization mode recording two specific SRM transitions (382 → 192, DP −65, CE −22, CXP −11; 382 → 162, DP −65, CE −32, CXP −9). 20 μL of the derivatized samples was injected onto a Chiralpak QN-AX column (150 mm × 4.0 mm, 5 μm) from Chiral Technologies (Illkirch, France) and eluted under isocratic conditions employing 20 mM aqueous ammonium acetate (adjusted to pH 4.5 with AcOH)/ACN 60:40 (v/v) at a flow rate of 1 mL/min.

2.3.2. Chiralpak QN-AX HILIC method

In this method a Chiralpak QN-AX column was employed as stationary phase. The mobile phase was composed of 10% (v/v) buffer in water (channel A) and 10% (v/v) buffer in ACN (channel B), respectively. The utilized buffer contained 100 mM formic acid in water adjusted to pH 3.5 with NH_4OH solution. Linear gradient elution from 100% (B) to 65% (B) in 20 min was carried out at a flow rate of 1 mL/min. The column was then re-equilibrated with 100% (B) for 13 min.

2.3.3. Polysulfoethyl A HILIC method

In this method a Polysulfoethyl A (150 mm × 4.6 mm, 5 μm) column from PolyLC (Columbia, USA) was used as stationary phase. Mobile phase conditions were as follows: (A) 10% buffer in water and (B) 10% buffer in ACN. The buffer consisted of 100 mM NH_4OH, pH adjusted to 5.0 with AcOH.

A linear gradient from 100% (B) to 100% (A) in 30 min at a flow rate of 500 μL/min was applied. The column was re-equilibrated with 100% (B) for 13 min, thereby starting with a flow rate of 1 mL/min which continuously decreased to 0.5 mL/min until the end of re-equilibration.

Between 19.0 and 21.0 min of the gradient run the effluent was diverted to waste in order to avoid contamination of the ion source with AlaGln.

2.3.4. Gemini C18 RP method

A Gemini C18 column (150 mm × 3.0 mm, 3 μm) from Phenomenex (Aschaffenburg, Germany) was used as stationary phase. The column was protected with a guard column (4.0 mm × 3.0 mm) containing the same stationary phase. Mobile phase conditions were as follows: (A) 0.1% FA in water and (B) 0.1% FA in ACN. A linear gradient from 5% (B) to 15% (B) in 30 min at a flow rate of 300 μL/min was applied. After the gradient was finished, the system was allowed to re-equilibrate with starting conditions (95% (A), 5% (B)) for 10 min. The effluent from the column was diverted to waste during the first 10 min of the run in order to avoid contamination of the ion source by eluting the major constituents such as AlaGln.

2.4. Preparation of standard solutions

Standard addition was performed by adding 100 μL multicomponent spiking standard to 1 mL diluted sample solution. Concentration increments of spiking standards were chosen to match expected intrinsic concentrations of individual analytes in the sample.

2.4.1. Preparation of calibrants for HILIC method with Chiralpak QN-AX

The sample (unstressed or stressed infusion solutions) was diluted 1:50 with mobile phase (B). The concentrations of spiking standards were 0.5, 1.0, 2.0, 5.0, 10, 25, 50 μg/mL for AlaGln

Table 1
List of investigated compounds along with their respective analysis methods and specific MS-parameters. With only one exception (qualifier of cyclo(GlyTyr)) all transitions were measured in the positive polarity mode. Cyclo(GlyTyr) showed better ionization efficiencies in negative mode. Fragments in italic were used as quantifier transitions.

Analyte	*m/z* precursor	*m/z* product ion	DP[a] (V)	CE[b] (V)	CXP[c] (V)	tr[d] (min)	Method	Period
AlaGln epimers (DL/LD)	218.1	*130.0*	51	*27*	*8*	7.2	Chiralpak QN-AX	1
		84.2		41	4			
AlaAlaGln	289.1	*147.2*	46	*19*	*8*	7.4	Chiralpak QN-AX	1
		130.1		33	6			
cyclo(AlaGlu)	201.1	*183.0*	46	*15*	*10*	8.4	Chiralpak QN-AX	1
		155.0		21	8			
pyroGluAla	201.2	*84.1*	56	*29*	*14*	17.6	Chiralpak QN-AX	2
		90.1		17	14			
AlaGlu	219.1	*148.1*	56	*19*	*8*	15.5	Chiralpak QN-AX	2
		84.1		41	14			
AlaGluAlaGln	418.3	*218.1*	71	*23*	*12*	15.4	Chiralpak QN-AX	2
		147.3		27	8			
AlaGlu(AlaGln)	418.3	*147.3*	71	*27*	*8*	19.7	Chiralpak QN-AX	2
		201.0		35	10			
cyclo(GlyTyr)	221.1	*107.1*	61	*31*	*6*	5.5	Polysulfoethyl A	1
	219.1	113.0	−90	−20	−7			
cyclo(AlaGln)	200.0	*155.2*	46	*23*	*8*	11.0	Polysulfoethyl A	2
		183.1		15	10			
cyclo(AlaGlu)His	338.1	*156.1*	56	*23*	*8*	15.8	Polysulfoethyl A	2
		110.1		45	6			
pyroGluAlaHis	338.1	*156.1*	56	*23*	*8*	15.2	Polysulfoethyl A	2
		110.1		45	6			
TyrGly	239.1	*136.0*	36	*23*	*8*	14.3	Polysulfoethyl A	2
		91.0		51	14			
AlaGlu(His)	356.1	*156.1*	56	*23*	*8*	22.4	Polysulfoethyl A	3
		110.1		47	6			
AlaGluHis	356.1	*156.1*	56	*23*	*8*	23.7	Polysulfoethyl A	3
		110.1		47	6			
AlaGlu(Arg)	375.2	*175.1*	81	*31*	*10*	23.8	Polysulfoethyl A	3
		70.0		69	10			
AlaGluArg	375.2	*175.1*	81	*31*	*10*	25.1	Polysulfoethyl A	3
		70.0		69	10			
AlaGlu(Lys)	347.2	*84.1*	66	*67*	*14*	24.0	Polysulfoethyl A	3
		130.2		35	8			
AlaGluLys	347.2	*84.1*	66	67	*14*	25.1	Polysulfoethyl A	3
		130.2		35	8			
$(AcCys)_2$	325.0	*116.1*	41	*49*	*6*	16.0	RP-18 Gemini	1
		162.1		27	8			
pyroGluAlaMet	332.3	*150.1*	41	*17*	*8*	18.6	RP-18 Gemini	1
		104.1		29	6			
cyclo(AlaGlu)Met	332.3	*150.1*	41	*17*	*8*	22.2	RP-18 Gemini	2
		104.1		29	6			
cyclo(AlaGlu)-GlyTyr	421.2	*239.0*	66	*19*	*14*	22.6	RP-18 Gemini	2
		136.2		47	6			
pyroGluAla-GlyTyr	421.2	*239.0*	66	*19*	*14*	23.4	RP-18 Gemini	2
		136.2		47	6			

