Talanta 182 (2018) 83–91

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a capillary zone electrophoresis method to quantify *E. coli* L-asparaginase and its acidic variants

Han Yao[a], Jana Vandenbossche[a], Cari E. Sänger-van de Griend[b], Yorick Janssens[a], Cristina Soto Fernández[a], Xiaolong Xu[a], Evelien Wynendaele[a], Govert Willem Somsen[c], Rob Haselberg[c], Bart De Spiegeleer[a,*]

[a] *Drug Quality and Registration (DruQuaR) Group, Department of Pharmaceutical Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium*

[b] *Division of Analytical Pharmaceutical Chemistry, Faculty of Pharmacy, Department of Medicinal Chemistry, Uppsala University, Biomedical Centre, PO Box 574, SE-751 23 Uppsala, Sweden*

[c] *Division of BioAnalytical Chemistry, AIMMS Research Group BioMolecular Analysis, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV, Amsterdam, The Netherlands*

ARTICLE INFO

Keywords:
l-asparaginase
Capillary zone electrophoresis
Acidic impurities
Design of experiments (DoE)

ABSTRACT

A capillary zone electrophoresis (CZE) method with UV detection was developed for the quantification of the *E.coli* L-asparaginase (L-ASNase) and its acidic variants. During the initial method development, a variety of experimental conditions were screened. Subsequently, a Design of Experiments (DoE) was used to optimize the pH and concentration of the selected background electrolyte (BGE) containing both TRIS and boric acid. Optimization was performed taking into account both the separation efficiency of L-ASNase and its acidic variants as well as overall method robustness. A repeatable separation between *E.coli* L-ASNase and its acidic variants was achieved on a bare fused silica capillary in combination with a BGE consisting of both 400 mM TRIS and boric acid. The method was validated for linearity, accuracy, precision, LOD, LOQ and robustness. The recovery for L-ASNase was 97.9–104.4% with a precision RSD of 1.5–3.2%, while the recovery of impurities was 92.1–109.8% with a RSD of 1.7–4.6%. The quantification limit was 1.9% (m/m). Moreover, the CZE-UV method was applied to determine the degradation rate in the presence of ammonium bicarbonate, confirming the suitability of the method. The degraded, partially charged L-ASNase was evaluated for its *in-vitro* enzymatic activity showing an insignificant different enzyme activity compared to the unmodified sample.

1. Introduction

L-asparaginase (L-ASNase) is a tetrameric enzyme used in pediatric treatment of acute lymphoblastic leukaemia (ALL) [1]. Clinically used L-ASNase preparations are currently derived from two bacterial sources: *Escherichia coli* and *Erwinia chrysanthemi* [1], with a tetrameric molecular weight (MW) of 136,320 Da resp. 140,320 Da and an isoelectric point (pI) of 4.9 resp. 8.6 [2–4]. Like other biopharmaceuticals, L-ASNase is prone to modifications during its bacterial production as well as pharmaceutical processing and storage [5]. Also, during the preclinical and clinical trials, consistency in the nature and quantity of these modifications is of critical importance for a successful development of the originator medicines as well as of the biosimilars. The consistency of these modifications is also crucial for the novel L- ASNase formulations under development [6,7]. As these protein modifications are known to change the physicochemical, immunochemical, biological and pharmacological properties, which may contribute to adverse effects, it is important to characterize this heterogeneity [8].

