Sensitive LC MS Quantitative Analysis of Carbohydrates by Cs^+ Attachment

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The development of a sensitive assay for the quantitative analysis of carbohydrates from human plasma using LC/MS/MS is described in this paper. After sample preparation, carbohydrates were cationized by Cs^+ after their separation by normal phase liquid chromatography on an amino based column. Cesium is capable of forming a quasi-molecular ion $[M + Cs]^+$ with neutral carbohydrate molecules in the positive ion mode of electrospray ionization mass spectrometry. The mass spectrometer was operated in multiple reaction monitoring mode, and transitions $[M + 133] \rightarrow 133$ were monitored (M, carbohydrate molecular weight). The new method is robust, highly sensitive, rapid, and does not require postcolumn addition or derivatization. It is useful in clinical research for measurement of carbohydrate molecules by isotope dilution assay. (J Am Soc Mass Spectrom 2005, 16, 1805–1811) © 2005 American Society for Mass Spectrometry

Carbohydrate metabolism research in humans has become increasingly significant with the increasing incidence of obesity and diabetes. Examples of carbohydrates, the quantification and kinetic turnover of which have been investigated in metabolic studies, include glucose, glycerol, and sorbitol [1–12]. Quantitative measurement by isotope dilution assay is one of the traditional methods used to achieve the requisite sensitivity, accuracy, and precision for such in vivo investigations. That type of measurement is routinely solved by GC/MS chromatography because electron impact ionization (EI), widely used in GC/MS, is more efficient for uncharged analytes such as monosaccharides, compared with electrospray (ESI) or atmospheric pressure chemical ionization (APCI) techniques, used in LC/MS [5, 11, 13, 14]. Sample preparation for GC/MS analysis (including precolumn clean up) is frequently a prolonged and multi-step manual process because GC techniques require volatile and prepurified analytes. Sample preparation for large sample lots, which frequently occurs in clinical studies, is an important rate-limiting step for sample throughput, and thus, is both costly and labor intensive. For example, glucose analysis by GC/MS from the aldonitrile pentaacetate derivative requires five different chemical steps including protein precipitation, deionization, two steps of derivatization, and extraction before analysis [15]. Inexpensive derivatization reagents however, such as acetic anhydride, do not provide optimum volatility for carbohydrates, reducing sensitivity, limiting analysis to abundant species such as glucose. Increased sensitivity of carbohydrate detection by GC/MS has been achieved with specialty reagents for derivatization; however, they are significantly more expensive [8, 10, 16]. LC/MS-based methods are potentially advantageous because of the simplification of sample processing [4–6, 11]. The challenge in this type of approach has been the low efficiency of API or APCI ionization for neutral carbohydrates which has limited sensitivity of measurements.

There are two different approaches to LC/MS quantitative analyses of monosaccharides that have been reported. The first approach [17–19] is based on Cl^- attachment to carbohydrates by postcolumn addition of chloroform. Nevertheless, Cl^- attachment as a general single quadrupole method has serious limitations: (1) Chloroform is essentially immiscible in aqueous mobile phase; therefore, a second pump for delivery of chloroform as a postcolumn addition may be required. (2) It should be noted that older generation mass spectrometers (frequently in use in academic laboratories), are significantly less sensitive in negative- compared with positive ionization and detection mode. Kato et al. [17] and Kumagai et al. [19] reported sensitivities of ~1–10 ng of glucose, using $CHCl_3$ in water with atmospheric pressure chemical negative ionization. (3) Chloroform is reactive and easily forms dichloro, tetrachloro, and hexachloro free radicals and phosgene [20, 21] which generates a variety of different adducts, complicating interpretation in scan mode and lowering sensitivity in SIM mode.