[a] Declustering potential.
[b] Collision energy.
[c] Cell exit potential.
[d] Retention time.

epimers (DL and LD), AlaAlaGln, cyclo(AlaGlu), AlaGlu, AlaGluAlaGln, AlaGlu(AlaGln) and 4, 8, 16, 40, 80, 200, 400 μg/mL for pyroGluAla.

2.4.2. Preparation of calibrants for the HILIC method with Polysulfoethyl A

The sample was diluted 1:20 with mobile phase (B). The concentrations of spiking standards were 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20 μg/mL for TyrGly, AlaGluArg, AlaGlu(Arg), AlaGluHis, AlaGlu(His), AlaGluLys, AlaGlu(Lys) and 20, 50, 100, 200, 500, 1000, 2000 μg/mL for cyclo(GlyTyr) and cyclo(AlaGln).

2.4.3. Preparation of calibrants for the RP method with Gemini C18

The sample was diluted 1:5 with mobile phase (A). The concentrations of spiking standards were 0.2, 0.5, 1.0, 2.0, 5.0, 10, 15 μg/mL for pyroGluMet, cyclo(AlaGlu)Met, pyroGluGlyTyr, cyclo(AlaGlu)GlyTyr and 100, 250, 500, 1000, 2500, 5000, 7500 μg/mL for $(AcCys)_2$.

2.5. Validation

The three reported methods were validated according to the ICH guidelines (Q2 R1) [14,15]. Linearity, intra- and interday precision and accuracy as well as LOQ were determined. Standard addition was performed by spiking defined amounts of standard compounds to stressed sample solutions. Thus, matrix matched calibration functions were obtained by correcting for analyte contents already present in the samples. Standards were individually spiked and respective amounts can be found in Tables 2–4. Accuracy and precision were determined for three different concentration levels (low, middle, high; see Tables S1–S3 of Supplementary material) in spiked sample solutions (quality control samples) by triplicate analysis. Interday precision and accuracy were determined on three consecutive days using freshly generated calibration functions. The

Table 2
Calibration functions, linear range, and LOQ of impurities determined by the optimized HILIC method using a Chiralpak QN-AX column.

Compound	Linear range[a] (μg/mL)	LOQ[b] (μg/mL)	Spiked quantity[c] (μg)	Measurement range[d] (μg/mL)	Corrected calibration function[e]		
					Slope	Intercept	R^2
AlaGln epimers (DL/LD)	0.005–5.00	0.005	0.05–5.00	0.55–5.05	4.25E+05	98.17	0.9999
AlaAlaGln	n.d.[f]	0.005	0.05–5.00	0.28–4.78	8.76E+05	−54.68	1.0000
cyclo(AlaGlu)	0.50–5.00	0.500	0.05–5.00	0.16–4.66	8.10E+04	−15.90	0.9995
pyroGluAla	0.50–5.00	0.500	0.40–40.00	2.01–38.01	6.15E+04	0.89	0.9999
AlaGlu	0.025–5.00	0.025	0.05–5.00	1.26–5.76	4.08E+05	−187.46	0.9998
AlaGluAlaGln	0.025–5.00	0.025	0.05–5.00	0.14–4.64	4.59E+05	104.03	0.9986
AlaGlu(AlaGln)	0.10–5.00	0.100	0.05–5.00	0.21–4.71	1.41E+05	−3.14	0.9998

[a] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[b] LOQ was determined with standard solutions using an injection volume of 10 μL. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[c] 100 μL of spiking standard was added to 1 mL of 1:50 diluted sample.
[d] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[e] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.
[f] n.d. – not determined.

Table 3
Calibration functions, linear range and LOQ of impurities determined by the optimized HILIC method using Polysulfoethyl A column.

