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Novel, highly robust method of carbohydrate pre-purification by two-dimensional liquid chromatography prior to liquid chromatography/mass spectrometry or gas chromatography/mass spectrometry

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Abstract

We describe a novel two-dimensional liquid chromatography (2D-LC) method for fast and robust isolation and concentration of low abundant carbohydrates (sorbitol, glycerol) from biological matrices (plasma and urine). Off-line pre-purified fractions, enriched by analyte of interest, were analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS–MS). Initial 2D-LC automated sample pre-purification improved MS detection, eliminated matrix effects, and achieved high sensitivity (picogram detection limit) with a 6 min runtime and increased column lifetime. Using this method we have analyzed more than 1300 samples from biological matrices without column replacement.

Keywords: Human plasma; Carbohydrate analysis; 2D liquid chromatography; LC/MS

1. Introduction

Clinical research into carbohydrate metabolism using stable isotopes in human subjects has become increasingly significant with the increasing incidence of obesity and diabetes. These types of measurement are routinely achieved by GC/MS, because electron-impact ionization, widely used in GC/MS, is more efficient for uncharged analytes like glucose, fructose, sorbitol or glycerol, than electrospray or corona discharge ionization techniques, utilized by LC/MS [1,2]. GC/electron impact ionization (EI) MS analysis nevertheless has serious limitations: fragment ions that contain every isotopically labeled carbon atom of interest, may not be available at high abundance [3]. Routine GC/MS analysis of carbohydrates requires long runtimes and complicated sample preparation. Currently, quantitative analysis of carbohydrates by LC/MS is not widely used. At present, these applications have been limited due to low sensitivity or suboptimal linearity range, limited robustness and therefore are not ideally suited for quantification from large sample batches [4–6].

Sample preparation for GC/MS analysis (including pre-column clean up) is commonly a prolonged and multi-step process because GC techniques require volatile and pre-purified analytes. Sample preparation for large batches is the most important rate limiting step for sample throughput, and thus is both costly and labor intensive. For example, GC/MS analysis of glucose of the aldonitrile pentaacetate derivative requires five different chemical steps including protein precipitation, deionization, two steps of derivatization, and extraction prior to analysis [7]. This is a manual sample preparation process which cannot be completely automated. Significant simplification of the sample processing can be achieved using LC/MS methods and, therefore, identification of robust electrospray ionization methods for carbohydrates analysis is potentially of benefit.

Recently, we have developed new sensitive method for LC/MS analysis of carbohydrate from human plasma, based on multiple reaction monitoring (MRM) analysis (of a metastable complex of carbohydrate with a metal cation) on a

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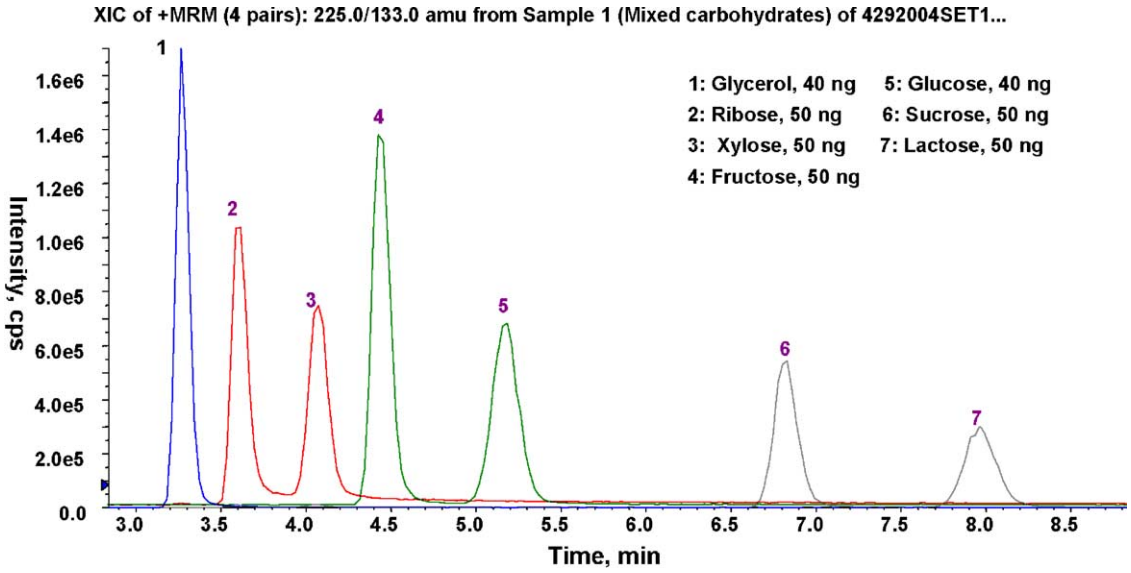

