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Two-step Ion-exchange chromatographic purification combined with reversed-phase chromatography to isolate C-peptide for mass spectrometric analysis

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Summary

A liquid chromatography with mass spectrometry on-line platform that includes the orthogonal techniques of ion exchange and reversed phase chromatography is applied for C-peptide analysis. Additional improvement is achieved by the subsequent application of cation- and anion-exchange purification steps that allow for isolating components that have their isoelectric points in a narrow pH range before final reversed-phase mass spectrometry analysis. The utility of this approach for isolating fractions in the desired “pI window” for profiling complex mixtures is discussed.

Keywords

C-peptide; Ion exchange chromatography; Isotope dilution assay; orthogonal separations; peptide purification

1 Introduction

Clinical chemistry applications of MS usually require careful sample preparation. In many cases, one is dealing with an analyte in a relatively low concentration. When biological fluids are involved, there is often a necessity for a long and tedious multi-step purification and concentration procedure. There are a variety of methods and approaches in protein purification and fractionation and success can be achieved in different ways. It is desirable that the most sensitive and critical stages are automated.

In recent years, progress has been made in profiling complex biological mixtures with so-called multi-dimensional methods [1–3]. Some of the multi-dimensional separations can be successfully applied to clinical chemistry problems. Moreover, the task seems to be much simpler since one only has to characterize one analyte or a relatively small number of analytes with somewhat similar physicochemical properties.

C-peptide is a marker of intact insulin secretory capacity that can differentiate true insulin deficiency characteristics of type 1 diabetes from the relative insulin insufficiency seen in

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type 2 diabetes [4–7]. MS characterization of C-peptide isotope dilution analysis (IDA) has been employed by several different groups and the results appear to demonstrate good agreement. After the work of Kippen et al. [8], who first used IDA for C-peptide quantitation using single quadrupole MS, other groups have also reported IDA–MS/MS procedures [9–12].

Rogatsky et al. [11,12] used a two-step chromatographic process which they referred to as 2D chromatography [13–15], although it was essentially the same separation mechanism of RP chromatography at each stage. Nevertheless, even in its reduced form, the 2D chromatographic approach proved to be effective. Recently, the utility of cation exchange chromatography has been demonstrated [16–18]. The authors did not use 2D chromatography per se, but a combination of ion-exchange (IEx) and RP chromatography were utilized.

In this communication we report the use of an anion exchange column, which is incorporated on-line and directly precedes the RP column. With the use of an anion exchange column instead of a cation exchange it became possible to achieve on-line 2D chromatography implementation (IEx – RP). Additionally, we used a two-step chromatographic procedure, a combination of cation exchange purification followed by anion exchange chromatography that allowed for isolating a C-peptide fraction with a reduction of impurities. The obtained fraction contained protein and peptides with pI values inside the pH range of two and a half pH units. We also discuss the possibility of generalizing this method of a narrow pH window for complex mixture profiling.

2. Materials and Methods

2.1. Reagents

Human C-peptide (57–87), synthetic analogue of the native peptide, unlabeled (Mw 3020) – was obtained from Sigma–Aldrich (St. Louis, MO); isotopically labeled [D8]-Val7,10 C-peptide was obtained from Bachem (Bubendorf, Switzerland). A laboratory synthesized peptide which contained 14 residues labeled with ^{13}C and ^{15}N , was provided by Dr. Daniel Stein from Albert Einstein College of Medicine, Bronx, NY. All other chemicals were purchased from Sigma–Aldrich.

2.2. Instrumentation

API-4000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) coupled to a Shimadzu Prominence LC system (Shimadzu Scientific Instruments, Columbia, MD) was used for analysis.

2.3 MS operating conditions

For RP chromatography, two mobile phases were used for elution: (A) 0.4% formic acid in water and (B) 0.4% formic acid in acetonitrile. The total flow rate was 0.35 mL /min. The column and autosampler plate were set at 40 and 4°C respectively. The MS parameters were as follows: positive ionization mode, resolution Q1 one unit, pause between masses 5 ms,

time for each mass 50 ms, declustering potential 70V, ion spray voltage 4000 V. The LC–MS system was operated under Analyst 1.6 software.