Impurity	Linear range[a] (μg/mL)	LOQ[b] (μg/mL)	Spiked quantity[c] (μg)	Measurement range[d] (μg/mL)	Corrected calibration function[e]		
					Slope	Intercept	R^2
cyclo(GlyTyr)	0.05–5.0	0.05	0.05–2.0	3.79–47.43	2.66E+05	663.35	0.9987
cyclo(AlaGln)	0.013–5.0	0.013	0.013–2.0	34.74–51.10	6.75E+05	1.00E+07	0.9897
TyrGly	0.025–5.0	0.025	0.025–2.0	1.49–3.29	8.81E+04	−0.23	0.9950
AlaGluHis	0.025–5.0	0.025	0.025–2.0	0.10–1.90	3.62E+05	−0.37	0.9995
AlaGlu(His)	0.025–5.0	0.025	0.025–2.0	0.67–2.47	4.97E+05	−0.80	0.9998
AlaGluArg	0.05–5.0	0.05	0.05–2.0	0.09–1.89	2.07E+05	0.34	0.9991
AlaGlu(Arg)	0.05–5.0	0.05	0.05–2.0	0.22–2.02	1.41E+05	−0.03	0.9996
AlaGluLys	0.05–5.0	0.05	0.05–2.0	0.11–1.91	2.58E+05	0.28	0.9992
AlaGlu(Lys)	0.05–5.0	0.05	0.05–2.0	0.17–1.97	3.86E+05	−0.32	0.9995

[a] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[b] LOQ was determined with standard solutions using an injection volume of 10 μL. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[c] 100 μL of spiking standard was added to 1 mL of 1:20 diluted sample.
[d] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[e] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.

LOQ was defined as the concentration at which the qualifier transition of the analyte yields a signal to noise ratio of at least 3 and the quantifier of at least 10.

3. Results and discussion

3.1. Preliminary study on stereoisomeric impurities of L-Ala-L-Gln

L-Ala-L-Gln, the most abundant component in the parenteral solution, has multiple chiral centers and stereoisomeric impurities may be formed during processing. Monitoring of diastereomers, formed by single step epimerization at the stereogenic centers of *N*-terminal Ala or *C*-terminal Gln amino acids appears to be more important than analysis of its enantiomeric impurity which is obtained rather via a two step epimerization than via a simultaneous inversion of both stereogenic centers (for reaction scheme see also Fig. S1 of Supplementary material). Hence, D-Ala-D-Gln is expected to be present at lower concentration than the epimers D-Ala-L-Gln and L-Ala-D-Gln.

Based on a reported chromatographic method for the separation of stereoisomers of AlaAsn [19], preliminary experiments were performed on a Chirobiotic T column. In spite of a successful separation

Table 4
Calibration functions, linear range and LOQ of impurities determined by the optimized RP method using a Gemini C18.

Impurity	Linear range[a] (μg/mL)	LOQ[b] (μg/mL)	Spiked quantity[c] (μg)	Measurement range[d] (μg/mL)	Corrected calibration function[e]		
					Slope	Intercept	R^2
$(AcCys)_2$	0.05–2.00	0.050	10.0–750.0	33.2–705.9	8.18E+03	0.87	0.9989
cyclo(GluAla)GlyTyr	0.025–2.00	0.025	0.02–1.50	0.09–1.43	3.30E+05	0.02	0.9925
pyroGluAlaGlyTyr	0.025–2.00	0.025	0.02–1.50	0.05–1.40	2.02E+05	0.0543	0.9929
cyclo(AlaGlu)Met	0.005–2.00	0.005	0.02–1.50	0.03–1.37	6.31E+05	−0.0385	0.9940
pyroGluAlaMet	0.005–2.00	0.005	0.02–1.50	0.02–1.36	8.52E+05	0.054	0.9929

[a] The linear range was determined in preliminary calibration experiments using neat standard solutions.
[b] LOQ was determined with standard solutions using an injection volume of 10 μL. The LOQ was defined as the concentration where the quantifier yields a signal to noise ratio of 10:1 and the qualifier at least 3:1.
[c] 100 μL of spiking standard was added to 1 mL of 1:20 diluted sample.
[d] The measurement range constitutes the concentration range that was effectively measured. It is calculated as the sum of the spiked quantity and the concentration of the analyte already present in the sample solution.
[e] Calibration was accomplished using standard addition. Generated calibration functions were corrected for the concentration of the analyte already present in the sample.

