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Control of impurities in L-aspartic acid and L-alanine by high-performance liquid chromatography coupled with a corona charged aerosol detector

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ABSTRACT

In this study a reversed phase ion-pair high-performance liquid chromatography (HPLC) method using charged aerosol detection (CAD) was developed and fully validated for the pharmaceutical quality control of L-aspartic acid (Asp). With a slight modification, the method also allows the evaluation of related substances in L-alanine (Ala). The method enables simultaneous control of related amino acids and of possibly occurring organic acids contaminants. A minimum limit of quantification of 0.03% could be achieved for all occurring related substances. Moreover, the detector sensitivity of the CAD was compared with an evaporative light scattering detector (ELSD). Depending on the analyte the CAD was found to be 3.6–42 times more sensitive than the ELSD. The HPLC method was applied to the purity testing of 8 samples of pharmaceutical grade and reagent grade Asp and of 12 samples of Ala supplied by various manufacturers. Both substances were found to be of high purity (greater than 99.8% for Asp and greater than 99.9% for Ala). Malic acid and Ala were the major impurities in Asp. Asp and glutamic acid (Glu) were the only detectable impurities in Ala.

1. Introduction

Amino acids belong to the most widely used biological compounds e.g. in the fields of nutrition, cosmetics, agriculture and medicine [1]. In the latter field amino acids are widely used in "classical" medicinal applications including the parenteral nutrition of patients with insufficient renal clearance, liver insufficiency, in the paediatric domain or the use of certain amino acids like tryptophan because of their specific pharmacological effects [2,3] in medicines against depression and as sleep inducing substances [4]. Moreover, they are also of interest for "alternative" medicinal treatment (e.g. amino acids in whitmania pigra used in traditional Chinese medicine (TCM) [5]).

Based on their significant use in the fields of nutrition and medicinal products a proper control of the quality of the amino acids is of crucial importance for the consumer or patient.

Unfortunately, due to their physico-chemical properties, i.e. the lack of a chromophor in most of the amino acids, their analysis and especially the purity control of low level impurities is a particular analytical challenge and no analytical method has yet been found which is superior to all the others [6].

This is probably one of the major reasons, why in Pharmacopoeia monographs [7,8] amino acids are still controlled by a thin layer chromatography (TLC) test for ninhydrin-positive substances, accompanied by a limit test for ammonia instead of a high-performance liquid chromatography method (HPLC) for related substances as it is a common standard for the quality control in most other compendial monographs of active pharmaceutical ingredients (APIs).

In industry the purity of amino acids is usually controlled using Amino-Acid-Analysers (AAA). The analysis is based on ion-exchange chromatography, normally using complex gradients, followed by post-column derivatisation with ninhydrin, dinitrophenylhydrazone (DNP) or other suitable reagents. The major disadvantage of these methods, apart from the fact that AAA-instruments are not broadly available outside some specialised laboratories, is that impurities other than amino acids are not detected. In some cases, especially for amino acids produced by enzymatic synthesis, an additional ion-exchange chromatography method is employed to control residues of organic acids used as starting materials.

However, the paramount importance of having a general test for related substances became evident in 1989 when it was hypothesized that one or more trace impurities produced during the manufacture of tryptophan might have been responsible for the outbreak of a disabling autoimmune illness called

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eosinophilia-yalgia syndrome (EMS) leading to the death of several patients [9,10].

Considering the above, it was concluded that the development of tailor-made related substances test for the individual amino acids would be a step forward in the quality control of amino acids. This specific related substances test should take into account the real impurity profile including – in contrast to AAA – also impurities other than amino acids.

In the recent past HPLC methods with evaporative light scattering detection (ELSD) have been described for the evaluation of amino acids [5,6]. The development of the ELSD dates from the late 1970s. The detector can be considered to be a quasi-universal detector which is more sensitive than other universal detectors including refractometry [6]. ELSD can be of great benefit to analytical HPLC methods when it is used for the detection of compounds in mixtures of similar concentrations, but the detector might not necessarily be sensitive enough for the control of low level impurities in an API.

Some years ago, the charged aerosol detector (CAD) was introduced by Dixon and Peterson [11]. Compared with the ELSD, the CAD detector was reported to have an about 10-fold increased sensitivity [11–15].

As it is the case for the ELSD, the response of CAD is not directly linear over a broad concentration range, and good linearity is obtained only in a logarithmic coordinate system [11,12,16]. However, the response of the CAD was reported to be linear over a limited range of about 2 orders of magnitude in different studies [17,18]. This allows to apply a linear calibration function in a limited concentration range.

Although an increasing number of papers about the CAD are being published in the literature, Nováková et al. [19] reported that pharmaceutical applications of the CAD are still rare.

Following the concept of developing specific methods for the impurities control for the individual amino acids, Asp and Ala were selected as examples.