Fig. 1. Retention time of various carbohydrates (40–50 ng on column) after chromatography on an Luna amino column (Phenomenex) detected by LC/ESI-MS–MS with Cs+ attachment. Actual LC/MS conditions listed in methods.

Sciex API 4000 triple quadrupole system [8]. This method is highly sensitive (picogram level detection limit), specific, does not require derivatization, and is highly useful for measurement of isotope dilution assays on intact carbohydrate molecules (Fig. 1).

This method achieves excellent results for carbohydrates, if their concentration exceeds a few hundreds pg/μl, such as glucose, fructose and galactose (in saliva). For low abundant analytes such glycerol and especially sorbitol, we encountered several difficult obstacles: (1) MS signal suppression, and (2) rapid deterioration of the amino chromatography column resulting in loss of separation between carbohydrates of similar molecular weight.

In this paper we describe the development of a novel, robust approach for off-line pre-purification and enrichment of low abundance carbohydrate samples prior to LC/MS [9]. The aims of this method were to eliminate limitations due to MS signal suppression and column deterioration [10,11]. Our rationale was that eliminating signal suppression from matrix effects would improve the sensitivity of MS detection. In addition, in the case of overlapping analytes, separation of analytes on an off-line column would allow efficient desalting, peak focusing and elution of a Gaussian shaped peak into the MS for quantification. This arrangement strongly decreases requirements for on-line column performance and results in increased column lifetime.

2. Experimental

2.1. Reagents and materials

All organic solvents were HPLC grade and were obtained from Fisher (Suwanee, GA, USA). Milli-Q grade water was produced by a Millipore system (Millipore, Bedford, MA, USA). Carbohydrates and sodium borohydride were obtained from Sigma (St. Louis, MO, USA). [U-$^{13}C_6$]glucose and and [1,1-$^{2}H_2$]sorbitol (99%) were obtained from Isotec (Miamisburg, OH, USA). U-$^{13}C_6$-labeled sorbitol was produced from sorbitol reduction of the corresponding labeled glucose. 0.5 mmol of [U-$^{13}C_6$]glucose was dissolved in 50 ml of methanol and 1.5 mmol of sodium borohydride was added. The mixture was stirred for 60 min at room temperature, and 4.5 mmol of hydrochloric acid added to react with excess sodium borohydride. Subsequent LC–MS analysis showed that the sorbitol compound contained 98.8% [U-$^{13}C_6$]sorbitol (M_r 188).

2.2. Sample preparation

Seventy-five microliters of internal standard was spiked with 125 μl of human plasma and vortexed for 45 s. Plasma proteins were precipitated with 1.3 ml of acetone/chloroform (9:4 mixture) and centrifuged 7 min at 14,000 rpm. The upper, aqueous layer containing carbohydrates was transferred into 2 ml auto-sampler vials containing a 300 μl conical insert. Vials were pre-warmed to 50 °C rior injection into the 2D-LC system.