The MS/MS version of the chlorine attachment approach has increased sensitivity compared to single quadrupole MS detection. The robust and sensitive MRM version of chlorine attachment was successfully

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implemented with 0.1% CH_2Cl_2 in 50% methanol/water. Carbohydrate-chlorine complex was monitored at Q1 and $[Carb\text{-}H]^-$ at Q3 [22]. Chloride attachment can successfully be accomplished with chloride salts [23]. However, the main disadvantage of the use of chlorine is that it has 2 natural isotopes of 35 and 37 nominal masses. Abundance of ^{37}Cl is 24.23%. This splits the analyte peak by a 3:1 ratio and reduces method sensitivity. A major disadvantage of this method is the incompatibility of direct quantitation of M + 2 isotopic labeled carbohydrates (such as 6,6 $^2H_2$ glucose), widely used in clinical research as an in vivo tracer of glucose metabolism [2].

The second approach is based on small cation attachment to carbohydrates such as Na^+ [11, 24–27] and Li^+ [33, 34]. The analytical method, based on Na^+ attachment [11], is simpler than chlorine attachment as it does not require postcolumn addition, and therefore, a second pump. This method utilizes "free" Na^+, which is a ubiquitous contaminant from glassware in aqueous mobile phases. Since Na^+ is monoisotopic, the Na^+ attachment method in contrast to chlorine attachment, provides only a single *m/z* peak. This method therefore is potentially applicable to isotope dilution assays. The disadvantage of this attachment ion is insufficient sensitivity in analysis of carbohydrates that are present at low abundance in clinical samples such as plasma glycerol and sorbitol due, at least in part, to undesirable adduct formation by $[M + 2Na]^{2+}$. Low sensitivity necessitates larger plasma volumes for analysis, which decreases column lifetime and overall robustness of the LC/MS method [28–30].

In the present study, a simple, sensitive, and rapid LC/MS/MS method for carbohydrate analysis is described. Uncharged carbohydrate molecules (Carb) were detected after Cs^+ attachment and electrospray ionization in positive mode. Cesium is capable of forming a cationic adduct $[Carb + Cs]^+$ with neutral carbohydrates in the positive ion mode of ESI mass spectrometry [26, 27, 31]. The mass spectrometer was operated in MRM (SRM) mode, and the transitions $[M + 133] \rightarrow 133$ for each analyte were monitored. The overall method time is only 6 min. This method is highly sensitive and specific. The new method does not require derivatization or postcolumn addition and is potentially useful in clinical research for measurement of carbohydrate molecules by isotope dilution assay.

Experimental

Reagents and Materials

All organic solvents were HPLC grade and were obtained from Fisher (Suwanee, GA). MiliQ grade water was produced by Millipore system (Millipore Corp., Bedford, MA). Carbohydrates were obtained from Sigma (St. Louis, MO). Stable isotope- labeled carbohydrates (99%) was obtained from Isotec (Miamisburg, OH).

Sample Preparation

Plasma samples stored at −80 °C were thawed at room-temperature and 125 μl ml was spiked with 75 μl of 14.1 μM U-^{13}C sorbitol (internal standard) and vortexed 45 s in 2.0 ml microcentrifuge tubes. Plasma proteins were precipitated with 1.3 ml of cold acetone/chloroform (9:4 mixture) and centrifuged 7 min at 14,000 RPM. 50 μl of the aqueous (upper) layer, (containing carbohydrates) was transferred into 2 ml auto-sampler vials and diluted 10-fold with 80% acetonitrile before autosampler injection. All human samples were collected through protocols approved by the Albert Einstein College of Medicine Committee on Clinical Investigations (IRB).

Liquid Chromatography/Mass Spectrometry Operating Conditions

API-MS detection was achieved using an Applied Biosystems/MDS SCIEX API 4000 triple quadrupole mass spectrometer (Applied Biosytems, Framingham, MA) equipped with an orthogonal Turboionspray source, Agilent G1376A capillary pump, 2 position/10 port Valco valve (Houston, TX), MetaChem Column heater (MetaChem Technologies, Torrance, CA), and Agilent G1367A well plate autosampler (Agilent Technologies, Wilmington, DE) .

A 3μ Luna Amino column, 2 × 150 from Phenomenex (Torrance, CA) equipped with a 4 × 2 mm guard was eluted at 200 μl/min with the mobile phase composed of acetonitrile/water (80/20) and 40 μM cesium acetate in isocratic mode. Column temperature was maintained at 35 °C. Typically, 4 μl of diluted plasma extract was injected on column.