For an appropriate design of the corresponding methods it was important to know the possible ways of production/synthesis. In principle, four different routes are used for the industrial production of amino acids. These are chemical synthesis, hydrolysis of proteins/peptides followed by chromatographic separation, enzymatic synthesis and fermentation [20–22]. For Asp a chemical synthesis was reported [23], but does not have practical relevance for industrial production. Moreover, different fermentation methods were described [24,25] and it is also possible to obtain Asp as a product of protein hydrolysis [22]. According to available information, enzymatic production of Asp starting from fumaric acid currently appears to be the predominant means of production [21,26,27].

As described above, a biological product like Asp can be obtained using rather different processes with numerous possible impurities. For this reason, the European Pharmacopoeia Commission has introduced the general monograph on products of fermentation [28]. This monograph applies general rules for the quality of a product obtained by fermentation, defined in a general manner. These include inactivation or removal of the producer micro-organism, purification processes, residues from the producer micro-organism, culture media, substrates and precursors. In practical terms, the related substances control in a monograph can be limited to certain specific impurities.

For Asp obtained by enzymatic production possible impurities are (a) fumaric acid as a starting material, (b) maleic acid as an impurity of fumaric acid, (c) malic acid which may be produced from fumaric acid by enzymatic reaction, and (d) alanine (Ala) as a decarboxylation product of Asp. In case of a production of Asp by protein hydrolysis glutamic acid (Glu) could possibly occur as a by-product. Since Glu and Asp are acidic amino acids, it is possible that Glu is not completely removed by a chromatographic purification step [22]. The amino acid Ala is also easily accessible by enzymatic synthesis using Asp as a starting material [27]. Therefore, the impurity profile of Ala produced in this way should be similar to the one described above, but also includes Asp as a potential impurity.

The aim of this study was to develop and validate an HPLC method using a CAD for the control of related substances in L-aspartic acid (Asp) and L-alanine (Ala). The method should ensure the appropriate control of possible impurities – often referred to as related substances – on an ICH [29] conform level for drug substances with an average daily dose above 2 g—hence, a reporting threshold of 0.03%.

Several batches of pharmaceutical grade Asp and Ala obtained by different manufacturers/suppliers together with samples of reagent grade Asp and Ala were tested using this new method.

To the best of our knowledge, this is the first time that a single related substances method simultaneously covering related amino acids as well as other process related impurities (organic acids) has been successfully employed. This method is therefore considered to be an important improvement compared with the TLC test for ninhydrin-positive substances currently published in the Pharmacopoeias. Also compared with the AAA-method used by amino acid manufacturers the described LC-CAD method is considered to be very favourable.

2. Experimental

2.1. Reagents and chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Methanol puriss. p.a. and perfluoroheptanoic acid (PFHA) 99% were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). The organic acids, Glu, and L-glutamine (Gln) were of 99% purity. For citric acid and L-asparagine (Asn) the monohydrates were used. The reagents were either supplied by Sigma–Aldrich (St-Quentin Fallavier, France), Fluka (St-Quentin Fallavier, France) or Acros (Noisy le Grand, France).

Test samples of aspartic acid and alanine were kindly provided by Merck (Darmstadt, Germany), Kyowa Hakko (Tokyo, Japan), Degussa Rexim (Radebeul, Germany), Ajinomoto (Leuven, Belgium), Amino GmbH (Frellstedt, Germany), and Shanghai Kyowa (Shanghai, China). Reagent grade standards of the two amino acids were purchased from Sigma, Aldrich, and Fluka (St-Quentin Fallavier, France). Hydrogen peroxide 30% was supplied by Merck (Darmstadt, Germany). Nitrogen +99% was delivered by a Peak Systems NM18LA nitrogen generator (Lab Gaz Systems, Massy, France).

2.2. Apparatus

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St-Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography. Detection was performed by a Corona CAD Detector (ESA Bioscience Inc., Vendor: Eurosep Instruments Cergy Pontoise, France).

Evaporative light scattering detection was performed using a Polymer Laboratories PL-ELS 2100 Evaporative Light Scattering Detector (Marseille, France). The Inertsil ODS 3 column was purchased from Interchim (Montlucon, France).

2.3. Method

2.3.1. Aspartic acid

The separation was performed on an Inertsil ODS 3 column (150 mm × 4.6 mm; particle size 5 μm) at a column temperature of 30 °C. A mixture of 96 vol.% of 1.0 mmol/L PFHA in water and 4 vol.% of 1.0 mmol/L PFHA in methanol was used as mobile phase.