2.3. 2D-high-performance liquid chromatography (offline)

Offline 2D-LC sample pre-purification was performed using a 1100 series liquid chromatography system (Agilent Technologies, Wilmington, DE, USA), equipped with a G1312A binary, G1311A quaternary pumps, G1367A autosampler, G1314 UV detector, G1362A refractive index (RI) detector, G1316A column compartment with two position.

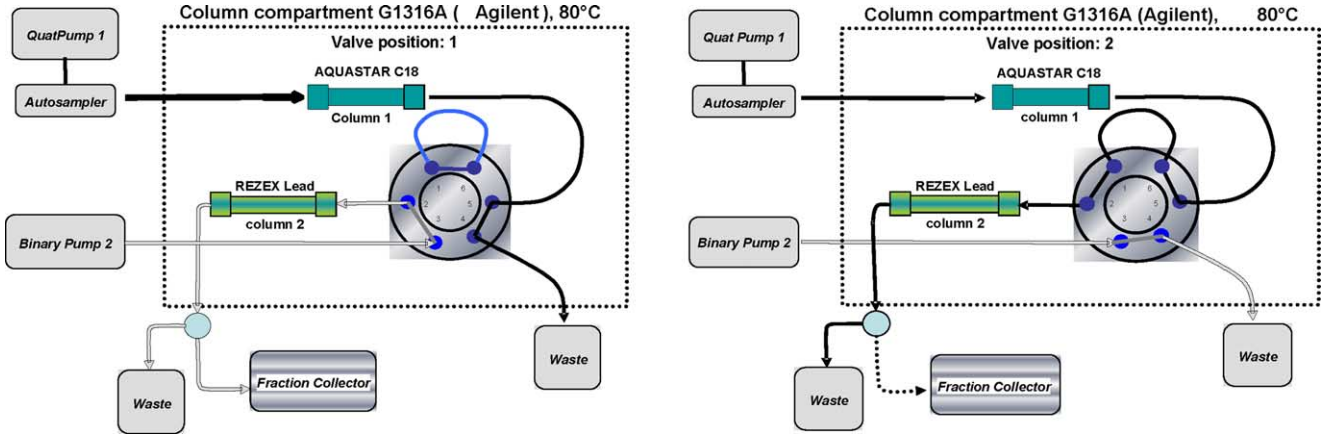

Fig. 2. Plumbing diagram of 2D off-line purification method. Position A: valve position 1. Injection of sample while columns run separately. Position B: valve position 2. Columns switched together for 9 s while void containing sugars from column 1 are transferred to column 2. Position A. Regeneration and re-equilibration of column 1 while column 2 runs to the fraction collector.

Six port Rheodyne thermostabilized valve, and G1322A degasser. Fractions were collected by a Gilson FC 203B fraction collector.

In the first step (column position 1), 40 μl of plasma extract was injected onto the first column, 10 mm × 4 mm 5 μm HyPurity Aquastar C18 Guard cartridge (Thermo Electron Corp., Bellefonte, PA, USA), which was used as the first LC dimension (Fig. 2). The cartridge was flushed at 0.5 ml/min with pure Milli-Q grade water at room temperature. After 14 s, the valve was switched to position 2 and for 9 s the first column was connected to the second column, 5 cm × 7.8 mm of Rezex Lead (Carbohydrate Anion Exchange Guard Column), from Phenomenex (Torrance, CA, USA). The second column was run isocratically with Milli-Q water at 0.5 ml/min and 80 °C. Nine seconds is enough time to introduce the carbohydrate fraction, eluted in the void volume of the first column, to the second ion-exchange column. At the end of this time, the valve was switched back to position 1 and the columns continued to run separately. The C18 column was regenerated with 95% acetonitrile followed by equilibration with pure water. Fractions from the second dimension containing the pre-purified factions were dried in a Speedvac system and stored at 4 °C prior to MS analysis.

