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Impurity profiling of L-asparagine monohydrate by ion pair chromatography applying low wavelength UV detection

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ABSTRACT

L-asparagine is a non-essential amino acid being used for a variety of pharmaceutical applications. The compound may be produced following synthetic or fermentative pathways leading to the formation of distinct impurities such as organic acids, other amino acids, dipeptides, or cyclic amino acid derivatives. Analysis of the respective analytes is challenging due to the lack of a chromophore, thus the monograph of the European Pharmacopoeia describes a thin layer chromatographic test for detection of other amino acids. Thus, a sensitive and robust liquid chromatographic method was developed and validated applying detection at 210 nm for determining the related substances. Separation and quantification of the analytes was achieved on a reversed phase C_{18} column using a mobile phase composed of a mixture of a phosphate buffer, sodium octanesulfonate, and acetonitrile in an isocratic elution mode. In contrast to the currently used thin layer chromatographic test, the method is capable of separating and quantitatively assessing expected ninhydrin-positive and −negative impurities from synthetic and enzymatic production pathways.

1. Introduction

Amino acids (AAs) such as L-asparagine play an important role in pharmaceutical applications as nutritive supplements and drugs. L-asparagine is a non-essential AA which is a significant factor during the development of the brain [1] and for regulating the ammonia levels of the human body [2]. Being a polar, ionisable, and structurally similar analyte in comparison to other AAs it is challenging to develop good analytical procedures for evaluating the impurities in L-asparagine.

Currently the European Pharmacopoeia (Ph. Eur.) describes a thin-layer chromatographic test using ninhydrin as spraying reagent for impurity profiling. Though this procedure is able to detect other AAs, it can be considered obsolete because it is blind for ninhydrin-negative impurities and not only lacks sensitivity but also resolving power [3]. Nowadays the Amino Acid Analyzer, as described in chapter 2.2.56 of the Ph. Eur. [4], is applied. It makes use of cation exchange resins using lithium or sodium buffers applying a pre- or post-column derivatization with ninhydrin or fluorescent reagents, respectively [4]. Even though it is being considered more sensitive it cannot detect other impurities than AAs, too. In addition, it is a rather expensive method as it needs special instrumentation. Derivatization methods producing fluorescent compounds through reaction with 9-fluorenylmethyl chloroformate (FMOC) [5] or *o*-phthalaldehyde [6] are selective for primary amines; however, organic acids which might be present as impurities cannot be detected. Since most AAs lack typical UV/Vis chromophores, one needs to benefit from the absorbing properties of the carboxylic acid moiety at 210 nm when detecting them without preceding derivatization [3,7–9]. Of note, the poor sensitivity in low wavelength detection may be compensated by highly concentrated sample preparations for impurity profiling [3].

The synthetic pathway of L-asparagine has to be considered for impurity profiling (see Fig. 1). L-asparagine (**8**) can be produced through the reaction of L-aspartic acid beta methyl ester (**4**) with ammonia [10,11]. Alternatively, an excessive fermentative approach using recombinant microorganisms and disabled negative feedback mechanisms and degradation processes [12] can be applied: The ATP-dependent enzyme asparagine synthetase catalyzes the transfer of ammonia from L-glutamine (**5**) to L-aspartic acid (**2**) and thereby produces L-glutamic acid (**6**) and L-asparagine (**8**) [13]. However, all production pathways use L-aspartic acid (**2**)

Abbreviations: AA, amino acid; FMOC, 9-fluorenylmethyl chloroformate; ICH, International Council for Harmonization; LOQ, limit of quantitation; Ph. Eur., European Pharmacopoeia; R^2, regression coefficient; RSD, relative standard deviation; S/N, signal-to-noise ratio; RRT, relative retention time.

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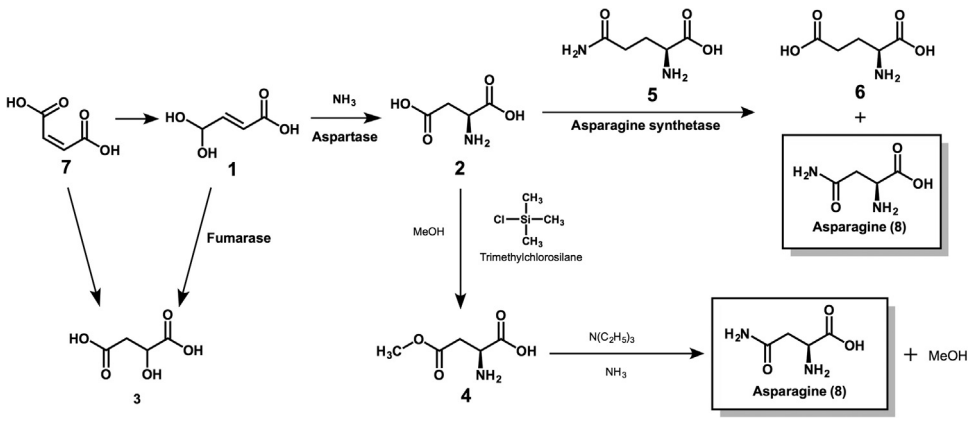

Fig. 1. Production pathway of L-asparagine (8) from L-aspartic acid (2) showing its potential organic acid impurities 1, 3, and 7 [13–17] as well as L-glutamine (5) and L-glutamic acid (6) as impurities from the enzymatic approach [12]; TMCS = Trimethylchlorosilane.

as a precursor: it is therefore inevitable to have an analytical procedure capable of analyzing ninhydrin-negative organic acids which might be present in L-aspartic acid (**2**).