To reduce the baseline noise the column was flushed over night at a flow rate of 0.2 mL/min using methanol/water (50/50, v/v). Thereafter the column was conditioned for about 3 h using the mobile phase. The method runtime was set to 6 times the retention time of Asp at a mobile phase flow rate of 1.0 mL/min. The injection volume was 40 μL. Detection was performed using a CAD at a gas pressure of 35 psi in the 100 pA detection range. Additionally, a mixture of all organic and amino acids at a concentration level of 0.1% (relative to the concentration of the test solution) was injected using an ELSD (nebulizer and drift tube temperature at 50 °C, detector gas-flow at 1.0 standard litre per minute). For the purity testing 10 mg/mL solutions of Asp and Ala in water were used. Due to the limited solubility the Asp solutions had to be heated to 60 °C and stirred for about 20 min to obtain complete dissolution. The solution was found to be stable for about 4–6 h before re-precipitation occurred. An aqueous solution containing 0.01 mg/mL of malic acid, fumaric acid, Asp, Gln, Glu, Ala and 0.05 mg/mL of succinic acid was used for peak identification and as an external standard solution for quantification. A semi-quantitative estimate of the concentrations of unidentified impurities eluting before Asp was made using the instrument response obtained for malic acid. A solution of the same concentrations but also containing 10 mg/mL of Asp was used to check for appropriate resolution.

2.3.2. Alanine

For the purity testing of Ala the above method was slightly modified in that the concentration of PFHA was increased to 1.5 mmol/L. This was found necessary to obtain resolution of Ala and Glu at high concentrations of Ala. It was not necessary to heat the test solution to obtain dissolution of 10 mg Ala per millilitre. For peak identification and quantification the same solution as described under Section 2.3.1 was employed. Moreover, the same strategy regarding the quantification of possible unspecified impurities was applied.

3. Results and discussion

In the reversed phase LC method developed, different principles of separation contribute to obtain sufficient resolution between the substance to be examined and possibly occurring related substances. Whilst the amino acids interact with PFHA and are separated by ion-pair chromatography, the organic acids interact directly with the stationary phase. However, coverage of the C18-surface of the LC-column with PFHA decreases the selectivity of the column for the separation of the organic acids. For this reason, the amount of PFHA in the mobile phase was kept to a minimum. The applied method represents the best compromise for the separation of amino and organic acids under the same chromatographic conditions. Sufficient method selectivity was ensured injecting the spiked test solution described under Section 2.3.1. A minimum resolution of 2.0 between the peaks due to fumaric acid and succinic acid and a peak-to-valley ratio of minimum 1.2 for the separation of Asp and Gln (calculated according to chapter 2.2.46 of the European Pharmacopoeia [7]) were applied as acceptance criteria for the chromatographic separation.

To make this method also applicable to the control of impurities in Ala the concentration of the ion-pair reagent was increased from 1.0 mmol/L to 1.5 mmol/L. Since this modification was checked during robustness testing of the Asp method and did not show a negative impact on method performance, the method described under Section 2.3.2 is considered to be valid for the purity control of Ala. The stability of an Ala test solution during 48 h was checked and the test solution was found to be stable. To determine a suitable runtime for the Ala purity testing, one of the batches was analysed using a runtime of 60 min. Since – apart from a system peak eluting at about 29 min – no impurities were found to elute after the peak due to Ala, the runtime was reduced to 30 min. As described above, a minimum resolution of 2.0 between the peaks due to fumaric acid and succinic acid was appropriate as a resolution requirement. However, the peak-to-valley ratio between of Asp and Gln was replaced by a resolution between Glu and Ala, as being the most critical separation in this method and was set to minimum value of 15.

3.1. Method validation

The above described separation method for Asp has been validated considering the corresponding ICH guideline for the "Validation of Analytical Procedures" [30]. Some important validation parameters are discussed below.

3.1.1. Specificity and system suitability criteria

Under the selected chromatographic conditions, the method was shown to be sufficiently selective to separate Asp and Ala from each other as well as from their possible impurities and several structurally related compounds such as Glu, Gln, fumaric acid, maleic acid, malic acid, citric acid, and succinic acid Fig. 1a/b.

The peak eluting at about 31 min (Fig. 1a) and at about 29 min (Fig. 1b) was identified as a system peak detected also in injections of the mobile phase as a blank. In a 0.1 mg/mL solution a slight separation between Asn and Asp could be achieved, but the method was not capable of separating Asn from Asp in the presence of high concentrations of Asp (Fig. 2). However, the manufactures of Asp did not state Asn to be an impurity of Asp [31].