Mass Spectrometry Operating Conditions

The mass spectrometer was operated in positive mode. The analyte-dependent and ESI source parameters were selected to optimize formation of $[Carb + Cs]^+$ metastable complexes in the source and maximize its transmission into Q2. The various ions monitored in Q1 were: *m/z* = 313, 319 for unlabeled and U-^{13}C glucose; 316 and 321 for 1-^{13}C and U-^{13}C sorbitol; 225 and 230 for unlabeled and d_5 glycerol; and 351.1 and 356.1 for unlabeled and d_5 glycerol triacetate, respectively. 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 with a dwell time of 350 milliseconds. Q1 was opened-up to low-resolution. Q3 was set to high-resolution. The ionspray voltage was 5500 V. The nebulizing gas (gas 1) was operated at 40 psi and the turbo gas (gas 2) temperature was 100 °C at 35 psi, with the interface heater turned ON. Curtain and collision gases operating pressures were maintained at 10 and 9 psi, respectively. The declustering potential (DP) was optimized at 27 V; entrance potential (EP) was 10 V; collision energy (CE) was 15 V, and collision cell exit potential (CXP) was 11

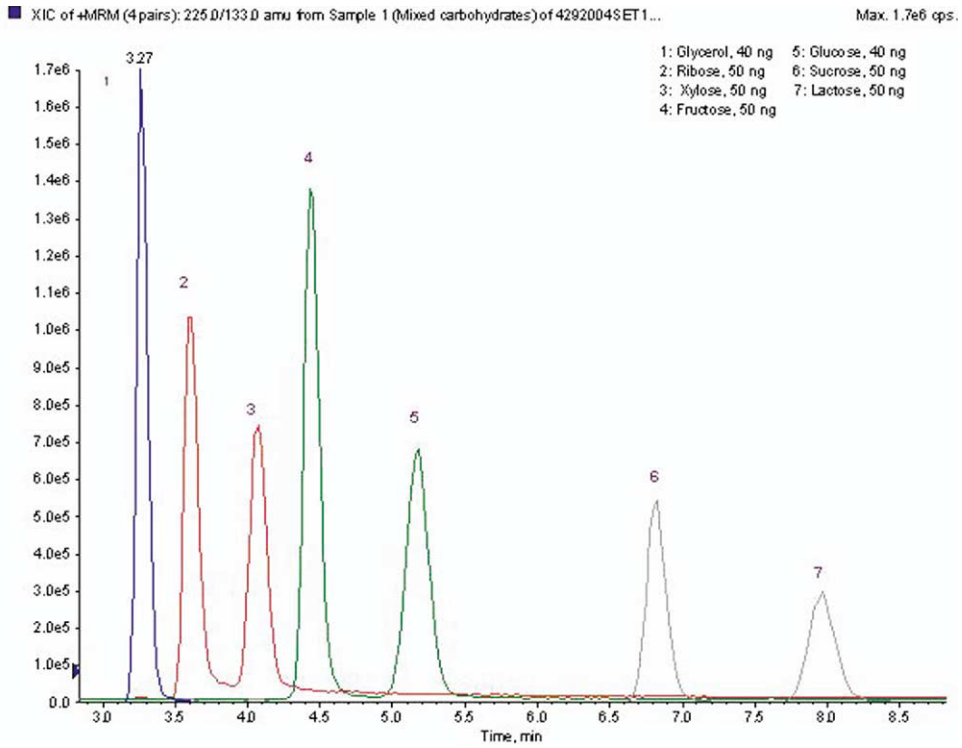

Figure 1. LC MS/MS spectrum of a carbohydrate mixture

V. Deflector was preset to 220 V and CEM was operated at 2000 V. The system was operated under control of Analyst 1.4 software (MDS SCIEX, Concord, ON Canada).

Results and Discussion

LC/MS Method Development

A variety of carbohydrate molecules were detectable after Cs^+ attachment and electrospray ionization in positive mode (Figure 1).

