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Quantitative determination of endogenous sorbitol and fructose in human erythrocytes by atmospheric-pressure chemical ionization LC tandem mass spectrometry

H.R. Liang [a,*], T. Takagaki [b], R.L. Foltz [a], P. Bennett [a]

[a] *Tandem Labs, A Division of NWT Inc., Salt Lake City, UT, USA*
[b] *Pharmacokinetics Research Laboratories, Dainippon Pharmaceutical Co. Ltd., 33-94, Enoki-cho, Suita, Osaka 564-0053, Japan*

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Abstract

Evaluation of different extraction methods for quantification of endogenous sorbitol and fructose in human red blood cells (RBCs) and matrix effects in ESI and APCI showed that protein-precipitation followed by mixed-mode solid-phase extraction was more effective extraction method and APCI more effective ionization method. Then the LC/APCI-MS/MS method was fully validated and successfully applied to analysis of clinical RBC samples. The concentrations of endogenous sorbitol and fructose were determined using calibration curves employing sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$ as surrogate analytes. The method has provided excellent intra- and inter-assay precision and accuracy with a linear range of 50.0–10,000 ng/mL (correlation coefficient >0.999) for sorbitol-$^{13}C_6$ and 250–50000 ng/mL (correlation coefficient >0.999) for fructose-$^{13}C_6$ in human RBCs.

Keywords: Human red blood cells; LC/APCI-MS/MS; Endogenous sorbitol and fructose; Mixed-mode solid-phase extraction; Matrix effects

1. Introduction

It is important to monitor the levels of monosaccharides in various human matrices in certain diseases such as uremia and diabetes. Analysis of monosaccharides by liquid chromatography is complicated by the fact that these compounds do not possess a UV chromophore; consequently they are usually analyzed by derivatization before detection with UV [1,2]. Other detectors are also used to analyze neutral carbohydrates, such as evaporative light-scattering detector [3], refractive-index, [4–6] and pulsed-amperometric detectors [7–11] and fluorometric detector [12]. Gas chromatography/mass spectrometry (GC/MS) has been used for analysis of monosaccharides but the methods require derivatization and laborious sample preparation [13–17].

Atmospheric-pressure ionization (API) with single or tandem MS has been applied to characterize and identify some carbohydrates [11,18–20]. Most recently, monosaccharides have been characterized by atmospheric-pressure photoionization [21]. Thermospray LC/MS has been applied for the determination of some carbohydrates [22,23]. Anion-exchange LC ion-spray or electrospray ionization (ESI) MS has also been reported [24,25], but in these experiments the high salt content from the mobile phase had to be eliminated prior to entry into the ion source. Atmospheric-pressure chemical ionization (APCI) LC/MS via negative-mode with post-column addition of $CHCl_3$ is another way to analyze monosaccharides [26,27], but the LC/MS methods generally have not provided the sensitivity needed for proposed clinical studies. Attachment of anions to some neutral monosaccharides has been shown to enhance sensitivity in

* Corresponding author at: Developmental Bioanalysis, Charles River Discovery and Development Services, 57 Union Street, Worcester, MA 01608, USA. Tel.: +1 508 890 0159; fax: +1 508 753 1834.
E-mail address: hairui.liang@us.crl.com (H.R. Liang).

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API MS [28–31]. However, there have been no publications on the quantitative determination of endogenous sorbitol and fructose in human red blood cells by LC coupled to API-tandem MS (LC/MS/MS). The goal of this study was to develop a sensitive and selective LC/MS/MS method for quantitative determination of endogenous sorbitol and fructose in the presence of their isomers in human red blood cells.

2. Experimental

2.1. Reference materials, biological matrix and chemicals

Fructose-$^{13}C_6$, sorbitol-$^{13}C_6$, fructose-$^{13}C_3$ and sorbitol-D_2 were purchased from Omicron Biochemicals Inc. (North Ironwood Drive South Bend, IN). Fructose, sorbitol, glucose, galactose, mannose, sorbose, mannitol, and dulcitol were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fig. 1 shows the chemical structures of labeled sorbitol and fructose. Blank human red blood cells were purchased from Biochemed Pharmacologicals (Winchester, VA). All chemicals were of analytical-reagent grade: methanol, CH_2Cl_2, CH_3Cl, CCl_4, 1-chlorobutane, $CaCl_2$ and acetonitrile from EM Science (Gibbstown, NJ); and formic acid from VWR Scientific Products (West Chester, PA).