Typical resolution values found for the separation between fumaric acid and succinic acid ranged from 3.0 to 4.8. For the peak-to-valley ratio between Asp and Gln values from 2.0 to 3.9 were determined. Using an increased concentration of 1.5 mmol/L of PFHA as described under Section 2.3.2 the resolution between fumaric acid and succinic acid slightly decreased but was still found to be above 2.5. Succinic acid and Gln were not considered to be related substances of Asp and Ala. Both compounds were not found in the batches tested. However, the resolution between fumaric acid and succinic acid was selected to ensure selectivity for the compounds not reacting with the ion-pair reagent (organic acids). Moreover, this criterion ensures that succinic acid as the organic acid eluting closest to Asp is separated from the principle peak. Since succinic acid was found to show a low response in the CAD, the test indirectly checks adequate method sensitivity. The second resolution criterion for the Asp method (Section 2.3.1) was chosen to demonstrate sufficient resolution of the compounds forming ion-pairs with PFHA. Gln was selected because it is the compound eluting closest after the Asp peak. For Ala (method 2.3.2) this criterion is not meaningful and was replaced by a resolution requirement between Glu and Ala.

3.1.2. Linearity and range

This study confirmed that the response of CAD is not linear over a broad concentration range [16], but may be linear over a limited range of about 2 orders of magnitude [17,18]. This finding could also be confirmed in this study. A verification of the linearity using mixtures containing the above amino and organic acids at 2.5 μg/mL, 5.0 μg/mL, 10 μg/mL, 14 μg/mL, 20 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL revealed an exponential relation between the concentration and the detector response. The response curve of malic acid is presented as an example in Fig. 3a.

However, for a range from 2.5 μg/mL to 20 μg/mL (corresponding to 0.025–0.2% of the concentration of the Asp test