The cationic adduct $[Carb + Cs]^+$ was successfully monitored by both Q1 SIM ([M + 133]) and MRM mode ([M + 133] → 133). We found that implementation of MRM mode does not significantly increase signal to noise (S/N) compared to SIM. The difference in S/N between MRM and SIM was only about 20% for pure samples and only 2- to 3-fold for dirty biological samples. These results suggest that analysis by Cs^+ attachment may be successfully implemented on more affordable single quadrupole mass spectrometers. Carbohydrate analysis from pure formulations might utilize a shorter column, which would increase sample throughput.

Methodologically, direct measurement of analyte is preferable. Detection of the uncharged carbohydrate M can only be performed by a neutral loss experiment, which is (by definition) an indirect measurement, resulting from the subtraction of 133 from [M + 133], and is performed in scan mode of both Q1 and Q3. In contrast, MRM is a direct measurement; by using selected ion monitoring fixed to the precursor and product ions in Q1 and Q3, instrument duty cycle/dwell time is maximized [32], resulting in superior sensitivity compared to scan mode.

Cesium is the preferred monoisotopic cation for attachment as its large mass coupled to the analytes of interest raises the *m/z* monitored in Q1 out of the range where background is high because of a variety of low molecular weight contaminating species.

As mentioned previously, Na^+ attachment may cause undesirable adduct formation such $[Carb + 2Na]^{2+}$ (11;25). We detected only insignificant amounts of $[2Carb + Cs]^+$ or $[Carb + 2Cs]^{2+}$ for glycerol, sorbitol, or glucose adducts. Absence of $[Carb + 2Cs]^{2+}$ might be explained by the increase in Coulomb repulsion due to the large size of Cs^+. The formation of the $[2Carb + Cs]^+$ complex was maximal at 10–15 μM Cs^+ and efficiently suppressed by increasing Cs^+ concentration (data not shown). In contrast, smaller cations such as Li^+ and Na^+ bind tightly to carbohydrates [25–27]. Due to their high coordination affinity they decompose to the respective cation coordinated carbohydrate fragments [26]. In contrast, large cations, such as Cs^+ and Rb^+, form lower affinity carbohydrate complexes [26, 27]. As a result, they preferentially undergo cation elimination without carbohydrate decomposition [26]. Thus, monitoring of Cs^+ loss in our method takes advantage of the efficient Cs^+ dissociation (from the cation carbohydrate complex) instead of the preferential carbohydrate fragmentation that occurs with smaller cations during CID. Despite the weak binding of Cs^+ to carbohydrates, our method reports much higher sensi-