2.2. Instrumentation

This study employed a Beckman GS-6R Centrifuge (Beckman Instruments, Fullerton, CA) and a TurboVap (Zymark Corp., Hopkinton, MA). Chromatography was performed on a Shimadzu SCL-10A controller with a CL-10AD pump and a CTO-10A column oven (Shimadzu Scientific Instruments, Inc., Columbia, MD). The autosampler was a PE Series 200 (Perkin Elmer, Hopkinton, MA). API 4000 tandem mass spectrometers were from MDS Sciex (Concord, Ontario).

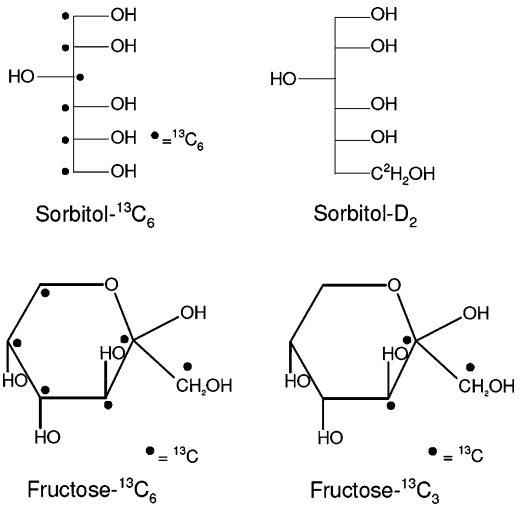

Fig. 1. Chemical structures of labeled sorbitol and fructose.

2.3. Preparation of calibrators, internal standards and quality control samples

Two sets of stock solutions of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ were prepared from independent weighings; one for calibration standards and one for quality control samples. All solutions were prepared in water and stored at 1–8 °C.

The internal standards, sorbitol-D_2 and fructose-$^{13}C_3$, were weighed and dissolved in the required volume of water to give concentrations of 0.1041 and 0.9937 mg/mL, respectively, and diluted to 5000 ng/mL with water for use the internal standards stock solution.

A sufficient amount of blank human red blood cells was thawed in an ice bath. Low QC (150/750 ng/mL of sorbitol-$^{13}C_6$/fructose-$^{13}C_6$), medium QC (6000/30,000 ng/mL of sorbitol-$^{13}C_6$/fructose-$^{13}C_6$), high QC (8000/40,000 ng/mL of sorbitol-$^{13}C_6$/fructose-$^{13}C_6$) and dilution QC (60,000/300,000 ng/mL of sorbitol-$^{13}C_6$/fructose-$^{13}C_6$) were prepared in pooled human red blood cells. All the above QCs were vortex-mixed thoroughly and stored in a −70 °C freezer. Calibration standards were prepared fresh daily in blank human red blood cells. Calibration stock solutions were spiked directly into aliquots of cold blank human red blood cells in an ice bath and vortex mixed well. The calibration standards were allowed to equilibrate for approximately 10 min in an ice bath before extraction. The calibration curve ranges were 50.0–10,000 ng/mL for sorbitol-$^{13}C_6$ and 250–50,000 ng/mL for fructose-$^{13}C_6$.

2.4. Extraction by protein precipitation

Blank human red blood cells were thawed in an ice bath. Standard curves were freshly prepared by adding standard spiking solutions into blank-red blood cells and vortexed well. Two hundred microlitres of sorbitol-D_2 and fructose-$^{13}C_3$ (5000 ng/mL) were added to each sample as internal standards (except Blanks). Four hundred microlitres of cold water was added to each Blank sample and 200 μL was added to each QC0 sample. Samples were vortex mixed for approximately 1 min using the maximum speed (10) on the multi-tube vortexer. Four millilitres of acetonitrile was added and the tubes were capped and vortex mixed. Samples were sonicated for approximately 5 min and centrifuged for 10 min at 3750 rpm. The supernatant for each sample was transferred to a clean tube and evaporated to dryness at 60 °C on the TurboVap under nitrogen for 60–90 min. One millilitre of methanol:water (50:50, v/v) was added to each tube and the samples were vortexed for approximately 1 min before sonication for approximately 5 min.