2.4. Samples

Plasma samples were stored frozen at -70°C . For quantitation, the internal standard (IS) was added immediately after thawing. After mixing with methanol (up to 1:4 ratio, v/v), samples were subjected to centrifugation (5 min at 12,000g) to remove precipitated proteins [16,17]. The supernatant was applied to SepPak C₁₈ disposable cartridges (Millipore, Billerica, MA). The eluent obtained was further subjected to ion exchange purification step with HiTrap HP SP cartridges (GE Healthcare).

2.5 Ion exchange sample purification (Cation exchange – Offline purification)

When samples were subjected to cation-exchange purification with HiTrap HP SP cartridges, the methanol content was reduced by at least 20% and the pH brought to 3.95 by ammonium formate. The eluate was collected as described earlier [16]. The pH of the sample was re-adjusted to pH 5 before 2D chromatography.

2.6. HPLC Anion exchange

A Mono Q column was equilibrated with ammonium formate 50mM, pH5. The sample load was typically 500 μL , by autosampler. Elution was achieved by decreasing pH stepwise (100% solution B, 0.4% formic acid). The anion exchange procedure was performed with a modular Shimadzu LC system containing two binary pumps. 50 mM ammonium formate pH 5.0 was used for sample application (solution A). For elution, a 100% of solution B (0.4% of formic acid in water) was used. Mono Q HR 5/5 was purchased from GE Healthcare.

2.7. RP-HPLC

RP-HPLC was performed using a Varian Pursuit C₁₈ column (50 mm $\times$ 2.1 mm (ID)), 300 Å pore size, Varian, Palo Alto, CA). For C-peptide isolation, a linear gradient of acetonitrile was employed, the details are given in Section 3.3.

3 Results and discussion

3.1 Optimization of the conditions for ion exchange chromatography

When using cation- or anion-exchange chromatography, for most analytes, there is an opportunity to retaining the substance of interest on the stationary phase by the appropriate selection of the pH of loading buffer (see Figure 1). Alternatively, the target protein can be allowed to go through the column without interaction while the “ballast” proteins and peptides can be effectively retained.

The charge versus pH curve, or “titration curve”, is a tool for selecting a purification strategy with ion-exchange chromatography. A theoretically modeled relationship [19,20] had been used for C-peptide purification using a cation exchanger [13]. Earlier we had calculated that its pI is around 3, which allowed for a very simple but effective purification scheme on a cation exchanger. Applying a starting solution with a pH slightly above the C-peptide pI to a cation exchanger will allow C-peptide to pass through the column while trapping most of the

impurities. This strategy was realized with the use of strong cation exchangers (disposable HiTrap SP cartridges or MonoS column)[16].

In contrast to a cation exchanger, an anion exchanger easily allows for a standard chromatographic procedure with a gradient/stepwise elution when working with C-peptide, since its pI is relatively low. Although both cation and anion exchange can be easily incorporated into a 2D HPLC separation set, the anion exchanger provides an essential advantage of additional sample concentration before C₁₈ column loading. For a Mono Q strong cation exchanger we determined that pH 5 and above was acceptable for sample loading.

The general scheme illustrating workflow is shown in Fig1. The procedure includes two on-line chromatographic separations performed sequentially, an anion exchange chromatographic step followed by RP. More exactly, with the anion exchanger, Mono Q, the isocratic elution is used; the eluate is then directed to the RP column and eluting with a standard gradient. What is important, is the off-line purification stage with a cation exchanger preceding the anion exchanger. This results in the isolation of a selected “pI window” of a mixture of components, see Figure 2. The exact details on how each process was organized are given in the following two sections. The last section discusses the possibility of using the above method of narrow window for profiling of complex mixtures.

