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Hydrophilic interaction liquid chromatography as second dimension in multidimensional chromatography with an anionic trapping strategy: Application to prostate-specific antigen quantification

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ABSTRACT

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS–MS) in selected reaction monitoring mode (SRM) has become a widely used technique for the quantification of protein biomarkers in plasma and has already proven to give similar results compared to the conventional immunoassays. To improve the lack of insufficient sensitivity for quantification of low abundance protein, we propose a new two dimensional liquid chromatography (2D-LC-SRM) method for the quantitation of prostate specific antigen (PSA) in human plasma. The method centers on anion exchange cartridge between reversed-phase chromatography and hydrophilic interaction liquid chromatography (HILIC) in an on-line arrangement. The use of the anionic cartridge allows an easier online transfer of the analytes between both dimensions. Moreover, it provides an additional selectivity since the more basic peptides are not retained on this support. This setup has been applied to the quantification of prostate specific antigen (PSA) protein in plasma on a previous generation of mass spectrometer, which enabled a limit of quantification (LOQ) of 1 ng/mL without any upfront immuno-depletion or intense off-line fractionation before the SRM analysis. The obtained LOQ is compatible with the required sensitivity for the clinically relevant plasma-based PSA tests.

1. Introduction

Biomarker discovery produces lengthy lists of candidate proteins that are detected differentially in cases versus controls samples and requires further verification and validation in large numbers of patient samples. Verification and validation of candidate proteins require targeted, multiplexed assays to screen and quantify proteins in patient plasma samples with high sensitivity, absolute specificity and sufficient throughput. At present, established immunoassay platforms, in particular ELISA, are the paragon for quantitative analysis of proteins. Selective reaction monitoring (SRM, also referred to as multiple reaction monitoring or MRM) coupled with stable isotope dilution mass spectrometry (SID-MS) using a triple quadrupole mass spectrometer has a long history of use in the area of quantitative mass spectrometry and has been a principal tool for quantification of small molecules in clinical chemistry for a number of decades [1]. Recently, SID-SRM/MS emerged as a possible core technology for protein quantification as it avoids the long and expensive development of specific antibodies [2–8]. Moreover, SID-SRM/MS assays have the necessary characteristics required for biomarker verification studies or new biotherapeutic drugs analysis (e.g. recombinant protein or monoclonal antibodies), namely: high specificity, sensitivity, multiplexing capability, and precision. The SRM method relies on the monitoring of precursor/product ion transitions forged from the gas-phase ionization of proteotypic tryptic peptide fragments, which serve as surrogates for the candidate protein. Standardization is usually carried out internally by spiking the sample with the corresponding synthetic stable-isotope labeled peptide or with isotopically labeled proteins [9,10]. In the first case, the standard peptide is identical to the one to be quantified except that it contains ^{13}C and/or ^{15}N. This is termed the AQUA strategy, for absolute quantification [11].

For small molecule quantitation in a biological matrix, interfering molecules such as salt and proteins are easily eliminated with sample preparation (e.g. protein precipitation or solid phase extraction). In the case of protein biomarker quantitation with a signature peptide, matrices are first submitted to enzymatic digestion. This leads to a more complex sample with hundreds of thousands of peptides, salt and endogenous small molecules components. This complex mixture can have a considerable effect on the way the

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analysis is conducted and compromise the quality of the results; such effects are called matrix effects. In order to reduce the matrix effect during the method development, some considerations may be used: protein depletion, cleaner sample preparations, more sensitive instruments, which allow less material to be injected. Value of SRM-based quantification has now been illustrated not only for proteins of average abundance i.e. in the microgram/mL range for plasma, but also in the low nanogram/mL range for a few biomarkers in undepleted and non-enriched human plasma [12–14] or urine [15,16]. For some analytes, lower limits of quantification can be reached after peptide fractionation by strong cation exchange combined with immunoaffinity depletion of the most abundant proteins (2.5 ng/mL) [17], or after immuno-enrichment of the peptide (0.3 ng/mL) [18] or protein (6 ng/mL) [19] targets. Thus, one of the challenges in SID-SRM/MS peptide research is to reach the sensitivity of ELISA (pg/mL).