2.4. Liquid chromatography/mass spectrometry operating conditions

Atmospheric pressure ionization (API) MS detection was achieved using a PE Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosytems, Framingham, MA, USA) equipped with an orthogonal Turboionspray source, Agilent G1376A capillary pump, 2-position/10-port Valco valve (Houston, TX, USA), MetaChem Column heater (MetaChem Technologies, Torrance, CA, USA) and Gilson 215 liquid handler, equipped with micro injector 841 (Middleton, WI, USA).

A 3 μm Luna Amino column, 150 mm × 2 mm from Phenomenex equipped with a 4 mm × 2 mm guard was operated isocratically at 200 μl/min with the mobile phase composed of acetonitrile/water (80:20) with 40 μM cesium acetate at 35 °C. The dried samples, obtained from 2D, were reconstituted in 80 μl of the same mobile phase and 2.5 μl was injected into a column by 5× loop overfilling.

The mass spectrometer was operated in positive mode with three MS–MS transitions monitored during LC separation in the MRM mode. A dwell time of 350 ms per transition was used. The analyte-dependent and ESI source parameters were selected to optimize formation of carbohydrate–Cs+ metastable complex in the source and maximize its transmission into Q2. In the collision cell this unstable complex was disjointed to Cs+ and an uncharged molecule of carbohydrate. The liberated Cs+ at *m*/*z* 133 was monitored at each transition. Q3 was set to high resolution; Q1 was opened-up to low resolution. Source temperature was maintained at 100 °C. Ion spray voltage was maintained at 5000 V, collision cell gas at 9 psi, curtain gas 10 psi, 1–40 psi, and 2–35 psi.

3. Results and discussion

The Cs+ attachment assay after the 2D off-line purification was highly sensitive with a detection limit below 12.5 pg of sorbitol on column, and was linear over a 10,000-fold concentration range. Full details of the optimum concentrations for Cs+ attachment during ESI-MS will be provided in a separate report. We have successfully analyzed over 1300 samples for plasma sorbitol concentration to date, and have successfully applied this method to measurement of other carbohydrates as well including glucose, and glycerol. In a group of normal non-obese non diabetic men and women ($n = 16$), measured concentrations of sorbitol were 0.40 ± 0.20 μmol/l (±1 S.D.). This level is consistent with prior reports of plasma sorbitol concentrations in healthy human beings [12,13].

Pre-purification of samples was established to be critical for robust measurement of low abundance carbohydrates such as sorbitol. Usually, LC/MS analysis of carbohydrates *from*