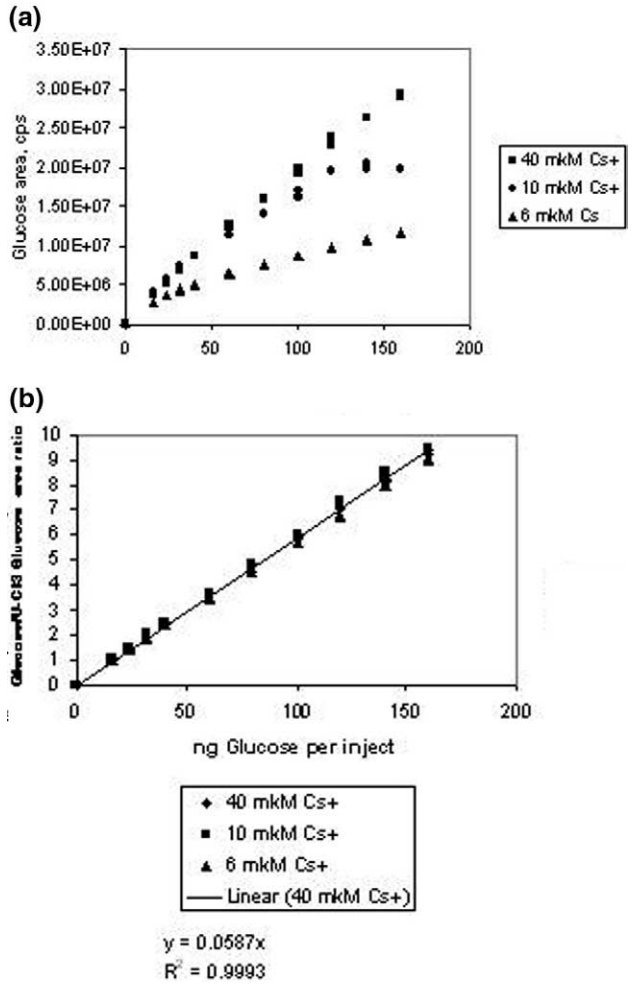

Figure 2. (**a**) Dependence of assay linearity on mobile phase [Cs^+] concentration. Decreasing of CsAcetate concentration in mobile phase decrease assay linearity range. (**b**) Use of internal standards for quantitation purposes eliminates concentration dependence on signal intensity.

tivity compared to analytical LC/MS methods based upon Na^+ or Li^+ attachment [11, 33, 34].

The cationized adduct $[M + Cs]^+$ is meta-stable and temperature-sensitive. Commonly used temperature ranges of 300–600 °C for turbo gas (gas 2) cause Cs^+ adduct decomposition. The best S/N was found at temperatures of 80–100 °C for the turbo gas. The SCIEX 4000 orthogonal source is equipped with an interface heater which is embedded in the ceramic of the interface plate. The consistent 100 °C warm interface surface contributes to the overall enhanced sensitivity of the API 4000 by enhancing desolvation without damage to many heat-sensitive molecules. In the absence of the heater (for a flow rate 0.2 ml/min), the optimal temperature of the turbo gas was found to be ~250 °C.

Carbohydrate Assay and Method Validation

The Cs^+ concentration in the mobile phase is an important factor for assay linearity if internal standards are not used. Since Cs^+ attachment to carbohydrate is a second-order reaction, the $[Carb + Cs]^+$ adduct formation curve has limited linearity and linearity range of the assay is dependent on both reagent and carbohydrate (analyte) concentration [17] (Figure 2a). The Cs^+ concentration was selected at 40 μM to maximize assay linearity range and as well minimize the formation of $[2Sorb + Cs]^+$ adduct (<0.025% at 60 ng sorbitol on column). Higher Cs^+ concentrations were not investigated due to potential fouling of the source. Use of internal standards for quantification purposes eliminated Cs^+ concentration dependence on signal intensity (Figure 2b) and strongly improved linearity and extended the quantitation range. At 40 μM Cs^+, the glucose assay was linear over a 10,000-fold concentration range (140 pg, 1400 ng) on column, $r^2 = 0.9993$.

Cs^+ attachment was also found to be a simple and sensitive technique for glycerol detection. Interestingly, direct LC/MS detection of glycerol by APCI without attachment was not successful. The Cs^+ attachment method by electrospray demonstrated excellent glycerol assay linearity to over 100 ng on column (r^2 = 0.9947, zero y-intercept) and good sensitivity (LOQ 69 pg on column) (Table 1). While this sensitivity is superior to other reported LC/MS assays for glycerol [11], detection methods by GC/MS, especially with chemical ionization, has been reported with sensitivities as low as <150 pg on column [9].

Cs^+ attachment was found useful not only for unmodified carbohydrates, but also for their derivatives. Interestingly, derivatization markedly increased sensitivity for glycerol triacetate compared to glycerol alone by ~15-fold (Table 1), with a limit of detection below 1 pg on column. Analysis of derivatized (acetylated) glycerol by Cs^+ attachment suggests that this detection method can serve as a unique tool to monitor the yield of carbohydrate acetylation, and by analogy, the efficiency of derivatization of carbohydrate species by other reagents, provided the underivatized compound can also be detected. Carbohydrate acetyl esters are classic GC analytes; underivatized carbohydrates are not volatile and therefore cannot be assayed by gas chromatography. In contrast, Cs^+ attachment LC/MS/MS assay allows monitoring of unreacted carbohydrate and its acetyl esters. Although the sensitivity of detection of other acetylated carbohydrates was not tested, our successful results with glycerol triacetate suggest that this approach has the potential to achieve

Table 1. Assay Detection and Quantification Limits

	LOD (pg on column)	LOQ (pg on column)
Glycerol	22	69
GlycerolTA	0.14	0.44
Sorbitol	2.5	8
Glucose	4	13

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to 3.3s/S and 10s/S. (s-is standard deviation of blank, S - is a slope of calibration curve).