3.2 Two-stage ion-exchange chromatography

After we have successfully used a cation exchange process to purify the C-peptide containing plasma fraction by using disposable HiTrap SP cartridges or a MonoS column, we decided to include an additional stage of fractionation on an anion exchanger. The purpose of adding an anion exchange step was dual: it provided higher purification and allowed for sample concentration before the final RP gradient elution. Figure 2 explains how such a two-stage ion-exchange purification occurs. One of the simplest ways of isolating a desired “pI window”, is to load the initial mixture on an anion exchanger with $\text{pH}=\text{pH}_1$ allowing all the ampholytes with $\text{pI}<\text{pH}_1$ to be absorbed on the charged matrix. The eluate is collected, the pH is re-adjusted to $\text{pH}=\text{pH}_2$ ($\text{pH}_2>\text{pH}_1$) and the procedure is repeated with a cation exchanger. At this point the mixture components with their $\text{pI}>\text{pH}_2$ are being removed from the solution. As a result, one gets a pH window which ideally contains only ampholytes (proteins and peptides) with their pI: $\text{pH}_1<\text{pI}<\text{pH}_2$. The procedure is very simple and can be performed with short disposable cartridges. It is clear, that the sequence is not critical in the above example. It has to be mentioned, that such a pI window can be theoretically obtained using different approaches with ion exchangers, in particular, the same type of ion exchanger can be used with a change of pH of eluent.

We used a two-step ion exchange procedure when the initial mixture was loaded first on a strong cation exchanger (HiTrap SP), at pH 3.9. At this stage, the impurities with their pIs above this value were removed from the solution, while the C-peptide passed freely through the column. The eluate was loaded on the HPLC column (anion exchanger MonoQ) and the elution was performed with acidic solution, $\text{pH}\sim 2.5$. As a result, a “pI window” of 2.5 pH units was isolated from the initial mixture, which included the pI of C-peptide (pI 3.0 approx.). This resulted in the fact that a highly purified sample was loaded on the C₁₈

column at the final stage. The latter is important for analyzing samples with extremely low C-peptide levels that requires high volume loads. The next section describes how the two column process was organized.

3.3 LC–MS on-line development (two column chromatographic process)

The 2D IEx–RP–LC–MS method was implemented on a Shimadzu HPLC system with two dual pump modules. Two columns were installed in a temperature controlled compartment. A high pressure switch allowed for flow re-directing after the first column, see Figure 2. At the stage of sample loading when C-peptide was adsorbed on the anion exchanger (Mono Q), the eluent was directed to waste (Figure 2B). The pH of the loading buffer was selected to be 5.5. When the pH changed to acidic and C-peptide started to be quickly eluted, the flow redirected to the second column, C₁₈ (Figure 2B). To avoid the necessity of pH adjustment, the same standard solution was used (buffer A: formic acid 0.4%, pH 2.5 approx.), which is slightly lower than necessary to achieve fast elution. Final separation was achieved in a linear gradient of acetonitrile, similar to what was described earlier [16]. Once C-peptide is eluted from Mono Q, the anion exchanger is subjected to regeneration. The details of the LC chromatographic method and the gradient profiles for the two columns are given in Figure 3. Briefly, a “narrow pI window” containing C-peptide is loaded onto MonoQ column, then the column is continuously washed by the same loading buffer for 5 min, while the eluent goes to waste (Fig. 4). With a sharp pH decrease the sample is transferred to the C₁₈ column (Fig. 5). For C-peptide elution, a linear acetonitrile gradient (20–50% in 15 min) is applied. The RP column is washed three times with acetonitrile gradient up to 100%. At the same time, the anion exchanger regenerates and re-equilibrates for the next cycle (Fig. 6). SIM for native C-peptide and IS are presented in Figure 7. A higher degree of purification results in very low background level.

