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Impact of ion-pairing agents on hydrophilic-interaction liquid chromatography high-resolution mass spectrometry's ability to separate intact glycoproteins

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ABSTRACT

High-resolution separations of glycoforms of glycoproteins that differ in the number of glycans are possible using hydrophilic-interaction liquid chromatography of intact proteins. To maximise the contribution of the hydroxyl groups of the sugars in the glycoprotein, it is necessary to shield the positively charged sites of the proteins with acidic ion-pair reagents in order to get effective separations. Here, we looked at how different IPRs with diverse physico-chemical characteristics, like hydrophobicity and acidity, affected the capillary-scale HILIC separation of intact. The use of fluoroacetic acid, difluoroacetic acid, trifluoroacetic acid, and heptafluorobutyric acid as diluents for sample preparation, solvents for loading samples onto a reversed-phase trap prior to the HILIC separation, and mobile-phase components for HILIC and HILICMS were all examined. To we used an acrylamide-based monolithic column to lessen the impact of ion-exchange interaction with the stationary phase (silica-based). We looked at how the various IPRs affected each phase in the separation of a mixture of proteins with varying sizes and hydrophilicity, as well as the separation of ribonuclease B's five glycoforms. It was established that the sample's IPR content had no impact on the separation or MS detection.

Keywords: Tetrodotoxin; Hydrophilic-interaction/ion-exchange; Mixed-mode solid phase extraction; LC-MS/MS; Human plasma

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INTRODUCTION

A low concentration of TFA and DFA in the mobile phase is advantageous, though, as it prevents the formation of adducts and increases signal intensity [1]. Horseradish peroxidase, a complex glycoprotein with a molecular weight of 40 kDa, was efficiently resolved under the optimal HILIC conditions [2]. Using high in-source collision energy during the MS analysis is one technique to lessen the effects that TFA has on the system. However, this could result in the in-source fragmentation of proteins with labile structures, which would make it difficult to analyse them whole [3]. The removal of TFA adducts has been accomplished using a variety of post-column strategies [4]. The most recent one was described by Wouters and involved a multi-channel microfluidic system that exchanged TFA ions for propionic or formic acid by selectively removing TFA ions [5]. TFA was tried to be replaced with another IPR or to have its concentration in the mobile phase decreased in a number of investigations [6]. Tengattini discovered that a good separation of glycoforms required at least 0.05% of TFA. Zhang were able to reduce the amount of TFA by using an updated stationary phase [7]. The silica particles were modified by coating them with a thick layer of polyacrylamide to reduce interactions between the free sialon groups and the cationic groups on the protein [8]. Lardeux recently came to the conclusion that TFA must be used for HILIC-MS separations after researching monoclonal antibodies [9]. In this article, we present findings from a study that intended to reduce the presence and consequences of TFA on intact proteins detected by HILIC-MS. We investigated the effects of various IRPs with various hydrophobicity's [10]. The sample was loaded onto a monolithic stationary phase made of acrylamide using a capillary setup with a trap-and-elute configuration. With the help of two samples a protein combination and the five major glycoforms of ribonuclease B the effects of the various IPRs were assessed. From sample preparation through MS analysis, every stage of the micro-LC separation process was considered. Finally, we showed that our improved procedure may be used to separate a difficult horseradish-peroxidase (HRP) glycoprotein, enabling us to keep track of its nine primary glycoforms. Thermo Fisher Scientific's Ultimate RSLCnano system, located in Breda, the Netherlands, was used for the LC analyses. It has a loading-pump system, a ten-port, two-position valve, and an auto sampler the separation performance was assessed using the following parameters: peak capacity,

retention duration, peak area, and asymmetry. Retention times showed no discernible trends or changes. Except for BSA and TfL, which varied slightly because to the more hydrophobic BSA and TfL, similar asymmetry values were seen for the various IPRs? We hypothesise that desorption of the proteins from the trap is a limiting factor and that the larger the protein, the higher is its retention on the C4 trap column. Additionally, a decreased retention on the RP trap-column due to a less hydrophobic loading pump solvent may result in sample loss.

DISCUSSION

Additionally, recovery values followed a similar pattern, with no IPR reagent or buffers acting as the loading pump solvent, High-resolution separations of glycoforms of glycoproteins that differ in the number of glycans are possible using hydrophilic-interaction liquid chromatography of intact proteins. The positively charged protein sites must, however, be protected by acid-pair reagents in order to maximise the contribution of the hydroxyl groups of the sugars in the glycoprotein and achieve effective separations. Here, we looked at how different IPRs with different physico-chemical characteristics, like hydrophobicity and acidity, affected the capillary-scale HILIC separation of whole proteins. Fluoroacetic acid, difluoroacetic acid (DFA), trifluoroacetic acid, and heptafluorobutyric acid were all tested as diluents for sample preparation, solvents for loading samples onto a reversed-phase trap prior to the HILIC separation, and mobile-phase components for HILIC and HILIC-MS. To we employed an acrylamide-based monolithic column to decrease the contribution of ion-exchange interaction with the stationary phase. We looked at how the various IPRs affected each phase in the separation of a mixture of proteins with varying sizes and hydrophilicity, as well as the separation of ribonuclease B's five glycoforms. It was established that the sample's IPR content had no impact on the separation or MS detection. A low concentration of TFA and DFA in the mobile phase is advantageous, though, as it prevents the formation of adducts and increases signal intensity. Horseradish peroxidase, a complex glycoprotein with a molecular weight of 40 kDa, was efficiently resolved under the optimal HILIC conditions. A method for separating polar chemicals based on hydrophilic partitioning, hydrogen bonds, dipole-dipole, and ionic interactions is known as hydrophilic-interaction liquid chromatography. Mass spectrometry and HILIC are widely combined to characterise tiny polar compounds and metabolites, and more recently, these techniques have been expanded to analyse entire proteins, in particular glycoproteins. A typical post-translational alteration known as glycosylation involves the attachment of saccharide chains to the protein. Glycans are the name given to these chains.

CONCLUSION

These days, polar stationary phases based on silica are used in HILIC's intact-glycoprotein analysis. With the addition of an ion-pair reagent, elution is carried out using shallow linear gradients with acetonitrile and water mixtures. Strong acids and other IPRs improve separation performance and lessen band broadening and peak tailing. Furuki investigated these phenomena in detail in glycopeptides separations and came to the conclusion that negatively charged IPR bind to the basic and positively charged groups of glycopeptides, diminishing the interaction of the molecule with the stationary phase. In this paper, we discuss protein capillary HILIC-MS using an ion pair reagent. Relatively large volumes of samples were loaded and injected into a monolithic capillary column using an online trap-column. To evaluate the impact of IPR on the separation of proteins, studies of IPR in the sample, loading pump, and mobile phase were conducted. Each step has been outlined in the three subparagraphs that follow, and the impact of IPR has been evaluated for each. The volumes involved appear to have a significant impact on the IPR's applicability in each stage of the HILIC-UV study. The final separation is unaffected by the IPR in the sample, however the loading solvent unexpectedly had a consistent impact on the protein regions. Finally, the IPR in the mobile phase exhibits variations in peak forms and selectivity as would be predicted.

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