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Protein digestion optimization for characterization of drug–protein adducts using response surface modeling☆

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ABSTRACT

The formation of drug–protein adducts *in vivo* may have important clinical and toxicological implications. Consequently, there is a great interest in the detection of these adducts and the elucidation of their role in the processes leading to adverse and idiosyncratic drug reactions. Enzymatic digestion is a crucial step in bottom-up proteomics strategies for the analysis of drug–protein adducts. The chosen proteolytic enzyme and digestion conditions have a large influence on the protein coverage of the modified protein and identification of its modification site. In this work, the enzymatic digestion conditions (pH, temperature and time) of trypsin and thermolysin were optimized specifically for the characterization of Human Serum Albumin (HSA) adducts. Using a Design of Experiments (DOE), it was found that of the three optimized parameters mainly pH and temperature showed strong effects on both responses. The optimized digestion conditions were different from those obtained from the suppliers or literature. Their application to HSA adducts resulted in improved protein coverage and signal intensity regarding the peptide containing the modification site, thereby highlighting the importance of a detailed optimization of digestion conditions.

1. Introduction

Drug–protein adducts are suggested to play a role as mediators of Adverse Drug Reactions (ADRs) and Idiosyncratic Drug Reactions (IDRs) [1]. Therefore, their detection and identification is crucial within the framework of drug safety [2]. In the last decade, several Liquid Chromatography–Mass Spectrometry (LC–MS) based strategies have been developed for the determination of drug–protein adducts [3], i.e., the screening of reactive drug intermediates trapped by small molecules such as glutathione (GSH) [4–6] and proteomics based methods analyzing the adduct formed by a drug and its protein target [7–11]. The latter strategies are mostly based on enzymatic digestion of the modified protein followed by LC–MS(MS) analysis of the resulting proteolytic peptides. These approaches allow for the detection of clinically relevant drug–protein adducts and their simultaneous identification thereby giving insight into the mechanisms underlying ADRs.

Two major factors influencing the success of such methods are protein coverage, linked to successful identification of the modified protein, and the detection of the specific peptides that contain the modification site. The latter defines the actual sensitivity of the method and, naturally, achieving high protein coverage increases the chance of detecting the modified peptides. The most delicate step in this respect is the digestion of drug–protein adducts. It is not only critical to choose the appropriate enzyme, but also to apply the right digestion conditions, such as buffer pH, digestion temperature and time. Enzyme suppliers usually provide optimal conditions for the delivered enzyme. In addition, a wide range of digestion conditions obtained with different substrates are available from literature and enzyme databases such as BRENDA (http://www.brenda-enzymes.org/). For example, the optimal digestion conditions of bovine trypsin (EC 3.4.21.4) according to several suppliers are 2–18 h digestion time (depending on the amount of protein) at a temperature of 37 °C in 50 mM ammonium hydrogencarbonate or 100 mM Tris–HCl, pH 8.5. However, other optima can be found in the literature, such as overnight digestion at 37 °C in 50 mM ammonium hydrogencarbonate buffer pH 7.8 [12] and 45 min digestion at 37 °C in 10 mM ammonium hydrogencarbonate buffer pH 8.5 [13], while BRENDA displays an optimal pH range of 7.0–8.7 and an optimal temperature range of 45–74 °C. The wide variety in published digestion optima complicates the selection of the correct digestion conditions based on literature data. Furthermore, digestion conditions often are optimized for specific protein targets, such as monoclonal antibodies [14], polyclonal ovine immunoglobulin G [15] and membrane proteins [16,17], or specific applications, such as on-line bioreactors

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[18] and are mostly focused on improving the peptide yield and protein identification rate. Taken together, this underlines the need for a detailed and systematic optimization of enzymatic digestion conditions for drug–protein adducts.

Optimization of chemical processes is traditionally carried out using a One-Variable-At-a-Time (OVAT) approach. Commonly, a limited number of OVAT experiments are carried out in which the levels of one variable are changed while the others are kept constant [19]. A major disadvantage of OVAT approaches is the disregard of interactions between variables. Therefore, this methodology often does not lead to the true optimum and may even lead to different end results depending on the starting point [20]. In order to avoid the local optima, more experiments need to be performed, which makes this approach more costly in terms of analysis time and consumption of chemicals [19]. In contrast, DOE techniques, such as the Response Surface Methodology (RSM), change combinations of variables simultaneously which does allow for incorporation of the interaction effects [21]. Another advantage of this technique is the concurrent optimization of multiple responses in order to find the optimal compromise between them. Additionally, RSM only requires a small subset of experiments from all possible variable combinations to cover the design space, which significantly reduces the number of necessary experiments. These advantages allow for a more efficient and more accurate determination of the optimum conditions.

In this study, a RSM approach was applied to the optimization of the three above mentioned conditions (buffer pH, digestion temperature and time) for digestion of HSA adducts with trypsin and thermolysin. These enzymes were selected because of their varying specificities and efficiencies [22]. HSA is the most abundant serum protein and often a target for reactive intermediates of drugs because of the free thiol on cysteine-34 (Cys34) [23]. A wide range of drugs, or their metabolites, including the *N*-acetyl-*p*-benzoquinoneimine (NAPQI) intermediate from acetaminophen [7] and several intermediates of diclofenac [3], are known to covalently bind to this site *in vivo*, thereby causing severe ADRs [1]. For the RSM optimization experiments, a model adduct was prepared by modification of HSA with monochlorobimane (MCB), which was selected for the simplicity of the adduct formation [24]. The two responses used to evaluate the optimization were the protein coverage of HSA and the peak area of the modified Cys34 peptide. For comparison, the digestion optima obtained from the RSM and selected literature conditions were applied to the digestion of NAPQI–HSA adducts.