2.5. Solid-phase extraction procedures

First, polymer anion exchange (PAX) and cation exchange (PCX) columns from Cerex, Hologent Technologies Inc. (Baldwin Park, CA) were successively attempted. Because the above procedure was time-consuming, ISOLUTE multimode mixed-mode solid-phase extraction columns (MM)-SPE from Argonaut Technologies, Inc. (Foster City, CA) were finally used. The MM-SPE columns were conditioned with 1 mL of methanol, then equilibrated with 1 mL of water. The above protein-precipitated supernatants were loaded onto the columns. The breakthrough was collected and combined with the eluent of 1.00 mL of methanol:water (50:50, v/v). The extracts were dried using a TurboVap at a temperature of 80 °C for approximately 90 min. Twenty-five microlitres of water was added to each extract and vortex mixed, followed by addition of 200 μL of acetonitrile:water (90:10) to each sample. Samples were mixed on a multi-tube vortexer for approximately one minute and sonicated for approximately ten minutes, then transferred to autosampler vials with polypropylene inserts and centrifuged at 3750 rpm for approximately five minutes.

2.6. LC conditions

The LC separations were performed on a Luna 5 μm NH_2 (100A column 150 mm × 4.6 mm) or a CapCell Pak 5 μm NH_2 column (UG-80A column 150 mm × 4.6 mm) from Phenomenex (Torrance, CA) with different compositions of mobile phase at different flow rates and oven temperatures using different gradient LC systems. The final LC conditions used for the analysis of the clinical samples were as follows: Luna 5 μm NH_2 100A column (150 mm × 4.6 mm); mobile phase (A): acetonitrile with 0.1% dichloromethane, mobile phase (B): methanol:water (50:50); oven temperature: 40°; flow rate: 800 μL/minute; gradient: 15% of B at zero time and 19% of B at 5 min. A switching valve was used to collect data for only 3 min and to allow makeup solution (50% MeOH with 0.5% formic acid) to clean the ion source for 5 min after each analysis.

2.7. MS conditions

The API 4000 was operated in the negative-ionization mode formed by ESI or APCI, and operated in multiple-reaction monitoring (MRM) mode under optimized conditions for detection of fructose, fructose-$^{13}C_3$, fructose-$^{13}C_6$, sorbitol, sorbitol-D_2, sorbitol-$^{13}C_6$ and their isomers including glucose, galactose, mannose, sorbose, mannitol and dulcitol.

2.8. Ionization suppression or matrix effects

The signal suppression (%) of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) due to the matrix effects was measured by comparing the peak areas of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) from neat solutions to those from extracted human red blood cells blanks post-spiked with sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$). Protein precipitation extraction with acetonitrile was used to extract the RBC blank samples.

2.9. Method validation

The quantitation range was 50.0–10,000 ng/mL for sorbitol-$^{13}C_6$ and 250–50,000 ng/mL for fructose-$^{13}C_6$ in human red blood cells. Two calibration curves over this range were prepared on three separate days. The curves were fit by a least-square 1/*x*-weighted linear regression method. Each analytical accuracy and precision run included calibration standards in duplicate with eight different concentrations; low, medium and high quality control samples (QCs) in replicates of six; two red blood cells blanks (no internal standard) and QC0s (red blood cells blank with internal standard). Dilution QCs were analyzed in replicates of six. The freeze-thaw stability of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ in human red blood cells was determined by subjecting low and high QC samples to three freeze-thaw cycles before processing. The room temperature (RT) matrix stability of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ in human red blood cells was determined by storing low and high QC samples for 6 h under RT and normal light conditions. The autosampler stability of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ was determined for an analytical run length of approximately 2 days at RT. A set of low and high QC samples was stored at RT for approximately 2 days after initiation of the run. These test QC samples were reinjected and quantitated against the original calibration curves and compared against theoretical. The processed extract stability of sorbitol and fructose was determined for low, medium and high QC extracts maintained at RT and normal light conditions for approximately 3 days prior to analysis. The data were acquired by re-injecting a previously analyzed set of calibration standards and QC samples. The extraction efficiencies of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ were determined by comparing peak areas of the analytes extracted from red blood cells with those of post-extracted blanks fortified with the analytes. The experiments were performed at low, medium and high concentrations to determine that there was no concentration bias.