Charge variants are an important type of protein heterogeneity and typically consist of acidic variants (*e.g.* sialylation and deamidation) and basic variants (*e.g.* succinimide formation and oxidation of some amino acid side chains [Met, Cys, Lys, His, Trp] and C-terminal Lys cleavage) [6,9]. Deamidation is one of the most frequent acidic degradation pathways and is common in Asn residues [10]. The Asn deamidation reaction results in a cyclic succinimide, which is further hydrolysed to form an Asp and isoAsp mixture (with a mass increase of 1 Da from 114 Da for Asn to 115 Da for Asp) with the release of ammonia [10]. This leads to a decrease of the isoelectric point (pI) due to the change in residue charge from neutral to the acidic carboxylate. Moreover, deamidation generally leads to loss of biological function, secondary and tertiary structure and stability [11,12]. However, deamidated variants of *Erwinia* L-ASNase (deamidated site: Asn281) are

* Corresponding author.
E-mail address: Bart.DeSpiegeleer@UGent.be (B. De Spiegeleer).

https://doi.org/10.1016/j.talanta.2018.01.048
Received 7 November 2017; Received in revised form 14 January 2018; Accepted 17 January 2018
Available online 20 February 2018
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reported to retain their *in-vitro* enzymatic activity [13]. No studies concerning about the enzyme activity of the deamidated variants of *E.coli* L-ASNase have been reported yet. Charge variants of proteins can be analyzed using a variety of techniques for distinct purposes, including isoelectric focusing like slab gel isoelectric focusing (IEF) and capillary isoelectric focusing (CIEF), capillary electrophoresis, ion-exchange chromatography (IEX), and mass spectrometric techniques [14–26]. A traditional method for evaluating the charge variants is the IEF [14,15]. IEF can provide high-resolution separation of similar charge variants, but it is time consuming, labor intensive and has an unsatisfactory quantitation precision. Other techniques reported were CIEF [14–17] and more recently imaged capillary electrophoresis (iCE) [18,19]. However, while they allow more quantitative evaluation, these techniques are still time consuming. Capillary zone electrophoresis (CZE) has recently been shown to be a suitable technique to characterize protein charge variants due to its high resolution and speed, low sample consumption and low cost availability of capillaries [20–22]. The charge variants of *Erwinia* L-ASNase, such as the deamidated variants and the non-deamidated acidic variants, have been characterized in the recent years by using IEX, CIEF, liquid chromatography tandem-mass spectrometry (LC-MS/MS) of proteolytic digests, and structural techniques including circular dichroism (CD), small-angle X-ray scattering (SAXS) and ion-mobility mass spectrometry (IM-MS) [25–29]. The charge variants of *E.coli* L-ASNase has been much less studied compared to that of *Erwinia* L-ASNase; some investigations have been reported such as the characterization of succinylated charge variants by using ultraviolet spectroscopy, fluorescence spectroscopy and CD, and the identification of deamidated variants by LC-MS/MS of proteolytic digests [25,30]. Regarding to the CZE-UV method, up till now, only one CZE-UV method for *Erwinia* L-ASNase is briefly reported without analytical details [13]. Moreover, the acidic variants were not separated. No CZE-UV methods for determining *E.coli* L-ASNase and charge variants have ever been reported.

In this study, a systematic development of a CZE-UV method for the quantification of L-ASNase as well as of the acidic variants of *E.coli* L-ASNase is presented. After an initial screening investigation, the method was optimized using experimental designs (DoE) following the quality-by-design (QbD) concept to define the design space. The optimized method was further formally validated and applied on different deamidated L-ASNase samples, which were finally evaluated for their functional enzyme activity.

2. Experimental

2.1. Materials

Hydrochloric acid, TRIS (Trizma®base), boric acid, sodium hydroxide, ammonium bicarbonate, potassium iodide, mercury (II) iodide and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Potassium phosphate monobasic and L-asparagine monohydrate were obtained from Merck (Darmstadt, Germany). Ammonium chloride was purchased from UCB (Brussels, Belgium). All chemicals were analytical grade. Paronal® samples, *i.e. E.coli* L-ASNase preparation without excipients (label claim of 10,000 IU per vial; Kyowa Hakko Kiri Co., Ltd.) (Tokyo, Japan), were obtained from Takeda Pharmaceutical Co., Ltd. (Brussels, Belgium). In the absence of a formal reference standard for the selective assay of L-ASNase, batch 10858381 was used as laboratory reference material, which contained 79.1% of active pharmaceutical ingredient (API), 14.4% of impurity 1, 4.5% of impurity 2% and 2.0% of impurity 3, based on peak deconvolution and area normalization (see Section 2.5) [31–33].