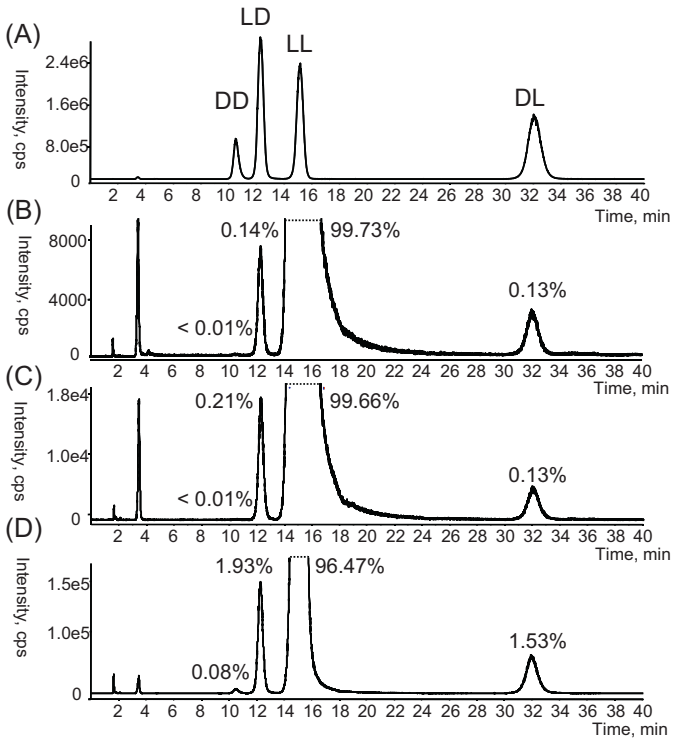

Fig. 2. Stereoselective separation of DNP-derivatized AlaGln isomers in parenteral infusion solutions on Chiralpak QN-AX under isocratic conditions. DNP-derivatives elute in the order DD < LD < LL < DL. MRM transition: 382 → 192 (DP −65, CE −22, CXP −11). (A) Standard solution of the four stereoisomers of AlaGln; (B) nutritional infusion solutions (not stressed); (C) stressed nutritional infusion solution stored for 12 months at 40 °C; (D) stressed nutritional infusion solution stored for 9 months at 60 °C.

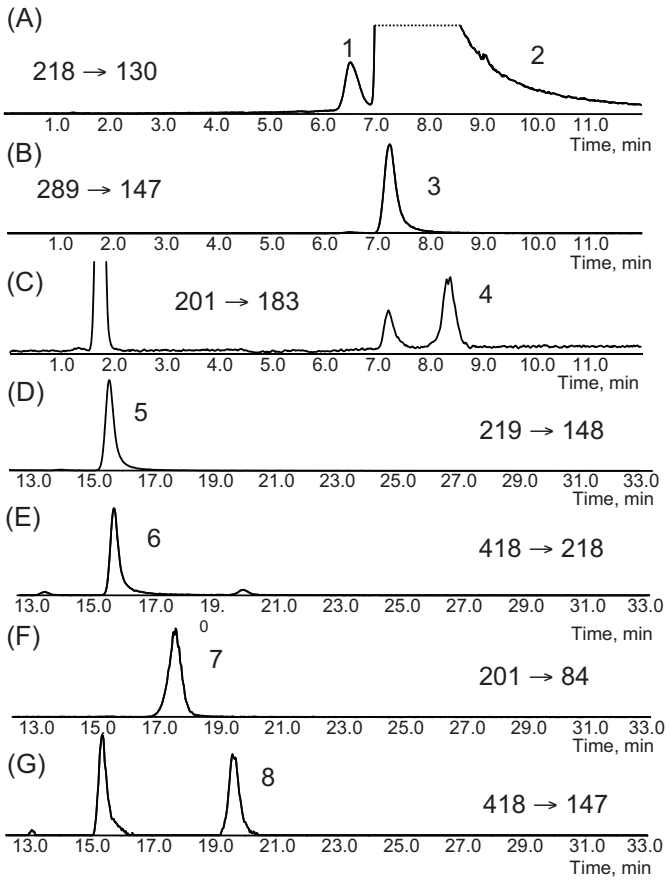

Fig. 3. HILIC–ESI-MS/MS chromatograms of the separation of AlaGln degradation products on Chiralpak QN-AX. Peak annotation: (A) 1, AlaGln epimers (DL, LD); 2, L-Ala-L-Gln; (B) 3, AlaAlaGln; (C) 4, cyclo(AlaGlu); (D) 5, AlaGlu; (E) 6, AlaGluAlaGln; (F) 7, pyroGluAla; (G) 8, AlaGlu (AlaGln).

of all four stereoisomers (elution order of D-Ala-L-Gln < L-Ala-L-Gln < L-Ala-D-Gln < D-Ala-D-Gln with MeOH/H_2O (90/10, v/v) at flow rate of 0.5 mL/min), the method was not very well suitable for trace level quantitative analysis, especially of those isomeric purities that eluted close to the major LL-form, due to insufficient resolution. Further experiments were carried out on a tert-butylcarbamoylquinine based chiral stationary phase (Chiralpak QN-AX column) known to exhibit stereoselectivity for *N*-derivatized amino acids and peptides according to an anion-exchange retention principle [20,21]. Thus, AlaGln dipeptides were derivatized with Sanger's reagent. The resultant *N*-dinitrophenyl-AlaGln (DNP-AlaGln) isomers were fully baseline resolved with adequate peak shapes (Fig. 2A).

Three infusion solutions subjected to different treatments, i.e. not stressed (Fig. 2B), stressed at 40 °C for 12 months (Fig. 2C) and stressed at 60 °C for 9 months (Fig. 2D), were analyzed. The relative peak areas of the four stereoisomeric forms are indicated in Fig. 2.

The LD- and DL-stereoisomers could be detected at levels above the reporting threshold in each of the three infusion solutions, while the DD-enantiomer was always present at levels below the reporting threshold of 0.05% except for the harshly stressed solution stored at 60 °C for 9 months (0.078%).

As the reporting threshold of DD-isomer was not exceeded in the reference solution (40 °C/12 month), the DD-isomer was excluded from further considerations as a relevant impurity.