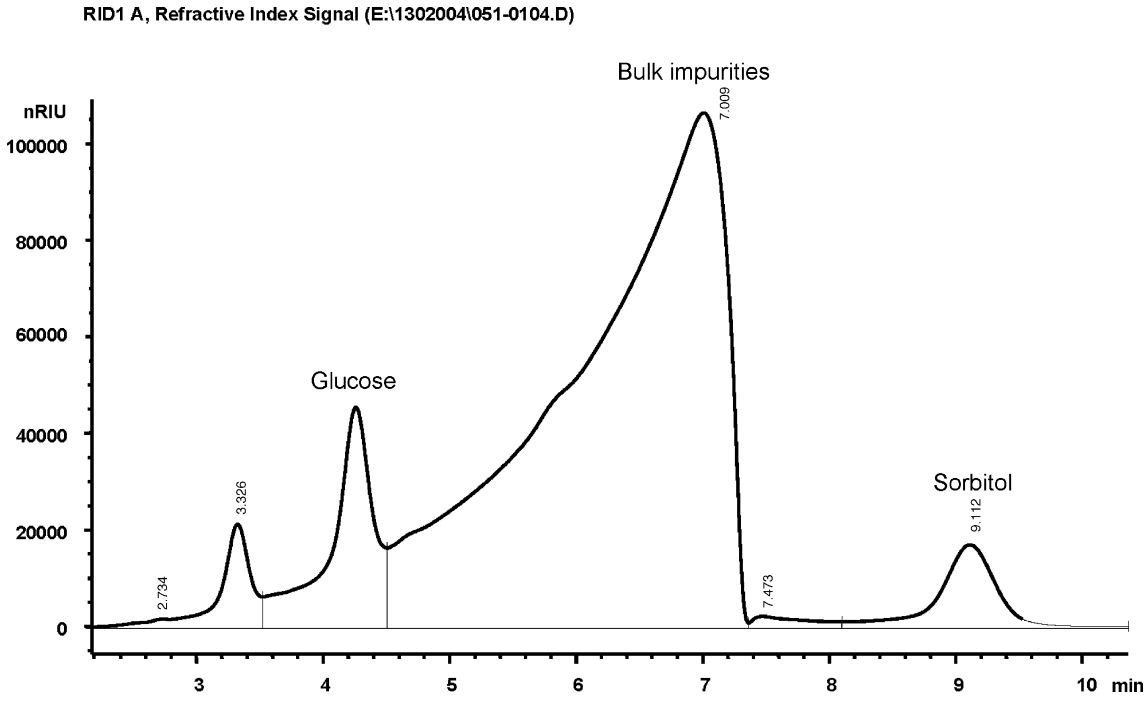

Fig. 3. Demonstration of bulk impurities arising from acetone extracts of plasma samples. Purification performed on 2 guard cartridges of Rewex Pb++ (Phenomenex) 50 mm × 7.8 mm. Detection by refractive index.

plasma is performed on amino columns without a prior clean-up step: the plasma acetone extract is directly injected onto the column. This approach has the advantage of requiring minimal labor. A major disadvantage of this approach however, is that the acetone extract of plasma is still full of contaminating compounds. During the MS analysis this results in significant matrix effects with high background and signal suppression. This minimal pre-purification approach successfully works *only* for high abundant carbohydrates such as glucose (Fig. 3). For low abundant carbohydrates, such glycerol or sorbitol [6], such a minimalist approach will concentrate impurities as well as the analyte of interest. We observed not only signal suppression, but also that the dried residue from the acetone extract was difficult to re-solubilize in organic and/or polar solvents, leading to inconsistent recoveries [14].

Difficulties in sorbitol analysis, instead of other carbohydrates, are due to its low concentration in plasma, and poor chromatographic efficiency of separation from glucose (which is the main plasma carbohydrate). These analytes have similar retention times on amino based carbohydrate columns and thus are poorly resolved from each other. The plasma sorbitol concentration, as noted above, is less than 1 μM and glucose concentration is ∼5 mM (>5000-fold concentration difference), therefore even minimal overlap of the $m+2$ (182 u) glucose isotope peak on the sorbitol peak can easily contaminate the sorbitol 182 u signal (Fig. 4). In this situation for LC, the sorbitol peak is buried under a huge glucose peak. While new amino columns were able to provide partial separation between glucose and sorbitol (Fig. 4), column performance rapidly deteriorated when acetone extracts were used. Thus, given the dramatic difference in abundance between glucose and sorbitol, even a 1% contamination of glucose into sorbitol, and assuming that ∼1.5% of glucose molecules contain molecules of M_r 182 due to ^{13}C natural abundance, can be calculated to raise the measured M_r 182 analyte concentration by 75%. We detected significant deterioration in amino column performance to separate sorbitol from glucose after only 50 injections (data not shown). An alternative approach to sorbitol analysis is to use GC/MS