2.10. Data management and quantitation

To quantify sorbitol and fructose using the stable isotope-labeled surrogate calibrators, it was necessary to edit the final, electronic-results file prior to importing the results into the Watson® DM laboratory information management system (LIMS). Edits were performed for each run in the following manner: the analyte name identifiers in the files were changed for all unknown samples from sorbitol to sorbitol-$^{13}C_6$ and from fructose to fructose-$^{13}C_6$. These edits were required prior to importing the results to Watson® DMLIMS in order

to quantify the endogenous levels of sorbitol and fructose using the corresponding stable-isotope labeled calibrators.

The chromatographic peaks of unlabeled and stable isotope-labeled of sorbitol and fructose were integrated using Analyst software (version 1.2) with a smooth factor of one to three. Quantitation was based upon linear regression analysis of calibration curves (weighted $1/x$) using the calibrator to internal standard area ratio vs. target concentration utilizing Watson® DMLIMS software (version 6.1.1.04).

3. Results and discussion

3.1. Formation and fragmentation of Cl-adduct anions of fructose and sorbitol in ESI- and APCI-MS/MS

In negative-ion ESI and APCI MS, fructose and sorbitol showed no significant signals due to deprotonated molecular ions $[M-H]^-$. Therefore we investigated mechanisms for formation of Cl-adduct anions in ESI and APCI. In the initial method development, we attempted to add 0.5 mL of 1 M $CaCl_2$ into 95.5 mL of reconstitution to form Cl-adduct ions. However, it was not a successful approach probably because the addition of inorganic salt suppressed the signals. Alternatively, we added different amount of ammonium chloride in a mobile phase. Hypothetically, advantage could be the constant and controlled flow of chloride ions and, possibly, an improved signal and limit of detection. In addition, this compound is, in contrast to the halides of alkali metals, highly volatile (it is already strongly subliming at room temperature) and should therefore, be compatible with LC/MS. However, the results showed that the addition of different amount of ammonium chloride in mobile phase did not assist to form Cl-adduct ions effectively. Then we attempted post-column addition of $CHCl_3$/MeCN (50/50) at a flow rate of 200 μL/mL as previously reported [26]. However, the signals of sorbitol and fructose were continually suppressed due to the elevated background, so that 100 ng/mL of sorbitol and fructose in neat solutions were undetectable when using ESI or APCI. Four chlorinated solvents (CH_2Cl_2, CCl_4, 1-chlorobutane and $CHCl_3$ in MeCN or MeOH) were investigated systematically for their ability to form Cl-adduct precursor ions and deprotonated product ions. Of those, CH_2Cl_2 in methanol produced the best results. The effect of percentage (0.1, 0.5, 1, 5, 10 or 20%) of CH_2Cl_2 in methanol was investigated further. Increasing the percentage of CH_2Cl_2 increased the baseline background, but not the intensity of the desired ions. The best sensitivity of fructose-$^{13}C_6$ and sorbitol-$^{13}C_6$ was achieved by adding 0.1% CH_2Cl_2 to the mobile phase when using ESI or APCI.

3.2. Improvement of signal-to-noise ratios and reproducibility

The apparent major causes of the high background were: (1) addition of Cl-containing compounds; (2) stickiness of polyols in the ion source; (3) tuning parameters, including source temperature, electrode-needle positions, curtain gas, and nebulizer current. Moreover, the high background baseline was cumulative. If the background baseline could be lowered, the sensitivity would be much improved. Thus, effective methods were developed to improve the signal-to-noise ratios and reproducibility. The lowest amount of CH_2Cl_2 added to the mobile phase (0.1%) gave the lowest background baseline. Other parameters that contributed to improved signal-to-noise ratios were low source temperature and nebulizer current or ion-spray voltage, high curtain-gas flow, and favorable needle positions. These changes also eliminated arcing, which happens more often in negative mode than in positive mode. A switching valve was used to collect data for only 3 min and to allow makeup solution (50% MeOH with 0.5% formic acid) to clean the ion source for 5 min after each analysis. As a result of these changes the reproducibility of peak areas of sorbitol and fructose improved dramatically and the background baseline was lowered substantially.