3.2. Chiralpak QN-AX HILIC method

The majority of impurities listed in Table 1 are hydrophilic peptide (-like) impurities that elute in RPLC unresolved with or close to the void volume, which is susceptible for ion suppression and may be associated with a low detection sensitivity due to the high water content of the eluent and poor ionization efficiency [22]. A HILIC separation mode using a Chiralpak QN-AX column, on contrary, provided sufficient retention and the required diastereoselectivity for the epimers (DL, LD isomers) of L-Ala-L-Gln without derivatization. It allowed the combined determination of both epimers and a distinction of the two epimers was not necessary at this point. A chromatogram of a spiked sample is shown in Fig. 3A. The epimeric impurities (DL and LD) coelute as minor impurity peak in front of the major ingredient compound L-Ala-L-Gln. This facilitates accurate peak integration and provides a lower LOQ than for the case where it elutes on the tailing edge of the main component.

Besides the epimers of L-Ala-L-Gln, a number of other impurities were analyzed with the HILIC method developed on the Chiralpak QN-AX column (see Table 1). For example other critical solute pairs that demanded separation owing to their isobaric nature are the constitutional isomers cyclo(AlaGlu) and pyroGluAla as well as AlaGluAlaGln and AlaGlu(AlaGln). Successful separation of these compounds was also achieved as illustrated in Fig. 3. On the other hand, the tripeptide AlaAlaGln and cyclo(AlaGlu) coeluted with L-Ala-L-Gln. To examine the specificity of the employed SRM transitions a single standard of L-Ala-L-Gln at a content corresponding to that in the formulation was injected and the SRM transitions of AlaAlaGln and cyclo(AlaGlu) were monitored. In the SRM traces of AlaAlaGln and cyclo(AlaGlu) no peak could be found at the retention time corresponding to L-Ala-L-Gln which demonstrated that the employed transitions were specific for these two compounds and interference of L-Ala-L-Gln on the signals of AlaAlaGln and cyclo(AlaGlu) could be precluded.

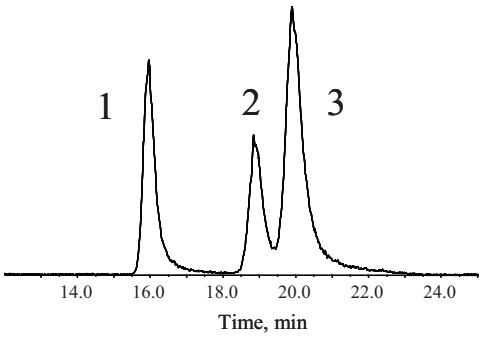

Fig. 4. TIC chromatogram illustrating the separation of: 1, AlaGlu; 2, GluAla; 3, Glu(Ala) on Chiralpak QN-AX.

In preliminary investigations, it was demonstrated that besides AlaGlu also structural analogs namely GluAla and Glu(Ala) were formed in harshly stressed solutions (e.g. 60 °C/9 months). Thus, selectivity of the present method was examined, in order to exclude interference of these isobaric impurities with quantification of AlaGlu. It becomes evident from Fig. 4 that neither GluAla nor Glu(Ala) interfered with the determination of AlaGlu because both are adequately resolved.

After validation of assay specificity, other validation parameters have been assessed including linear range, and LOQ (Table 2). Intra- and inter-assay precision and accuracy are summarized in Table S1 (see Supplementary material).

Calibration functions have initially been constructed with plain standard solutions in the range of 0.005–5.0 μg/mL. Linearity with $R^2 > 0.9938$ was observed for all target solutes in the range specified in Table 2. From these dilution series, LOQs could be determined for the individual compounds as concentrations at which the signal-to-noise ratio was 10:1 (see Table 2). Except for pyroGluAla and cyclo(AlaGlu), the LOQs were adequate and allowed the determination of all impurities below the reporting threshold. For pyroGluAla and cyclo(AlaGlu) the LOQ was above the reporting threshold. However, this was of no concern because pyroGluAla was always present in the investigated infusion solutions at concentration levels significantly above the LOQ. Concentrations of cyclo(AlaGlu) were near the determined LOQ in the infusion solutions. However, intra and interday precision and accuracy results were acceptable at the lowest concentration (Table S1, see Supplementary material). Thus, the applicability of the method was not compromised by the lower sensitivity for these compounds.

Furthermore, calibration functions have also been set up in a narrower but more relevant concentration range by standard addition into an unstressed infusion solution. Slopes of these calibration curves were compared with those obtained by neat standard solutions (i.e. standards spiked into L-Ala-L-Gln solution with a concentration level matching the one in infusion solutions), in order to assess whether the MS responses in the sample solutions are affected by potentially coeluting non-detected sample constituents. The matrix matched calibration function was completely overlapping with that in neat standard solution for AlaGlu(AlaGln) and only minor deviations were found for the other compounds (see Fig. S2 of Supplementary material). This indicates potentially coeluting compounds do not affect ionization efficiency and quantitative results for these solutes. In spite of it, matrix-matched calibration by a standard addition procedure was considered to be more accurate and more reliable, and was thus further employed for the validation process and the analysis of the samples from stress testing.

Intra- and interday precision and accuracy have been determined by three replicate injections of quality control standards (infusion solution spiked at three concentration levels; low, middle and high) on three different days.

Considering established acceptance criteria of 10% for intra- and interday precision, excellent results were obtained for all analytes at the tested concentration levels, with RSD values mostly lower than 2% but never above 7% for intraday precision. Interday precision measured on three different days mainly ranged between 1 and 5% and was always lower than 10%. Accuracy was assessed by % recoveries of spiked sample solutions after correction of the intrinsic impurity content of the utilized infusion solutions. The acceptance criterion for accuracy was set to a range of 95–105%. Both intra- and interday accuracies at the middle and the high concentration levels were always within the acceptance range, with a few outliers in the low concentration levels (see Table S1 of Supplementary material). In general, it may be concluded that the assay is conforming to the requirements.

