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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.