Recently, our group proposed a new technique coined multiple reaction monitoring cubed (MRM^3) which enabled targeting protein in non-depleted plasma down to few ng/mL after a simple cleaning of the trypsin hydrolysate by solid phase extraction on a mixed-cation exchange cartridge [20–22]. Compared to conventional SRM, MRM^3 improves the sensitivity and extends the dynamic range of quantification by increasing the specificity of detection. Another new technique called photo-SRM allows an increase of the specificity. After tagging with a DABSYL chromophore group, cysteine-containing peptides [23,24] or estrogen compounds [25], only derivatives compounds are fragmented by visible laser irradiation. Although MRM^3 and photo-SRM methods are very selective, they do not solve the issue of matrix effect during mass spectrometry ionization. As previously mentioned peptide fractionation by strong cation exchange, depletion of the most abundant proteins or immuno-enrichment allows matrix effect reduction. However, these methods are time consuming and/or expensive and limited by the long development time and exorbitant cost required to obtain high quality antibodies. Because of its high resolving power, two-dimensional liquid chromatography (2D-LC) has recently received a great deal of attention [26,27] [28]. All peptide separation techniques used in 1D-LC–MS (reverse phase (RP), hydrophilic interaction liquid chromatography (HILIC) [29] and strong cation exchange (SCX)), can be applied in 2D-LC. Approaches in proteomics combine however mainly RP in the second dimension (SCX × RP, RP × RP or HILIC × RP). Recently, Shi et al. [30,31] proposed an off-line 2D reversed phase LC fractionation strategy employing high and low pH mobile phases. In their PRISM strategy (high-pressure, high-resolution separations coupled with intelligent selection and multiplexing), the eluate from the first dimension was split: the majority (90%) was collected in a 96 wells plate and the other 10% was directed to a mass spectrometer for online SRM monitoring of the internal standard in order to determine in which wells are the peptides of interest. After data analysis, the wells containing the peptides of interest were pooled and analyzed by low pH nanoLC-SRM/MS. This approach allowed the quantification of PSA at the pg/mL or ng/mL range respectively with or without immuno depletion of the most abundant proteins in human serum. Surprisingly, HILIC is seldom used as a second dimension, although the RP × HILIC configuration is very attractive in combination with MS detection since the high percentage of organic solvent in the eluting phase provides enhanced sensitivity for small molecules [32] and peptides [33]. As it's already known, the combination of HILIC and RP chromatography provides a high orthogonality [34]. Hence, a good separation of peptides is to be expected and a lower matrix effect observed. HILIC is a separation technique using a polar stationary phase as known from normal phase (NP) chromatography. However, here, the mobile phase is much more polar than in the NP-mode. The used solvents in HILIC (water, acetonitrile) are similar to the used solvents in reversed phase chromatography. Yet the elution strength of the solvents and the elution order of the analytes are reversed compared to the RP mode. Moreover, RP and HILIC buffers are not directly compatible and so online hyphenation of these stationary phases will be difficult. Limited miscibility of the mobile phase buffers and/or too large differences in the elution strengths in the two dimensions is more critical in HILIC than in RP. This is probably why the HILIC chromatography is often positioned in first dimension for peptide analysis in off-line [35,36] or in on-line [37] configuration. Post-column dilution of first dimension fractions with appropriate solvent (e.g. acetonitrile prior to HILIC [38] or water prior to RP [37]) is necessary to reduce the eluent strength and to avoid band broadening or splitting in the second separation dimension. Recently, d'Attoma et al. [39] compared two on-line 2D-LC setups (RP × RP and RP × HILIC) for peptide separation without diluting the sample between both dimensions. Authors concluded that the injection volume cannot exceed 9% of the dead volume of the second dimension column in RP × HILIC. Although this approach is promising for qualitative analysis, it is not compatible for sensitive quantitative analysis. Indeed the high flow rates used in second dimension (2 mL/min) will decrease the electrospray ionization yield. Moreover the small column diameter in the first dimension (1 mm) restricts the loading capacity of the stationary phase in comparison to 2.1 mm column. Such diameter allows good analytical performances for protein quantification in complex matrices [40]. As previously noticed [33], HILIC increases sensitivity in LC–MS as observed in small molecules but the major drawback for protein quantitation is a very limited loading capacity: less than 1 μL of serum on the HILIC column versus 10 μL on the C_{18} column with the same internal diameter. In order to take advantage of the gain in sensitivity, a RP × HILIC configuration was preferred. The benefit of this setup is a smaller impact of the low loading capacity of the HILIC column as the sample is fractionated in the first C_{18} chromatography dimension. However with the RP × HILIC setup, the problem of mobile phase incompatibility between both dimensions has to be solved. In order to avoid the dilution step, a strategy consists of trapping the compound from the first dimension on a solid phase extraction (SPE) cartridge. This approach has been recently considered by Greibrokk's team [41] for HILIC × RP configuration. However the sample has still to be diluted in order to be retained on the C_{18} cartridge and an additional pump is then required. Contrary to a simple dilution before the second dimension, this configuration has the advantage that the analytes are not diluted between both dimensions since they are concentrated on the cartridge before their elution by the second dimension mobile phase. The use of a cartridge with the same retention mechanism of first dimension but with a higher retention can avoid the use of additional pump [42] (RP × RP cartridge × HILIC). Nevertheless non-negligible amounts of water will stay in the C_{18} cartridge. This water will be transferred to the second dimension and may disturb column equilibration and lead to peaks distortions.

In this paper, an analytical RP-SPE-HILIC-MS system for peptide quantification with HILIC chromatography in second dimension is presented. In order to avoid the use of an additional pump a strategy with mixed ion exchange SPE cartridge was selected. Another advantage of this strategy is that it allows the use of a conventional flow rate (300 μL/min) in the second dimension. This flow rate is more compatible with ESI sources than the several mL/min used in comprehensive 2D LC analyses. The analytical system was tested for the quantification of prostate specific antigen (PSA) (UniProt accession number P07288) in plasma which is a biomarker of prostate cancer [43]. PSA was previously used as a biomarker model to demonstrate that SRM-SID-MS was an alternative to ELISA. In our previous paper, serum samples were depleted, digested and fractionated off-line by strong cation exchange [44]. The presented method allowed the quantification