3.3. Polysulfoethyl A HILIC method

In the course of ongoing investigations a number of other impurities could be identified. In a screening, a Polysulfoethyl A column operated under HILIC conditions showed promising results, and finally allowed for adequate separation and analysis of the majority of remaining impurities.

Table 1 provides MS acquisition data and retention time information for the compounds analyzed using the Polysulfoethyl A column which is a strong cation exchange stationary phase well suited for HILIC separations of hydrophilic peptides [23,24]. It showed selectivity for structural isomers such as AlaGluX and AlaGlu(X) (X = Arg, His, Lys) as well as cyclo(AlaGlu)His and pyroAlaGluHis (Fig. 5) which need to be separated in order to allow unequivocal quantification. In this context, it is worthwhile to mention that this chromatographic system also allows separation of AlaGluX and AlaGlu(X) from the corresponding peptide with retro-sequence GluAlaX. For example, the three peptides AlaGlu-Arg/AlaGlu(Arg)/GluAlaArg were baseline resolved (see Fig. 6) and the corresponding set with His replacing Arg showed a similar separation. Preliminary experiments, however, proved that the retro-sequence GluAlaX is of no relevance and below the reporting threshold value or not at all found.

These peptides have, in addition to their terminal amino groups, a basic side chain for ionic interactions with the negatively charged sulfonic acid group of the stationary phase. Thus, a mixed mode HILIC/SCX mechanism may be at work which may be the key for the intriguing selectivity. However, also cyclic peptides such as cyclo(AlaGln) and cyclo(GlyTyr) lacking a free primary amine are well retained on this phase and well resolved from the parent dipeptides in accordance to a HILIC retention mechanism. cyclo(GlyTyr) is stemming from the parent GlyTyr constituent which substitutes Tyr due to its better solubility and a rapid in vivo hydrolysis to free Tyr and Gly [6]. The retro-peptide TyrGly was well resolved from GlyTyr as well as from cyclo(GlyTyr). In this case, preliminary experiments suggested that TyrGly should be of relevance as impurity being present in the stressed samples presumably above the reporting threshold value.

Calibration results (with neat standard solutions over extended range and standard addition to an infusion solution, respectively) as well as sensitivity data (LOQ) are summarized in Table 3. Acceptable coefficients of determination $R^2 > 0.994$ were obtained for the calibration functions in any case. LOQ (signal-to-noise = 10:1) of 0.05 μg/mL or lower for all analytes confirm appropriate method sensitivity for assessing concentrations at reporting threshold levels.

For all analytes intraday precision at the three tested concentration levels ($n = 3$) was always lower than the acceptance criterion of 10% RSD (Table S2 of Supplementary information). Similar results

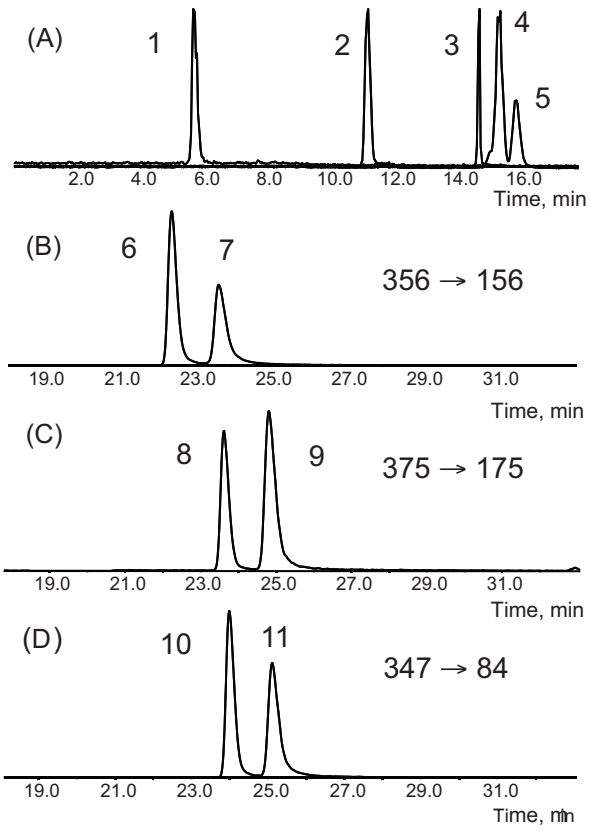

Fig. 5. HILIC–ESI-MS/MS chromatograms of the separation of degradation products on Polysulfoethyl A column. Peak annotation: (A) overlaid MRM traces of: 1, cyclo(GlyTyr) (221 → 107); 2, cyclo(AlaGln) (200 → 155); 3, TyrGly (239 → 136); 4, pGluAlaHis (338 → 156); 5, cyclo(AlaGlu)His (338 → 156); (B) 6, AlaGlu(Arg); 7, AlaGluArg; (C) 8, AlaGlu(His); 9, AlaGluHis; (D) 10, AlaGlu(Lys); 11, AlaGluLys.

were obtained for interday precision, which ranged between 0.6 and 8.3% RSD for all analytes, except for AlaGlu(Arg) for which %RSD values of 14.9 and 12.7% were determined at the spiking levels of 0.02 and 0.1 μg, respectively. Intra- and interday accuracy values were mostly within the acceptance interval of 95–105% and always between 80 and 120% even at the lower concentration level confirming applicability of the method.