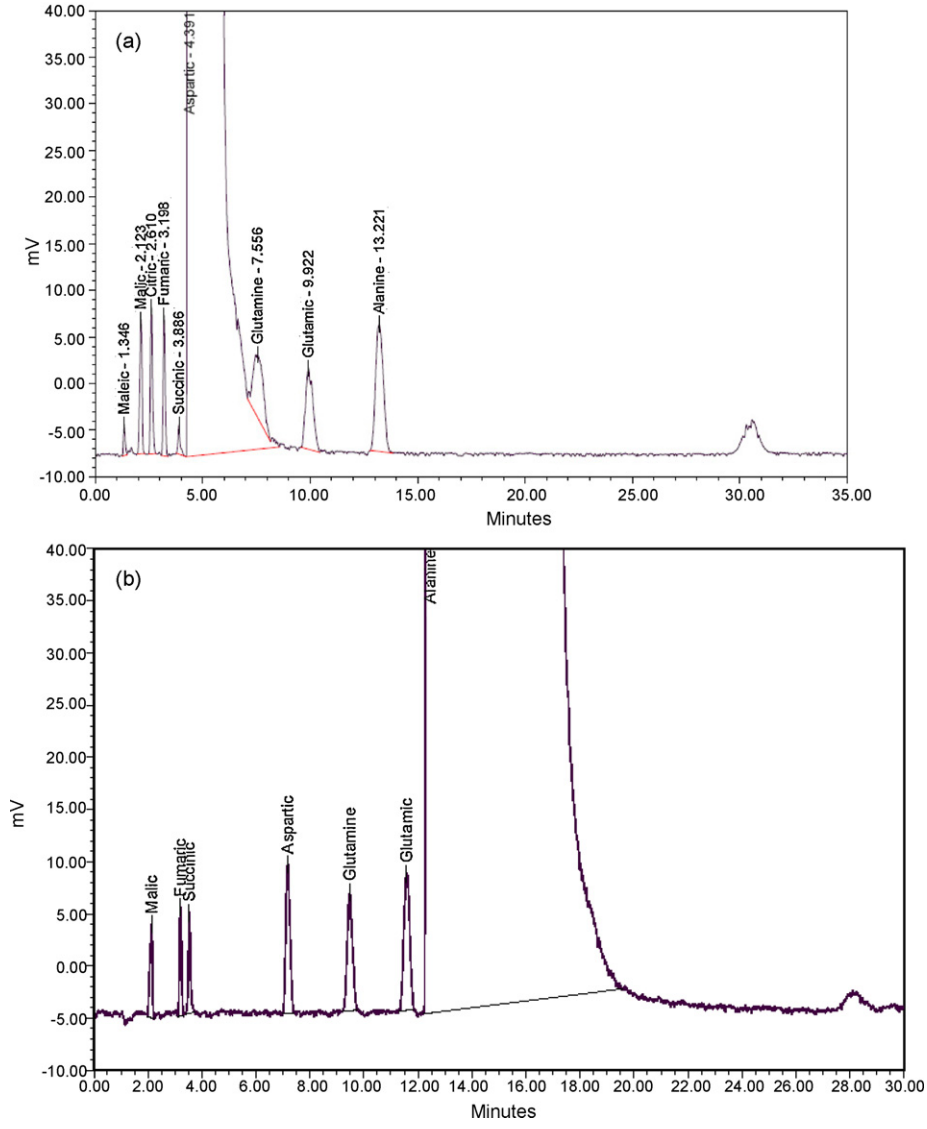

Fig. 1. (a) Chromatogram of a 10 mg/mL solution of Asp spiked with 0.1% of the below components. Conditions: Inertsil ODS 3 column (150 mm × 4.6 mm; particle size 5 μm), column temperature of 30 °C. Mobile phase: 96 vol. of 1.0 mmol PFHA in water and 4 vol. of 1.0 mmol/L PFHA in methanol, detection by CAD. Further conditions are described under Section 2.3.1 Elution order: maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Asp, Gln, Glu, Ala. (b) Chromatogram of a 10 mg/mL solution of Ala spiked with 0.1% of the below components. Conditions: Inertsil ODS 3 column (150 mm × 4.6 mm; particle size 5 μm), column temperature of 30 °C. Mobile phase: 96 vol. of 1.5 mmol/L PFHA in water and 4 vol. of 150 mmol/L PFHA in methanol, detection by CAD. Further conditions are described under Section 2.3.2: elution order: maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Asp, Gln, Glu, Ala.

solution) the detector response was found to be sufficiently linear ($y = ax + b$) to allow a quantification using a 0.1% dilution of the compounds as external standard (see Fig. 3b). For the CAD response of 8 compounds at 5 concentration levels, the coefficient of determination (r^2) was between 0.9957 and 0.9998.

3.1.3. Limit of quantification (LoQ) and relative response factors (RRF)

Table 1 shows the limits of quantification of the CAD – calculated based on the concentration exhibiting a signal-to-noise ratio of 10 – was extrapolated from the detector response of 2.5 μg/mL solutions of the different components (Fig. 4—mixture of organic acids

Table 1
Limits of quantification of the different components under method conditions using by CAD and ELSD. The relative response factors are given for the CAD.

Compound	LoQ ELSD (ng on column)	LoQ CAD (ng on column)	LoQ CAD (in %—referred to an Asp solution of 10 mg/mL)	RRF CAD
Asp	800	24	0.006%	1
Ala	800	24	0.006%	1.9
Glu	1333	32	0.008%	1.1
Gln	667	24	0.006%	1.2
Fumaric acid	800	40	0.01%	0.59
Maleic acid	444	124	0.03%	0.13
Malic acid	500	40	0.01%	0.55
Citric acid	800	40	0.01%	0.67
Succinic acid	1333	240	0.06%	0.14

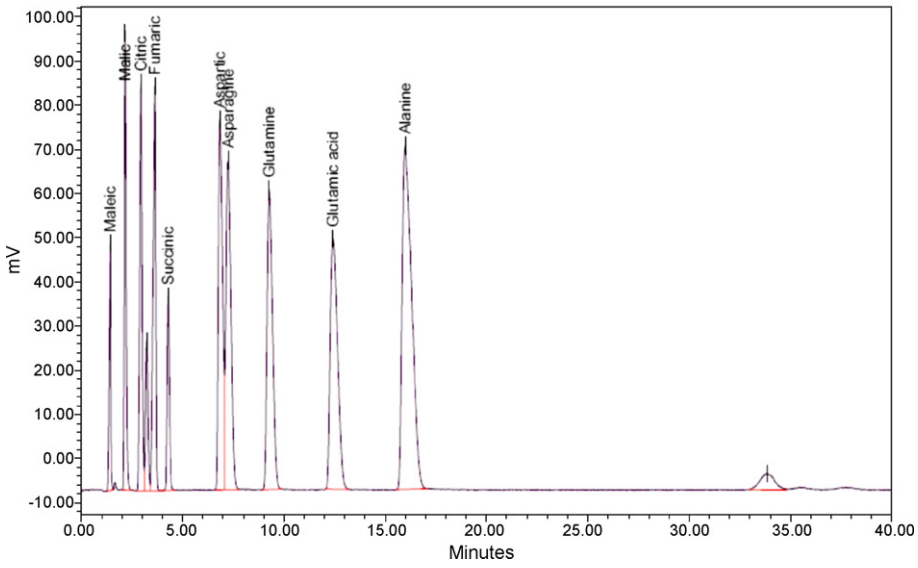

Fig. 2. Chromatogram of a mixture of 0.1 mg/mL of maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Asp, Asn, Gln, Glu and Ala. Conditions: see Fig. 1a.