2. Materials and methods

2.1. Reagents and materials

Human serum albumin (HSA), monochlorobimane (MCB), guanidine-HCl (G-HCl), ethanol, DL-dithiothreitol (DTT), iodoacetic acid (IHAc), thermolysin from *Bacillus thermoproteolyticus rokko* (EC 3.4.24.27), Tris–HCl, silver nitrate, sodium hydroxide, acetaminophen and the HPLC peptide standard mixture were purchased from Sigma Aldrich (Schnelldorf, Germany). Ammonium hydrogencarbonate, hydrochloric acid (HCl) 37% and diethyl ether were obtained from Riedel-de Haën (Seelze, Germany). Methanol, formic acid (FA), acetonitrile and chloroform came from Biosolve (Valkenswaard, The Netherlands). Trypsin from bovine pancreas (EC 3.4.21.4) was supplied by Roche (Almere, The Netherlands), acetone by Interchema (Oosterzee, The Netherlands), Bradford reagent by Biorad (Veenendaal, The Netherlands) and the synthetic peptide H-Pro-Pro-Pro-Pro-OH (Pro4) by Bachem (Weil am Rhein, Germany). Illustra NAP-25 gel-filtration columns with a bed volume of 2.5 mL, prepacked with G-25 DNA grade Sephadex, were obtained from GE Healthcare (Diegem, België). Water was purified by a Millipore (Amsterdam, The Netherlands) Milli-Q unit.

2.2. Design of Experiments

A RSM was applied for the optimization of digestion conditions of both enzymes with respect to the digestion of HSA adducts. A face-centered Central Composite Design (CCD) with uniform precision was created using JMP® 8.0.1 from SAS Institute Inc. (Cary, NC, USA). The CCD design was used to maximize two responses (protein coverage and peak area of the adducted Cys34 peptide) by optimization of three factors (buffer pH, digestion temperature and digestion time). The factor ranges were selected based on protease supplier's instructions and the BRENDA enzyme database. The applied factor ranges were pH 6–10, 24–50 °C and 1–12 h for trypsin and pH 5–9, 30–80 °C and 0.5–8 h for thermolysin. The complete DOE consisted of 40 randomized experiments per enzyme, including 6 center points and 1 replicate.

2.3. Sample preparation

2.3.1. Preparation of MCB–HSA adduct samples

For the RSM experiments, the MCB–HSA adduct was formed by adding a 50-fold molar excess of a 0.1 M solution of MCB in methanol to 5.5 mL of a 7.52 μM HSA solution in 50 mM ammonium hydrogencarbonate buffer pH 7.4. The reaction mixture was kept at 40 °C for 4 h after which the excess MCB was removed with a NAP-25 gel filtration column using 2 M G-HCl at pH 8.5 as the eluting buffer. The 35 cysteine residues of the denatured HSA were reduced by the addition of a 50-fold molar excess of 1 M DTT and alkylated using a 75-fold molar excess of 1 M IHAc. The reduced and alkylated MCB–HSA sample was split into three aliquots before being desalted using NAP-25 columns. As eluting buffers, three 50 mM ammonium hydrogencarbonate solutions were used with pH values corresponding to the three levels of the RSM design. A 100 μL aliquot of the desalted MCB–HSA was then digested with either trypsin or thermolysin (0.01 mg/mL in 0.1 mM HCl) using protein:enzyme ratios of 100:1 and 50:1, respectively. The enzymatic digestion was stopped with the addition of 10 μL of 10% FA. From a 12.3 μM internal standard (IS) solution of Pro 4 in water, 20 μL was added to the digested MCB–HSA samples to achieve a final concentration of 1.23 μM. The final volume of the samples was adjusted to 200 μL with water. The RSM experiments for trypsin and thermolysin were performed on different days and with different batches of the MCB–HSA adduct. A series of confirmation experiments were performed in triplicate to test whether the determined optimum digestion conditions lead to the predicted responses. These experiments were performed using the same batch of MCB–HSA for both enzymes, in order to guarantee comparability of the results.

2.3.2. Preparation of NAPQI–HSA adduct samples

The optimum digestion conditions obtained from the RSM of both enzymes were compared to literature conditions using the NAPQI–HSA adduct, which was prepared according to Hoos et al. [7]. This HSA adduct sample subsequently received the same treatment as described above for the MCB–HSA adducts applying either the optimum RSM digestion conditions or conditions obtained from literature. The selected literature values for trypsin digestion were taken from Aldini et al. [12] and consisted of overnight (13 h) digestion at 37 °C in 50 mM ammonium hydrogencarbonate buffer pH 7.8 and a protein:enzyme ratio of 20:1. For thermolysin, the reference digestion conditions were obtained from Bark et al. [25] and consisted of 15 min digestion at 65 °C in 100 mM ammonium hydrogencarbonate buffer pH 7.5 and a protein:enzyme ratio of 50:1.