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A novel reagent significantly improved assay robustness in imaged capillary isoelectric focusing

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ABSTRACT

Imaged Capillary Isoelectric Focusing (icIEF) has been used as primary method for charge variants analysis of therapeutic antibodies and proteins [1], [9]. Proteins tend to precipitate around their pI values during focusing [14], which directly affects the reproducibility of their charge profiles. Protein concentration, focusing time and various supplementing additives are key parameters to minimize the protein precipitation and aggregation. Urea and sucrose are common additives to reduce protein aggregation, solubilize proteins in sample matrix and therefore improve assay repeatability [15]. However some proteins and antibodies are exceptions, we found urea and sucrose are not sufficient for a typical fusion protein (Fusion protein A) in icIEF assay and high variability is observed. We report a novel reagent, formamide, significantly improved reproducibility of protein charge profiles. Our results show formamide is a good supplementary reagent to reduce aggregation and stabilize proteins in isoelectric focusing. We further confirmed the method robustness, linearity, accuracy and precision after introducing the new reagent; extremely tight pI values, significantly improved method precision and sample on-board stability are achieved by formamide. Formamide is also proven to be equally functional to multiple antibodies as urea, which makes it an extra tool in icIEF method development.

1. Introduction

Imaged Capillary Isoelectric Focusing (icIEF) is current industry standard for charge variants analysis of antibodies and proteins [4] [10], [11]. Reproducible charge profile is essential for monitoring protein pI values and relative distribution of charge variants in protein drug process development, formulation and stability study [6] [7], [13]. Protein solubility in icIEF sample matrix directly affects the reproducibility of charge profiles. Some additives are commonly used in icIEF to reduce protein aggregation and solubilize proteins in sample matrix, such as urea and sucrose [15]. However we find a typical fusion protein is an exception, common additives are not sufficient to obtain robust charge profile. Such that we tried a novel reagent, formamide, which significantly improved assay robustness. Formamide, also known as methanamide, is an amide compound similar to urea with one less amine group (Fig. 1). Formamide was published as a type of denaturant in gel electrophoresis of DNA and RNA [3], but it was not reported for charge separation of proteins and antibodies in icIEF assay and therefore it is novel in icIEF field. Urea was known as a key reagent in iCE charge separation technology [15], however it is not always as sufficient for certain proteins and often encountering reproducibility issues. Our results show formamide, with similar structure and function as urea, is a good supplementary reagent in such circumstances.

2. Materials and methods

2.1. Materials

0.5% methyl cellulose solution, 1% methyl cellulose solution, iCE electrolyte kit, pI marker 4.65, pI marker 7.05, iCE3 Instrument with Alcott 720NV Autosampler and FC Column Cartridge are purchased from ProteinSimple. Urea powder is purchased from J.T.Baker. Pharmalyte 3–10 is purchased from GE Healthcare. Formamide is purchased from Sigma.

2.2. Methods

2.2.1. icIEF sample preparation

The master mix is prepared by combining the reagents as

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0003-2697/

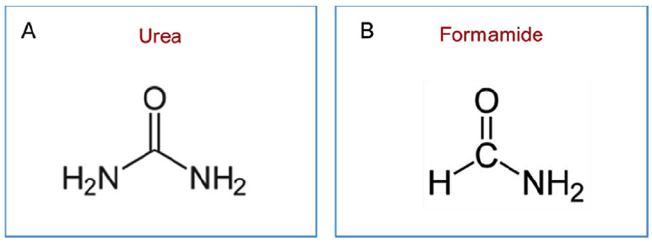

Fig. 1. Structure comparison of urea and formamide.

following: 700 μL of 1% methyl cellulose, 80 μL of Pharmalyte 3–10, 480 μL of formamide at 100%, 520 μL of deionized water, 10 μL of pI marker 4.65 and 10 μL of pI marker 7.05. Pipette mix the reagents gently and thoroughly using the 1 mL pipettes, prepare the master mix freshly before use. Dilute the test sample in water to 1.6 mg/mL and then add 20 μL of the diluted sample to 180 μL of the master mix, vortex mix gently.

Set the centrifuge to 10 °C, then centrifuge the mixture at 10, 000 rpm for 3 min. If the refrigerated centrifuges are not available, centrifuge the mixture at 6000 rpm for 10 s and keep them at 2-8 °C