3.4. RP method employing Gemini C18

A few of the remaining impurities which were less polar were investigated using the Gemini C18 phase. Thus, the pairs of constitutional isomers cyclo(AlaGlu)Met and pyro(AlaGlu)Met as well as cyclo(AlaGlu)GlyTyr and pyro(AlaGlu)GlyTyr could be separated

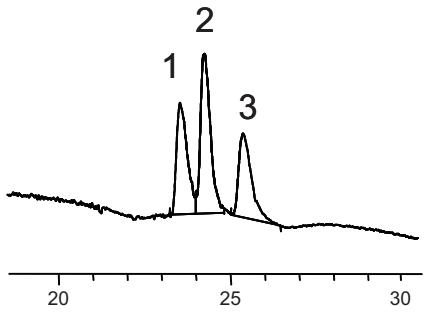

Fig. 6. HILIC-CAD (charged aerosol detector) chromatogram illustrating separation of: 1, GluAlaArg; 2, AlaGlu(Arg); 3, AlaGluArg in a standard solution on Polysulfoethyl A employing mobile phase conditions as specified in Section 2.

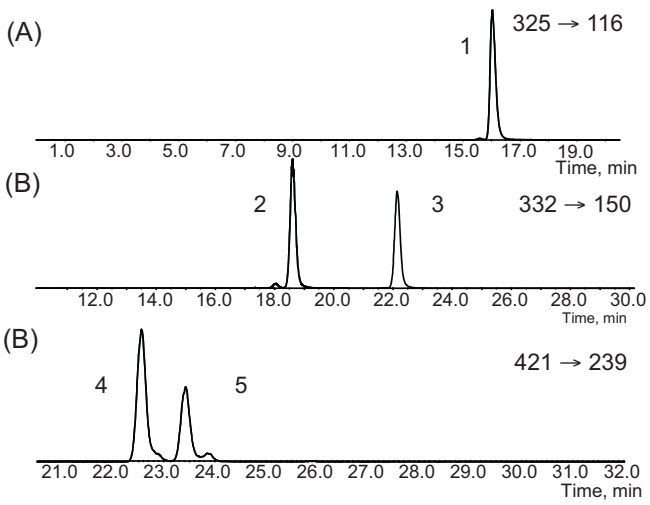

Fig. 7. RPLC–ESI-MS/MS chromatograms of the separation of degradation products on a Gemini C18 column. Peak annotation: (A) 1, $(AcCys)_2$; (B) 2, pyroGluAlaMet; 3, cyclo(AlaGlu)Met; (C) 4, cyclo(AlaGlu)GlyTyr; 5, pyroGluAlaGlyTyr.

according to hydrophobicity differences as illustrated in Fig. 7 and were finally accurately quantified by this method.

Preliminary calibration functions were set up with neat standard solutions. Coefficients of determination $R^2 > 0.999$ were obtained for the investigated extended calibration range. LOQs (signal-to-noise ratio > 10:1) were determined by dilution of standard solutions and ranged between 0.005 and 0.05 μg/mL (see Table 4) being in any case below the reporting threshold of the respective impurities.

Detailed results for validation can be found in Table S3 of Supplementary material. Intraday precision ($n = 3$) was always better than the acceptance limit of 10% RSD. The same was valid for interday precisions at the medium and high concentration levels (except for $(AcCys)_2$ for which it was 10.9%). However, interday precision for pyroGluAlaMet and cyclo(AlaGlu)Met was slightly above the limit of 10% at the lowest concentration level (again $(AcCys)_2$ showed much worse results). Accuracies, both intra-assay as well as interday, were quite acceptable except for the lowest QC level. Hence, the applicability of the RP method has to be restricted to a higher concentration range.

Stronger variations at the lowest concentration levels might be attributed to ESI spray instabilities caused by the high water content in the RP mode. HILIC elution modes, in contrast, seem to offer an advantage since the high organic content in the HILIC mode provides better sprayer efficiency and stability due to lower surface tension [22].

A particular problem represented $(AcCys)_2$. Several significant outliers were found with regard to both precision and accuracy (see Table S3 of Supplementary information). This compound is formed by oxidation of *N*-acetyl-cysteine, which is contained as active compound in the infusion solutions. The unacceptable precision and accuracy values of $(AcCys)_2$ at the lowest examined QC level might be explained by the redox instability and the susceptibility of this disulfide compound to uncontrolled and irreproducible redox-reactions in the ESI-sprayer. Considering middle and high concentration levels of $(AcCys)_2$ accuracy and precision were again within an acceptable range.

3.5. Application

The three validated methods were employed for the quantification of the discussed impurities in differently stressed infusion

Table 5
Concentration, standard deviation and % concentration of impurities relative to main precursor compounds identified and quantified in infusion solutions stored at 40 °C for different time spans (3, 6 and 12 months). Bold letters: >reporting threshold; italic: >identification threshold; underlined: >qualification threshold.