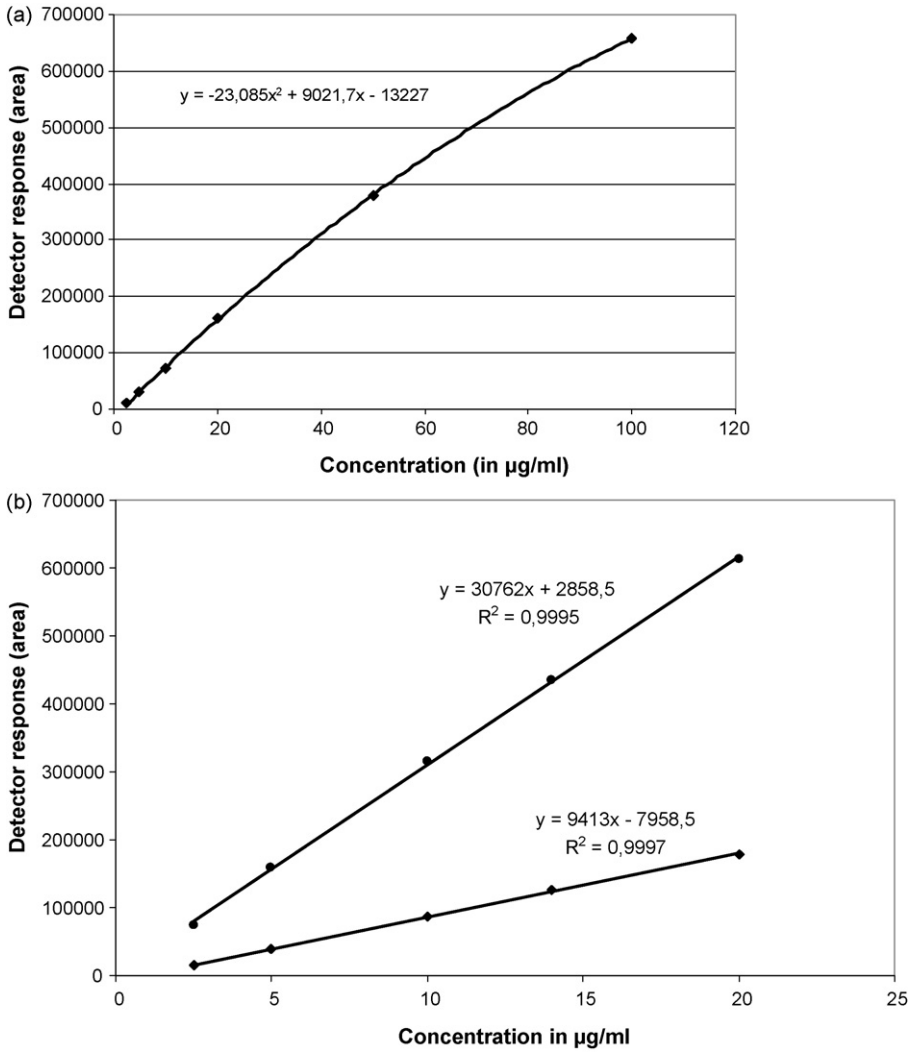

Fig. 3. (a) CAD-calibration curve of malic acid from 2.5 μg/mL to 100 μg/mL (8 concentration levels). Conditions: as described under Section 2.3.1. (b) CAD-calibration curve of malic acid (♦) and Ala (●) from 2.5 μg/mL to 20 μg/mL (linear fit – 5 concentration levels – r^2 malic acid: 0.9997 and Ala: 0.9995). Conditions: as described under Section 2.3.1.

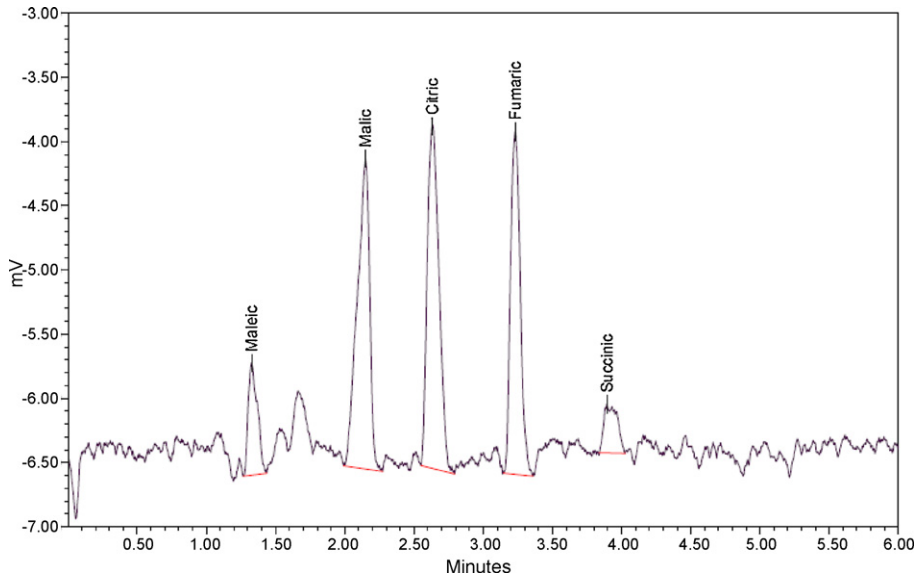

Fig. 4. Chromatogram of a 0.0025 mg/mL mixture of maleic acid, malic acid, citric acid, fumaric acid and succinic acid organic acids. Conditions: see Fig. 1a.