In a neat solution, limits of detection (defined as five times signal to noise ratio) was 5 and 10 pg for sorbitol and fructose, respectively. The sponsor of this project only requested 50 and 250 ng/mL of lower limit of quantification for sorbitol and fructose, respectively, in human red blood cells.

Table 1
Evaluation of matrix effects of fructose-$^{13}C_6$ and fructose-$^{13}C_3$ (sorbitol-$^{13}C_6$ and sorbitol-D_2) in ESI

($n=10$)	Fructose-$^{13}C_6$	Fructose-$^{13}C_3$	Sorbitol-$^{13}C_6$	Sorbitol-D_2
Peak areas of labeled fructose and sorbitol spiked in recon				
Ave	66736000	21833000	22485000	27881500
S.D.	1463711.04	555785.93	274357.4	184554.9
CV%	2.19	2.54	1.22	0.661
Peak areas of labeled fructose and sorbitol spiked in post-extracted blank human RBCs				
Ave.	13168500	2979050	4435550	4933700
S.D.	1505430	362250	414293	391595
CV%	11.4	12.1	9.34	7.93
Suppression%	−80.27	−86.36	−80.27	−82.30

The signal suppression (%) of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) due to the matrix effects was measured by comparing the peak areas of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) from neat solutions to those from extracted human red blood cells (RBCs) blanks post-spiked with sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$). Protein precipitation extraction with acetonitrile was used to extract the RBC blank samples. Concentration of fructose-$^{13}C_6$ ($^{13}C_3$): 5000 ng/mL; and sorbitol-$^{13}C_6$ (D_2): 2500 ng/mL; injection volume: 10 μL.

Table 2
Evaluation of matrix effects of fructose-$^{13}C_6$ and fructose-$^{13}C_3$ (sorbitol-$^{13}C_6$ and sorbitol-D_2) in APCI

(n = 10)	Fructose-$^{13}C_6$	Fructose-$^{13}C_3$	Sorbitol-$^{13}C_6$	Sorbitol-D_2
Peak areas of labeled fructose and sorbitol spiked in recon				
Ave	8244767	6434267	1791200	239043
S.D.	206631	167875	83045	10574
CV%	2.51	2.61	4.63	4.42
Peak areas of labeled fructose and sorbitol spiked in post-extracted blank human RBCs				
Ave	3358800	2859367	1028148	124541
S.D.	620879	509048	115313	23578
CV%	18.49	17.80	11.2	18.9
Suppression%	−59.26	−55.56	−57.3	−52.1

The signal suppression (%) of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) due to the matrix effects was measured by comparing the peak areas of sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$) from neat solutions to those from extracted human red blood cells (RBCs) blanks post-spiked with sorbitol-$^{13}C_6$ and D_2 (fructose-$^{13}C_6$ and $^{13}C_3$). Protein precipitation extraction with acetonitrile was used to extract the RBC blank samples. Concentration of fructose-$^{13}C_6$ ($^{13}C_3$): 5000 ng/mL; and sorbitol-$^{13}C_6$ (D_2): 2500 ng/mL; injection volume: 10 μL.

3.3. Separation of sorbitol and fructose along with their isomers

Some forms of sorbitol and fructose have exactly the same MRM transitions (e.g., Fructose-D_2/Sorbitol-D_0: 217 → 181; Fructose-$^{13}C_6$/Sorbitol-D_4: 221 → 185). Thus it is important to separate sorbitol from fructose. It is well known that any coeluting matrix components with a target compound will suppress the ionization response of the target when using ESI [32]. In human red blood cells, there are many isomers of sorbitol and fructose. Their major isomers include glucose, galactose, mannose, sorbose, mannitol, and dulcitol. The co-eluting isomers potentially influence the accurate measurement of the contents of sorbitol and fructose in human red blood cells. Therefore, in order to quantify accurately the contents of sorbitol and fructose in human red blood cells, it is important to achieve the baseline separation not only between sorbitol and fructose but also between sorbitol (fructose) and its isomers.