Impurity	Solution 40 °C/3 months			Solution 40 °C/6 months			Solution 40 °C/12 months		
	μg/mL	Std. dev. (μg/mL)	%[a]	μg/mL	Std. dev. (μg/mL)	%[a]	μg/mL	Std. dev. (μg/mL)	%[a]
AlaGln epimers (DL/LD)	*31.7*	0.6	*0.144*	38.0	n.d.[b]	0.173	78.2	3.3	0.355
AlaAlaGln	**16.7**	0.3	**0.076**	**17.5**	n.d.	**0.080**	*23.0*	1.0	*0.104*
cyclo(AlaGlu)	9.2	0.4	0.042	**15.6**	n.d.	**0.071**	76.7	4.9	0.348
AlaGluAlaGln	6.9	0.3	0.031	7.7	n.d.	0.035	**16.9**	0.5	**0.077**
AlaGlu(AlaGln)	**11.5**	0.3	**0.052**	**17.4**	n.d.	**0.079**	37.8	0.7	0.172
AlaGlu	63.6	1.2	0.289	116.5	n.d.	0.530	328.9	7.2	1.495
pyroGluAla	111.7	5.7	0.508	121.9	n.d.	0.554	309.7	8.3	1.408
cyclo(GlyTyr)	45.1	4.4	1.670	60.2	11.5	2.228	n.d.[b]	n.d.[b]	n.d.[b]
cyclo(AlaGln)	752.3	101.2	3.419	807.6	58.9	3.671	n.d.	n.d.	n.d.
TyrGly	31.1	9.9	1.150	27.4	9.8	1.016	n.d.	n.d.	n.d.
AlaGlu(His)	9.2	0.6	0.042	**11.9**	0.5	**0.054**	n.d.	n.d.	n.d.
AlaGluHis	1.1	0.1	0.005	1.3	0.2	0.006	n.d.	n.d.	n.d.
AlaGlu(Arg)	2.9	0.1	0.013	2.9	0.2	0.013	n.d.	n.d.	n.d.
AlaGluArg	1.0	0.2	0.004	0.9	0.2	0.004	n.d.	n.d.	n.d.
AlaGlu(Lys)	2.0	0.1	0.009	2.1	0.1	0.010	n.d.	n.d.	n.d.
AlaGluLys	1.0	0.1	0.005	1.0	0.2	0.004	n.d.	n.d.	n.d.
$(AcCys)_2$	147.8	21.5	14.778	239.0	n.d.[b]	23.897	4151.7	212.0	415.2[c]
cyclo(AlaGlu)GlyTyr	0.39	0.03	0.002	0.78	n.d.	0.004	3.25	0.23	0.015
pyroGluAlaGlyTyr	0.16	0.03	0.001	0.38	n.d.	0.002	1.14	0.05	0.005
cyclo(AlaGlu)Met	0.02	0.01	0.0001	0.03	n.d.	0.0001	0.13	0.005	0.001
pyroGluAlaMet	<LOQ	<LOQ	<LOQ	0.005	n.d.	0.00002	0.02	0.01	0.0001

[a] % concentration relative to the precursor compound present at higher concentrations in the infusion solutions.
[b] n.d. – not determined.
[c] $(AcCys)_2$ present in excess over parent compound AcCys.

solutions. In Table 5 the results of three solutions stored at 40 °C for different time spans (3, 6 and 12 months) are presented.

Quantitative results reveal that cyclo(AlaGln) (diketopiperazine derivative of AlaGln) is the most abundant degradation product of AlaGln. It is present at concentrations far above the qualification threshold of 0.15%. The same is true for cyclo(GlyTyr) which is the main degradation product of GlyTyr. Furthermore, it may be surprising that the impurity TyrGly was also found in concentrations above the qualification limit. Moreover, high concentrations of $(AcCys)_2$ were found in stressed sample solutions as expected due to the redox-instability of *N*-acetyl-cysteine. Its concentration was also determined to be above the qualification threshold of 0.15%.

Several isobaric peptide-like impurities were formed by condensation reactions with AlaGlu during storage at elevated temperatures. Thereby, it is striking that condensation reactions with the carboxylic function in the side chain seem to be preferred over condensation at the *C*-terminal end (see Table 5). Furthermore, higher contents of cyclo(AlaGlu)Y were found compared to pyroGluAlaY, which was unexpected because higher concentrations of the pyroGluAla precursor were detected in the solutions.

However, most of these condensation products were found in concentrations below the reporting threshold (0.05%) except for AlaGlu(His), AlaGlu(AlaGln) and AlaGluAlaGln for which a content above the reporting threshold was determined.

The obtained quantitative data allow unambiguous assignment of impurities below or above reporting, identification and qualification thresholds and provide a basis for shelf life estimation as well as for assessment of long-term stability.

4. Conclusion

Three HPLC–ESI-MS/MS methods using different stationary phases (Chiralpak QN-AX and Polysulfoethyl A under HILIC elution conditions and RPLC with a Gemini C18) were developed for the accurate analysis of impurities in nutritional infusion solutions for amino acid supplementation. Different separation mechanisms (HILIC, ion-exchange and hydrophobic interactions) were exploited for the separation and quantitative analysis of several impurities formed during stress tests. Critical pairs like stereoisomers, constitutional isomers or other isobaric compounds that cannot be distinguished by specific SRM transitions, could be chromatographically separated and thus accurately quantified. The assays were validated according to the ICH guidelines. Obtained validation results confirmed the applicability of the methods for the purpose of impurity profiling. Furthermore, investigations of stressed samples revealed that not only deamidation of AlaGln to AlaGlu and peptide hydrolysis of AlaGln occur, but also cyclization (diketopiperazine formation) and other condensation reactions take place, preferably with constituents present at higher concentration levels such as AlaGln and GlyTyr or amino acids carrying basic functional groups (Arg, His, Lys).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.020.

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