The most common approach involves fermentation and enzymatic reaction starting from fumaric acid (**1**) as a substrate of the enzyme aspartase [14–17]. Besides, the enzyme fumarase may convert fumaric acid (**1**) which is produced from maleic acid (**7**) to malic acid (**3**) [18], representing additional possible ninhydrin-negative impurities (see Fig. 1). Furthermore, dimerization products like dipeptides consisting of L-asparagine and L-aspartic acid and the cyclic dipeptide diketoasparagine can be expected (see Fig. 2).

Instead of using expensive ion-exchange resins [19] and/or complicated derivatization methods [5,6] in HPLC analysis, it was aimed to analyze these polar and ionisable impurities non-derivatized on an ordinary reversed-phase (RP) column. This can be achieved by using ion-pairing reagents such as perfluorinated carboxylic acids [20] or alkylsulfonates [21] at a low detection wavelength of 210 nm to address the carboxylic acid moieties of the organic acids as well as the amino acids [3].

2. Experimental

2.1. Chemicals and reagents

L-asparagine monohydrate, L-aspartic acid, and diketoasparagine were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM; Strasbourg, France). L-glutamine, L-glutamic acid, potassium dihydrogenphosphate (KH_2PO_4), phosphoric acid, dichloromethane, diethyl ether, FMOC-amino acids, diisopropylethylamine, 1,2-ethanedithiol, methanol, thioanisole, anisole, and acetonitrile (HPLC gradient grade) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), sodium 1-octanesulfonate from Alfa-Aesar GmbH & Co. KG (Karlsruhe, Germany), dimethyl formamide (DMF) from Fisher Scientific (Schwerte, Germany), and 2-chlorotrityl chloride resin (CTC) from Chem-Impex Wood (Dale, IL, USA). All chemicals used for quantification were of analytical grade or even better. Ultrapure water was produced by a water purification system from Merck Millipore (Schwalbach, Germany). All solutions were filtered through 0.45 μm cellulose acetate filters supplied by Macherey-Nagel GmbH & Co. KG (Düren, Germany) prior to use.

2.2. Apparatus

The HPLC-UV experiments were performed on an Agilent 1100 modular liquid chromatographic system consisting of an online vacuum degasser, a binary pump, an auto sampler, and a variable wavelength detector (Agilent Technologies, Waldbronn, Germany). A column oven from Beckman Coulter GmbH (Krefeld, Germany) was additionally used. Chromatograms were recorded and integrated using the Agilent ChemStation® software (Rev B.03.02). A Sigma 3K12 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), an ultrasonic bath from Bandelin electronic GmbH & Co. KG (Berlin, Germany), and an analytical balance from Mettler Toledo (Gießen, Germany) were used.

2.3. Chromatographic procedure

An octadecylsilyl (C_{18}) Microsorb®-MV column (250 × 4.6 mm, 5 μm particle size, 100 Å pore size, Agilent Technologies, Santa Clara, CA, USA) was used as the stationary phase. The chromatographic system was operated using an isocratic elution at 25 °C and a flow rate of 0.7 mL/min. The mobile phase was a 100 mM potassium dihydrogenphosphate buffer solution containing 10 mM sodium 1-octanesulfonate and 5 mL acetonitrile per liter. The pH of the solution was adjusted to 2.2 using phosphoric acid (85%) prior to the addition of acetonitrile. UV detection was carried out at a wavelength of 210 nm, and a sample of 20 μL of the respective solution was injected into the chromatographic system.

2.3.1. Preparation of solutions

A test solution containing 10 mg/mL of L-asparagine monohydrate was prepared dissolved in water. The solutions of the dipeptides for peak identification were prepared in the mobile phase and had an approximate concentration of 0.58 mg/mL. For the test solutions for linearity a solution containing 0.25 mg/mL of L-aspartic acid was diluted to 2.5, 5, 10, 20, 30, 40, 50, 60, 70, and 80 μg/mL. The reference solution for quantification and system suitability contained 0.025 mg/mL of L-aspartic acid, diketoasparagine, and L-asparagine monohydrate (calculated to correspond to anhydrous L-asparagine). All solutions were prepared immediately before use or thawed from storage at −20 °C before use. All solutions were sonicated to achieve complete dissolution.

2.4. Synthesis of the dipeptides

Dipeptides were synthesized using the fluorenylmethyloxycarbonyl (FMOC) amino acid coupling strategy (solid phase peptide synthesis – SPPS) [22]. In brief, the first FMOC-protected amino acid was added to the CTC resin in a 5-molar excess in comparison to its loading capacity with 0.2 M diisopropylethylamine (0.2 M solution in dichloromethane), and incubated under agitation at room temperature (RT) for 1 h. Eventually remaining unreacted binding sites of the resin were deactivated by adding