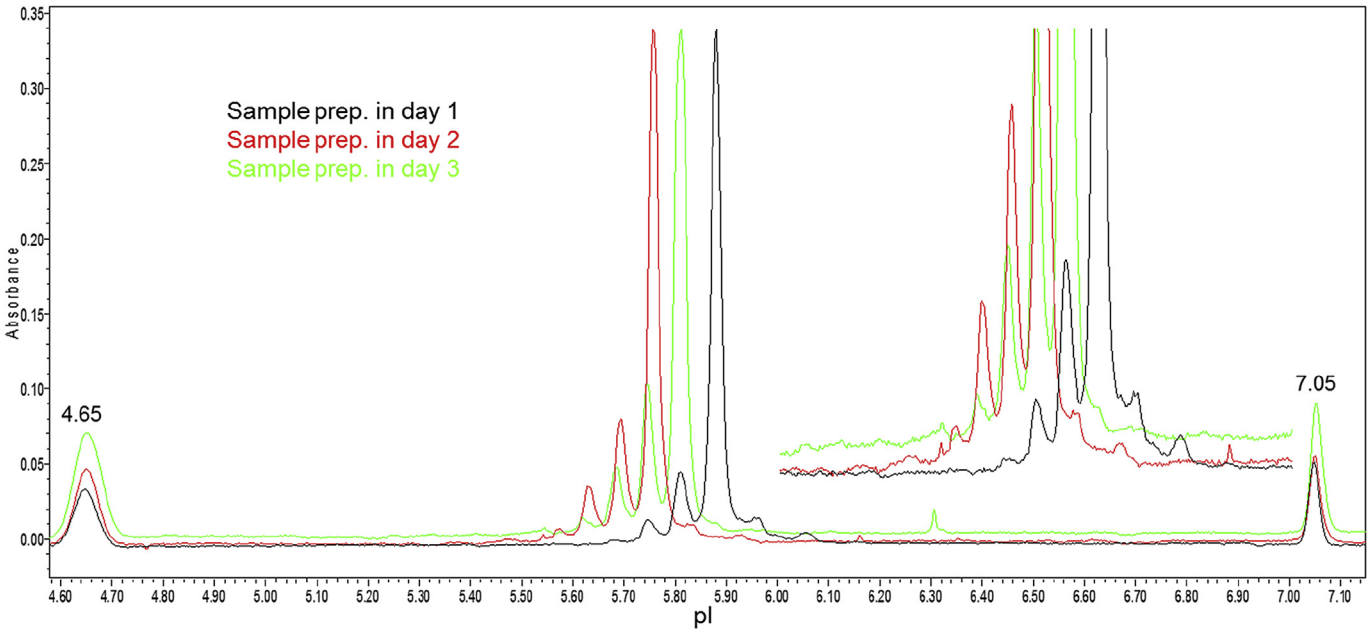

Fig. 2. Electropherogram overlay of Fusion protein A with 4 M urea. The sample matrix contains 4% pharmalyte 3–10, 0.35% methyl cellulose and 4 M urea, Fusion protein A was diluted in deionized water to 2.5 mg/mL and then further diluted in sample matrix to 0.25 mg/mL. Samples focus at 1500 V for 1 min and then at 3000 V for 8 min.

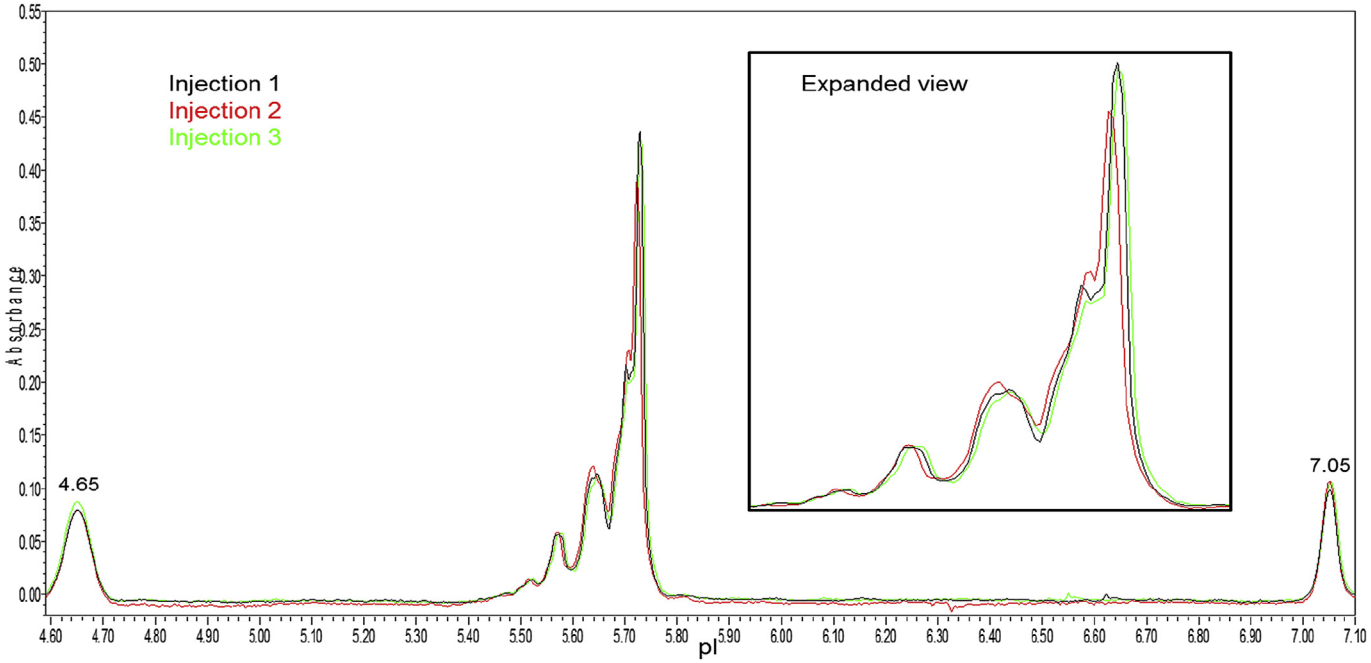

Fig. 3. Electropherogram overlay of Fusion protein A with 20% of sucrose. Sample matrix contains 4% pharmalyte 3–10, 0.35% methyl cellulose and 20% sucrose, Fusion protein A was diluted in deionized water to 2.5 mg/mL and then further diluted in sample matrix to 0.25 mg/mL. Samples focus at 1500 V for 1 min and then at 3000 V for 8 min.