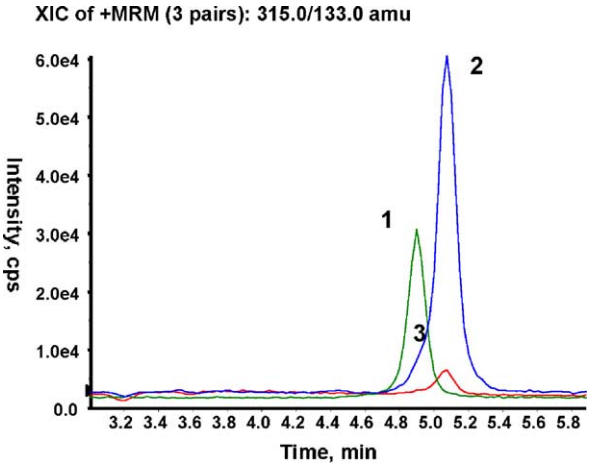

Fig. 4. Incomplete separation of sorbitol and glucose by normal-phase chromatography on a new Luna amino column. Detection was performed by API-MS–MS with Cs+ attachment. Peak identifications: 1, [U-^{13}C]sorbitol; 2, $m+2$ glucose; 3, $m+0$ sorbitol and overlapping $m+2$ glucose (shoulder). Sample: urine, 1/20 in acetone.

of sugar derivatives. Derivatization of hydroxyl functions in glucose and sorbitol, e.g. by acetic anhydride, creates two different products – penta- and hexaacetate derivatives that have different retention times. In contrast successful analysis of sorbitol by LC/MS in the absence of derivitization requires initial pre-purification from glucose.

To resolve these chromatography/mass spectrometry problems, we designed a new approach: off-line pre-purification of carbohydrates by utilizing a chromatography technique orthogonal to the normal phase (amino column). An alternative chromatography technique, commonly used for carbohydrate separation is based on cation exchangers, with calcium, sodium, silver, lead or hydrogen, and widely used for carbohydrate analysis of food products. The typical dimensions of these columns are 30 cm × 7.8 mm, and run-times generally exceed 30 min. Sample throughput is therefore quite restricted. We designed a high throughput pre-purification system which required less than 10 min per sample. Since short carbohydrate columns are not available, we built our method on one inexpensive guard cartridge, a 5 cm × 7.8 mm Rezex Pb++ from Phenomex.

The typical carbohydrate ion exchange matrix is not stable at high pressure and is incompatible with organic solvents. Contamination by salts such as is present in plasma extracts should be avoided – as they will exchange the cation from the column matrix and shift the analyte's retention time (Fig. 5). The acetone plasma extract also contains significant amounts of lipids (especially in our studies of fat metabolism). Lipids, especially phospholipids, tightly bind and rapidly poison anion-exchange columns. The typical methods of generic anion-exchange column regeneration – washing with a few column volumes of organic solvent (such as isopropanol), is not possible due to incompatibility issues.

To resolve this issue and obtain a robust method for protection of the analytical column from lipid contamination, we have developed a method of carbohydrate pre-purification from human plasma and urine, based on 2D-LC chromatography. The method consisted of high pressure column switching at 80 °C between an *unusual* combination of phases: the first, HyPurity Aquastar C18 (which is a stable in 100% aqueous conditions); and second, a carbohydrate ion exchange column. The Aquastar C18 very efficiently removes lipids, proteins etc from the injected sample and therefore protects the carbohydrate column. In this application, the C18 column is used like a guard pre-column but importantly also *works* orthogonal to the ion-exchange separation technique.

Carbohydrate ion exchange columns separate carbohydrates but do not concentrate. Typically the second dimension in a 2D method is a concentration step, chosen for focusing of the analyte from potentially a significant volume, transferred from the first onto the second column. Using the short guard on the first dimension and fast switching of the void volume – 75 μl only – did not cause peak broadening and, therefore did not require focusing in the second dimension. This arrangement of columns does not allow focusing and therefore is only useful when switched volumes remain low.

To ensure efficient trapping of hydrophobic contaminants from the acetone extract on the aqueous C18 column, the sample is heated to drive off the acetone completely. If this is not done, some lipids escape the reversed-phase column and can poison the downstream ion exchange column. The salts, contained in the acetone extract slowly exchange with Pb+ from the matrix, causing a slow decrease of the analyte's retention time arising from the loss in column binding capacity (Fig. 5). To avoid a drop in the retention time and incomplete analyte collection, the lead column was "recharged" by 5% lead nitrate every 10 injections. Also, we strongly recommend recharging a newly installed column to achieve and maintain maximum retention time for analytes of interest. The fractions of interest purified on the second column were collected into 1.5 ml micro centrifuge tubes (Eppendorf) by a fraction collector.