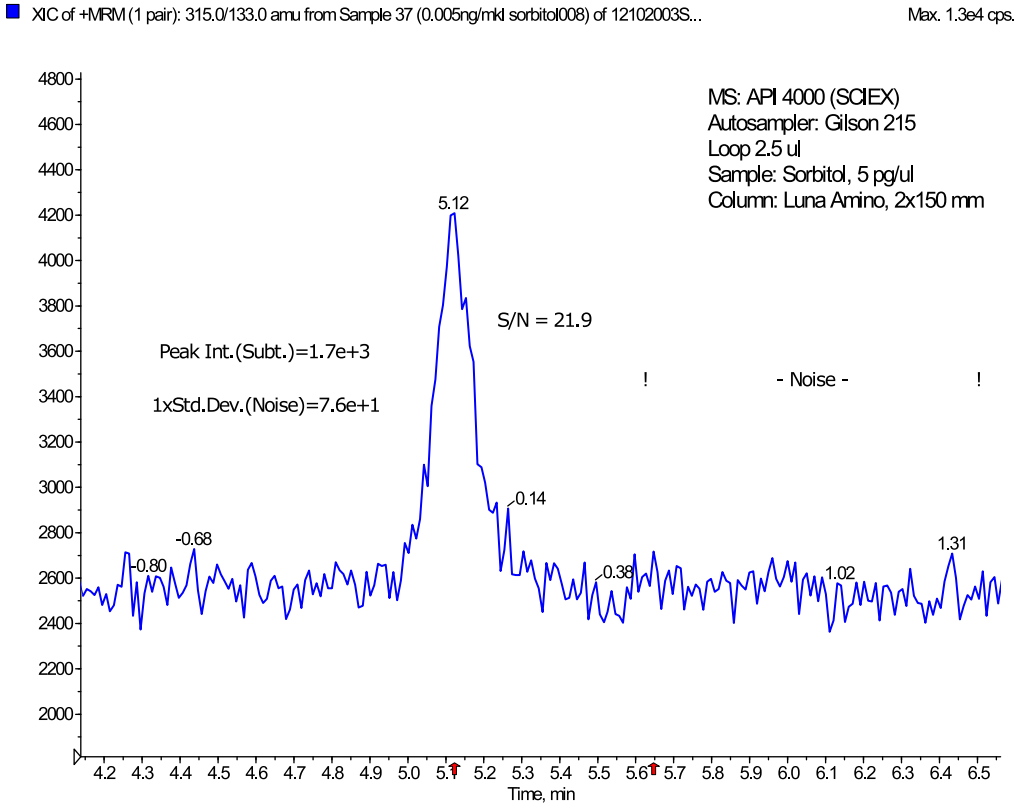

Figure 3. High sensitivity of Cs^+ attachment to sorbitol by LC/MS/MS.

ultra-high analyte sensitivities for extremely low abundance species and should be investigated further. It should be mentioned that glycerol triacetate elutes early near the void on amino columns. For routine analysis of glycerol triacetate alone from plasma samples, we found that a Synergy Fusion C18 column (Phenomenex), 2 × 75 mm, 4 μm is preferable for analysis to achieve adequate separation from the void peak. A binary gradient [water (A) and acetonitrile (B)] was developed, and the glycerol triacetate eluted in the range of 40–50% B with a retention time of 4.5 min.