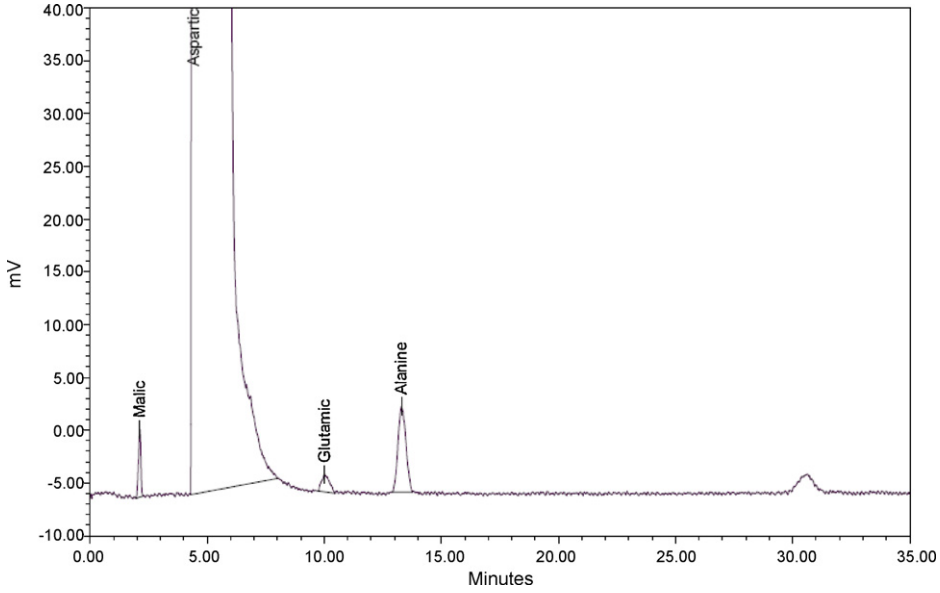

Fig. 5. Chromatogram of a 10 mg/mL test solution of Asp sample no. 3 in water. Conditions: see Fig. 1a and under Section 2.3.1.

at 2.5 μg/mL). The response factors of the different components relative to Asp were determined using the slope of the calibration curves obtained by linear regression from 5 calibration points from 2.5 μg/mL to 20 μg/mL.

Additionally, the limits of quantification were determined by evaporative light scattering detection on a mixture containing 0.01 mg/mL of all organic acids and amino acids separated by the method. All results are summarised in Table 1. The sensitivity of the CAD detector was found between 3.6 and 42 times higher than that of the ELSD. Interestingly, the difference in sensitivity between ELSD and CAD was greatest for the non-volatile amino acids, compared to the relatively volatile organic acids. Based on the results

Table 2
Batch results of the purity testing of 8 batches of L-aspartic acid.

Sample no.	Malic acid	Fumaric acid	Glu	Ala	Unspec. rel. ret. 0.33	Sum
Pharmaceutical grade						
1	0.017%	0.008%	–	0.007%	–	0.031%
2	0.047%	–	0.014%	0.052%	–	0.113%
3	0.051%	–	0.019%	0.067%	–	0.137%
4	0.034%	–	–	–	–	0.034%
5	0.052%	0.005%	–	–	–	0.057%
Reagent grade						
6	0.033%	0.046%	–	–	0.037%	0.116%
7	0.066%	0.008%	–	–	–	0.074%
8	–	0.059%	0.036%	–	–	0.095%