Sorbitol samples isolated free from glucose contamination were routinely assayed by LC/MS/MS. Since a Gilson 215

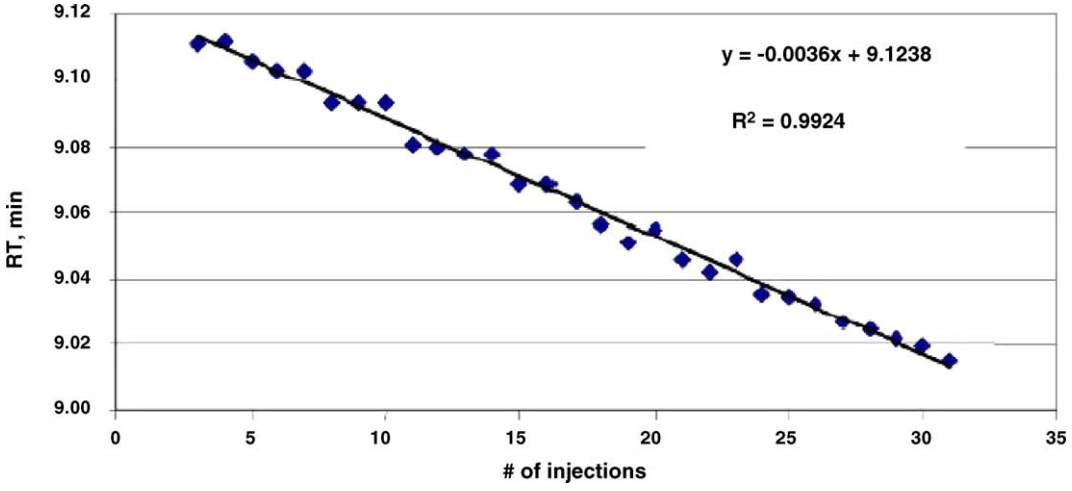

Fig. 5. Retention time shift on a Rezex Pb+ column after repeated injections of plasma extract containing residual plasma salts. Chromatography conditions given in Section 2.

liquid handler has a heavy-duty probe, the exact centering of the conical insert in a vial is important. After many trials we selected 2 ml vials with *fused* inserts, available from SUN-SRI (Wilmington, NC, USA).

A significant advantage of our method includes a significant improvement in the lifetime of the amino carbohydrate column used during LC/MS operation. The use of highly pre-purified carbohydrate samples also dramatically minimized the requirements for amino column performance; we were able to use our amino column for over a thousand injections without replacement. It should be emphasized, this method allows enrichment of low abundance analytes such as glycerol or sorbitol, and removal (if necessary) of glucose from the sample. Since glucose concentrations in plasma are a few thousand fold greater than other carbohydrates, this can cause an overload of LC (amino) or GC (capillary) columns and rapid contamination of columns and systems.

4. Conclusions

This work presents a new method of carbohydrate sample preparation prior to analysis by LC/MS. It requires less labor than typical published methods, provides high throughput during sample preparation, significantly improves column lifetimes, and provides reliable and completely automated pre-purification of thousands of samples prior to sensitive measurement by electrospray mass spectrometry.

"Common sense" or traditional approaches to HPLC method development suggest:

(1) Guard cartridges made from the same material as the main analytical column.
(2) The second dimension in a 2D-LC method is a focusing (concentration) step.

    Therefore, most 2D applications use ion-exchange chromatography in a first step, and reversed-phase chromatography in a second.
(3) The primary purpose of 2D method development is on-line analysis (by UV or MS) and usually is not designed for fraction collection.

In our method, these three common "rules" were reversed 180° allowing us to resolve a difficult problem in the analytical mass spectrometry of carbohydrates.

Acknowledgements

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