3.4 Possible applications of “pI window” isolation by ion exchange method for profiling complex mixtures

In this communication we applied to C-peptide the method step ion-exchange purification (Fig. 2) that results in isolating only a narrow fraction of components with the their pI values inside the selected pH window, pH. This can be done with the help of ion-exchange chromatography in several ways, as discussed previously. Once the desired sample is collected, the process can be repeated several times until the whole pH interval is covered (see Fig.2). With some restrictions the above approach can be applied to complex mixture profiling in one- or two-dimensional separations. The 2D analysis can be easily performed, for example, if the selected pI segment is further subjected to a hydrophobic interaction chromatography. Here one has to take into account that the isolation mechanism for the procedure described is not completely based on discrimination according to pI, as mentioned above, but still relatively close to that parameter [21]. In the case of just one target analyte, the usefulness of the described above approach becomes even more evident. Indeed, the pH window can be selectively narrowed. The exact conditions are to be determined experimentally. We successfully used window sizes up to 2 pH units. Again, it has to be emphasized that the retention time in ion exchange chromatography does not directly correlate with the pH–pI difference: many other parameters, like ionic strength, protein size, temperature etc. can affect chromatography results.

4 Concluding remarks

We described a 2D LC–MS method for human C-peptide analysis in serum. The chromatographic techniques included here are truly orthogonal: ion exchange and RP chromatography. The approach described here utilized anion exchange chromatography that brings a certain advantage for C-peptide analysis: it allows for sample analysis with very low analyte concentration. More generally, the combination of IEX and RP seems to be very promising in many MS applications. In addition, a sequential use of cation and anion exchangers can be used as an analog to IEF with many applications that include MS as a final stage analysis.

Abbreviations

IDA	isotope dilution analysis
IEX	ion exchange
pI	isoelectric point

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Figure 1.

The diagram illustrating LC-MS process and most important sample preparation steps. The two on-line chromatographic separations are performed sequentially, anion exchange chromatographic step is followed by RP and, further, by ESI-MS. A combination of cation exchange, which is a part of off-line purification, and anion exchange steps (on-line) allows for reducing the number of ballast components (only species with pI values in the selected window are collected (see Fig. 2 for details)).

Figure 2.

A two-step ion exchange chromatographic procedure (in this example a cation exchange precedes anion exchange) that isolates mixture components with pIs that belong selected pH interval. Multi-component system ampholytes (proteins or peptides) interacting with cation and anion exchangers. At any given pH₁, the analytes with their pI values located to the right (pI > pH₁) will be charged positively and thus will be retained by a cation exchanger. Similarly, for anion exchangers, the interaction takes place for the species having their pI below the selected pH value (pH₂). In this illustration, a sample is applied initially to a cation exchanger followed by an anion exchanger, so pH₁ > pH₂. In this picture, the same form curves (titration curves), uniformly dispersed across whole pH range, represent all different compounds in the sample solution.

Figure 3.

Potential application of a narrow pH window method for complex mixture profiling. As a result of sequential application of a pair ion-exchange separations, a fraction of initial sample is obtained that contained species with pI values belonging to the selected pH window. By repeating the above procedure, all necessary pH intervals can be covered. The collected fractions can be subjected to a second dimension analysis, as shown. With some limitations, the method can be considered as alternative for IEF.

Figure 4.
2D hardware design and valve positions. At position A, the flow from the IEx column is directed to waste.

Figure 5.
Valve position B. The flow from the IEx column is transferred to second column, C₁₈.

Figure 6.

2D chromatography separation method. Concentration gradient profiles for each column are shown. Dotted line represents Pump 1 gradient profile: a sharp increase of B solution concentration is used for a sharp pH decrease to provide fast C-peptide elution and then the column is re-equilibrated to the pH of sample loading (pH 5). A solid line shows Pump 2 gradient (acetonitrile concentration). C-peptide elution occurs at 40%, approx. and two more washing cycles are added to provide reliable C₁₈ column cleaning.

Figure 7.

Selected ion monitoring (SIM) for C-peptide plasma sample. 2D LC method: anion exchange purification on MonoQ followed by RP (C₁₈). Retention time for C-peptide is 27.96 min. The isotopically labeled standards ($m/z=1017.7$ and 1013.0) are co-eluted with the native C-peptide ($m/z=1007.7$). The insert shows the same full length 1 h SIM data.