Sorbitol was detected with particularly high sensitivity by the Cs^+ attachment method. 12.5 pg of sorbitol on column resulted in a signal to noise of ~22 (Figure 3). The limit of quantitation was 8 pg on column, which was almost double that of glucose and 8-fold greater than glycerol (Table 1). Similar to glucose, sorbitol was detectable with excellent linearity (R^2 = 0.9995; 11 points) over a wide concentration range (1–25 ng on column; data not shown). The concentraton of 1-^{13}C tracer to U-$^{13}C_6$ (internal standard, m+6) sorbitol ratio on the background of a 20 fold excess of unlabeled (m+0 sorbitol) was also highly linear in the presence of approximately 85 pmol of sorbitol (~16 ng) injected on column (Figure 4). The close correspondence between theoretical and observed molar ratio, as well as zero intercept, demonstrate that the assay is highly specific for the 1-^{13}C sorbitol tracer when potential contaminating species, e.g., from glucose, have been eliminated via chromatography.

Method Reproducibility

For reproducibility and accuracy measurements two QC standards using sorbitol were prepared at "low" and "high" concentrations. The low QC standard was prepared in a ratio of 1-^{13}C/U-^{13}C sorbitol of ~1:3. The high QC standard was prepared in a ratio of 1C^{13}/U-C^{13} of ~3:1. This represented 2.7 to 24 ng of 1-^{13}C sorbitol on column. These QC standards were measured in triplicate 8 separate times over 15 days. The data is summarized in Table 2 and was found to correlate highly with the reproducibility of an additional set of 40 injections of one QC (medium; ~1:1) standard. As can be seen in Table 2, intra- and inter-day % RSDs, as well as inter-day absolute errors, were at or

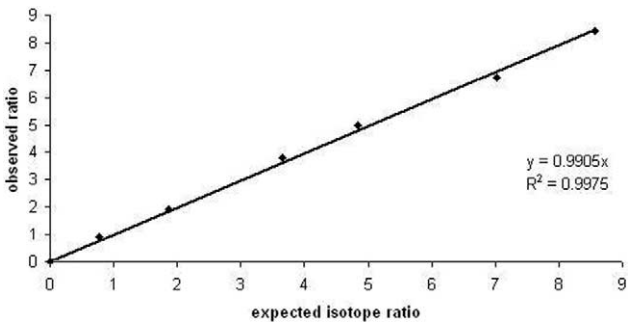

Figure 4. Cs^+ attachment isotope dilution assay of sorbitol; 1-^{13}C sorbitol detection (316/133) versus internal standard U-^{13}C sorbitol (321/133).

Table 2. Assay precision and reproducibility

	Daily mean isotope ratio	Intraday		Interday		
		STD	%RSD	STD	%RSD	ABS ERR%
Low QC	0.309	0.004	1.25%	.006	1.99%	1.52%
High QC	2.781	0.027	0.98%	.040	1.43%	1.20%
Interday QC n = 40	0.906			.018	1.97%	1.508%

below 2%. This degree of reproducibility was achieved well within the range of sorbitol concentrations we routinely measure from clinical plasma samples; tracer 1-^{13}C sorbitol concentrations were typically ~4 μmol/L which represented ~3 ng on column.

Method Accuracy

Accuracy was calculated for sorbitol both as the signal area absolute error relative to known concentration standards, and relative to an isotope ratio with an internal standard added to each sample. For a series of standards ranging from 1.6 to 24 ng on column, absolute mean error was 3.8% (range 1–10%). For isotope dilution assay with 40 pmol (~7 ng) on column the mean absolute error was 3% (range 0–4.4%).

Conclusions

We demonstrate a novel, robust, rapid, and sensitive method for quantitative analysis of common biological carbohydrates by LC MS/MS. The method is linear from the picogram to microgram range. The assay is extremely stable and exhibits measurement errors that are on par with the best methods described for GC/MS detection of isotope ratios in assays of glucose, sorbitol, and glycerol. The method does not require derivatization, minimizing sample handling, demonstrates rapid cycle times permitting high throughput analysis, and is applicable to carbohydrates in biological fluids. Ultra-high sensitivities were demonstrated by combining the Cs^+ attachment technique to derivatized highly polar carbohydrates such as glycerol. This method is an important advance in high sensitivity carbohydrate analysis and should become an attractive alternative to current methods of carbohydrate analysis by GC/MS or by ion chromatography.

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