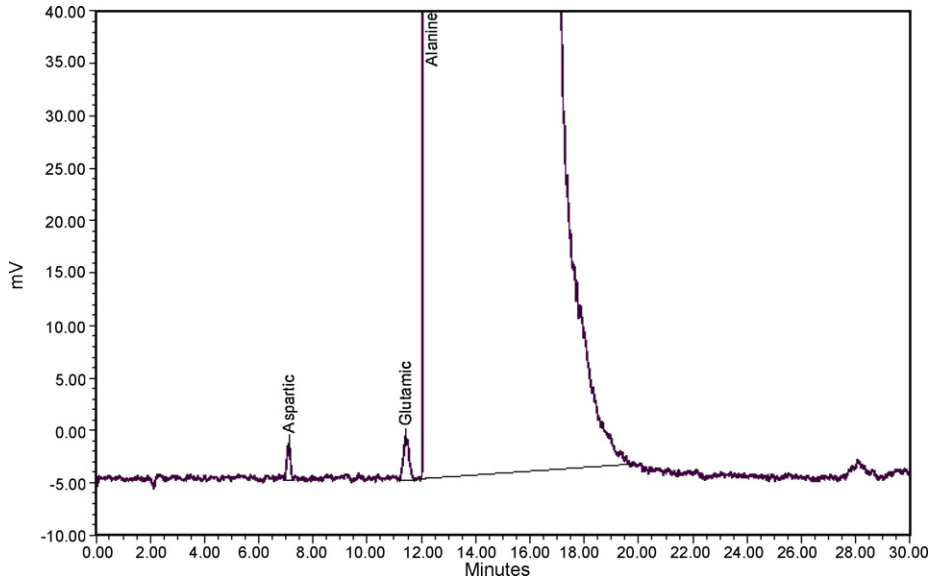

Fig. 6. Chromatogram of a 10 mg/mL test solution of Ala sample no. 6 in water. Conditions: see Fig. 1b and under Section 2.3.2.

obtained the ELSD is not an alternative to the CAD for the intended purpose of the method.

Several authors reported that the CAD response for different compounds is rather similar [11,12,16], which is not the case in this study. The relatively low response of the different organic acids may be explained by their lower chargeability compared with the amino acids. Moreover, organic acids are more volatile than amino acids. This phenomenon could also contribute to the reduction in detector response for the organic acids. For the higher area response of Ala compared with Asp, Glu, and Gln no obvious explanation was found.

3.2. Purity testing of L-aspartic acid

The method described under Section 2.3.1 was used to examine the purity of 5 batches of pharmaceutical grade Asp supplied by three different manufacturers. Moreover, batches of Asp purchased from reagent suppliers were tested. Since the linearity of the range was only verified starting from 0.025% upwards, a calculation of values below this threshold is strictly speaking not correct. However, since the error is considered negligible and inclusion of quantitative figures gives a better image of the batch quality, these results were also reported.

An overview of the results found is presented in Table 2. A chromatogram of the test solution of Asp sample 3 is presented in Fig. 5 for information. The peaks of the impurities occurring in the test solutions were identified by comparison of their retention times with the corresponding peaks in the reference solution.

All batches tested were found to be of greater than 99.8% (w/w) purity with malic acid and Ala as major impurities. In some of the batches additionally small amounts of fumaric acid and Glu were observed. In one of the batches of the reagent grade material an unknown impurity was found at a relative retention of about 0.33. The amount of Ala and Glu found in samples 1 and 3 was in full agreement with the results obtained from an amino acid analyser (data not shown).

3.3. Purity testing of L-alanine

To check the purity of 12 batches of pharmaceutical and reagent grade Ala supplied by 6 different suppliers, the modified method described under Section 2.3.2 was employed. An overview of the results found is presented in Table 3. Ala was found of greater than 99.9% (w/w) purity with only small amounts of Asp and Glu as detectable impurities. A chromatogram of an Ala test solution (sample no. 6) is given in Fig. 6 for information.

Table 3
Batch results of the purity testing of 12 batches of L-alanine.

Sample no.	Asp	Glu	Sum
1	0.015%	0.044%	0.059%
2	0.005%	0.048%	0.053%
3	0.009%	0.056%	0.065%
4	–[a]	–	–
5	–	–	–
6	0.029%	0.044%	0.073%
7	0.020%	0.038%	0.058%
8	–	0.051%	0.051%
9	–	–	–
10	–	–	–
11	–	–	–
12	–	–	–

[a] Not detected.

4. Conclusion

In this study a C18 reversed phase ion-pair HPLC method using 1 mmol/L of PFHA as an ion-pairing reagent and a CAD for the purity control of Asp was developed and validated. The method was capable of separating the major organic and amino acids known to occur as process related impurities. With a slight increase of the PFHA concentration from 1.0 mmol/L to 1.5 mmol/L, the method was also found to be suitable for the purity control of Ala. The limits of quantification for the potential impurities were found between 0.006% and 0.03% (referred to the concentration of a 10 mg/mL test solution), allowing control of impurities on an ICH [29] conform level for drug substances with an average daily dose above 2 g.

The HPLC method for Asp was also tested using an ELSD instead of a CAD for the detection of the impurities. The comparison with the CAD revealed the ELSD to be 3.6–42 times less sensitive than the CAD. Interestingly, the relatively low sensitivity of the ELSD was found to be more pronounced for the non-volatile amino acids compared to the relatively volatile organic acids. Based on the results obtained the ESLD is not an alternative to the CAD for the intended purpose of the method.

Whilst the detector response of the CAD was found to follow an exponential function over a broader concentration range, it was found to be linear in a range from 2.5 μg/mL to 20 μg/mL.

The HPLC method described in this study represents an easy to use alternative to amino acid analysis for the control of impurities in Asp and Ala. It has the additional benefit of controlling not only related amino acids but also other process impurities like organic acids. The method is considered suitable to be described as a Pharmacopoeial related substances control method to replace the currently described TLC test.

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