2.2. Sample and BGE preparation

For the initial development and the following optimization, 1 mg/mL L-ASNase in water was applied. In the initial development, BGEs of 500 mM/500 mM, 600 mM/600 mM and 800 mM/800 mM TRIS/boric acid at different pH values (pH 7.5, pH 8.0, pH 8.5, pH 8.25, pH 8.75 and pH 9.5), and 1 M/1 M TRIS/boric acid (pH 8.5 and pH 8.0) were prepared. Moreover, potassium tetraborate solution at fixed concentration of 50 mM (pH 7.0, pH 8.0, pH 9.0 and pH 10.0), boric acid solution (50 mM, adjusted to pH 10), and TRIS solution (25 mM, pH 8.0) and (50 mM, pH 8.0) were prepared. Additionally, 200 mM/200 mM TRIS/tricine (pH 8.24) was also prepared. All above BGEs were tested on the bare fused-silica capillary. In the entire study, 1 M hydrochloric acid or 1 M sodium hydroxide was applied, when the pH adjustment is required.

Regarding the method optimization, a three level central composite design (CCD) with three center points is employed to define the design space of the pH and concentration of BGE (Supplementary material Table 1) [34]. Each experimental block consisting of 11 runs, was performed in triplicate. The factors and their ranges were 1) concentration of TRIS/boric acid ranging from 100 mM/100–600 mM/600 mM, 2) pH of TRIS/boric acid ranging from pH 8.0 to pH 9.0. For these experiments, 100 mM/100 mM TRIS/boric acid (pH 8, pH 8.5 and pH 9), 350 mM/350 mM TRIS/boric acid (pH 8, pH 8.5 and pH 9) and 600 mM/600 mM TRIS/boric acid (pH 8, pH 8.5 and pH 9) were applied. For the final method, the running BGE consisted of 400 mM TRIS and 400 mM boric acid (pH unadjusted, the measured pH value was pH 8.4).

The final method was applied on different modified (degraded) samples to test the modification rate. 1.0 mg/mL L-ASNase samples were stored in 1% NH_4HCO_3 (m/v), and incubated at 37 °C for 0, 8, 16 and 24 h [13,35].

2.3. CE parameters

Proteomelab PA 800 plus CE instrument (Beckman Coulter, Brea, United States) was used coupled with a diode array detector. In the method development and validation parts, a bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used which possessed an effective length of 20 cm and internal diameter of 50 µm (outer diameter, 365 µm). Samples were kept at a temperature of 10 °C and injected at 0.4 psi for 4 s. The separation voltage was 9 kV, and the polarity was normal. UV absorbance was detected at 214 nm and the optical window was 200 µm. New fused silica capillaries were pretreated with 1 M NaOH, water, and BGE subsequently at 20 psi for 20 min. Between two consecutive injections, the fused silica capillary was flushed with 1 M NaOH, water and BGE for 2 min at 20 psi, respectively.

2.4. Method characteristics

2.4.1. Relative response factor of the acidic L-ASNase species

Neglecting differences in MW of the unmodified L-ASNase and the acidic species (*i.e.* amide *versus* acid), the relative response factor (RRF) of the acidic species relative to the unmodified L-ASNase is defined by [36]:

$$RRF = \frac{\text{area of acidic species}}{(\text{original area of unmodified L-ASNase}) - (\text{residual area of unmodified L-ASNase in modified sample})}$$

As quantitative evaluation of these related impurities can be accomplished using the internal normalization procedure, the raw data (*i.e.* peak area or height) are to be converted to mass units (*i.e.* as %). Therefore, the relative response factor (RRF) needs to be assessed: if this RRF falls between 0.80 and 1.20, then no consideration for it is normally required (*i.e.* an RRF of 1.0 can be used). In the absence of

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