The compositions of mobile-phase, percentages of organic modifiers (MeCN, MeOH and water), oven temperature, and different gradients were investigated to separate sorbitol and fructose along with their isomers. In addition, different vendor's NH_2 columns (Luna versus CapCell Pak) were

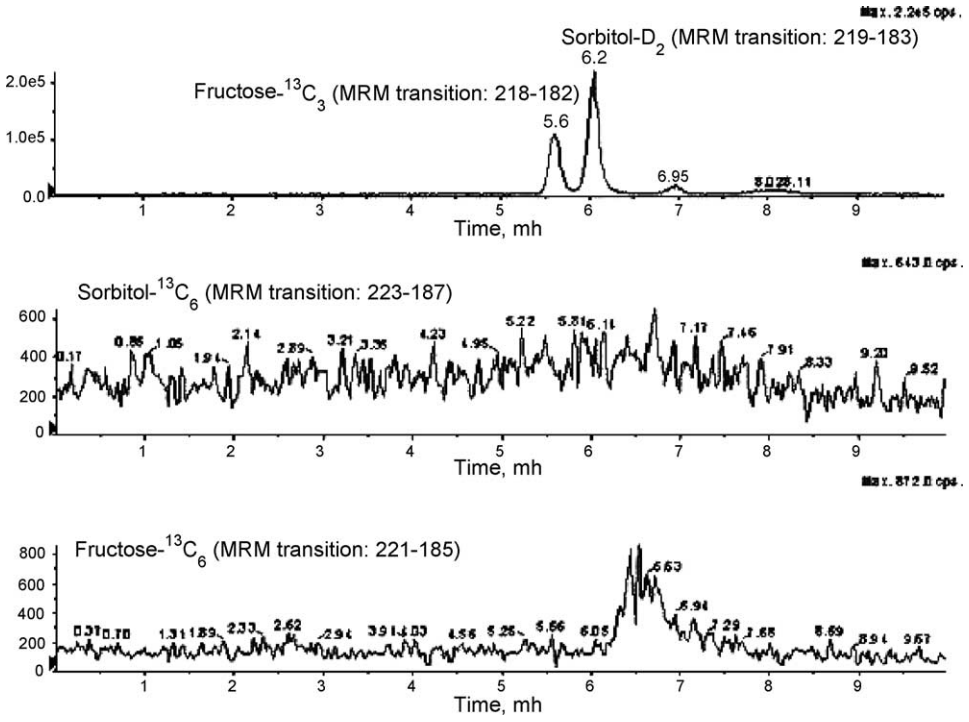

Fig. 2. Extracted ion chromatograms of human red blood cell blank with internal standards (sorbitol-D_2 and fructose-$^{13}C_3$) for sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ by LC/APCI-MS/MS. Experimental conditions: mobile Phase (A): acetonitrile with 0.1% dichloromethane, (B): methanol:water (50:50); flow rate: 800 μL/min; LC column: Luna 5 μm NH_2 100A column (150 mm × 4.6 mm); injection volume: 20 μL. For more detailed conditions, see Table 3. Extraction: 200 μL of nerve tissue blank spiked with internal standards was extracted by PPE and followed by SPE.

compared. The baseline separation was achieved between sorbitol and fructose, between sorbitol (fructose) and its isomers on a Luna 5 μm NH_2 100A column (150 mm × 4.6 mm) using a mobile phase containing (A): acetonitrile with 0.1% dichloromethane, and (B): methanol:water (50:50) at an oven temperature of 40° at a flow rate of 800 μL/min using a LC gradient of 15% of B at zero time and 19% of B at 5 min. A switching valve was used to collect data for only 3 min and to allow makeup solution (50% MeOH with 0.5% formic acid) to clean the ion source for 5 min after each analysis.

3.4. Evaluation of extraction methods and matrix effects in ESI and APCI

Protein precipitation extraction (PPE) method was used to extract the human red blood cell samples and the matrix effects of the extracted samples were evaluated in ESI and APCI. As shown in Tables 1 and 2, approximately 80% of the responses of fructose-$^{13}C_6$ ($^{13}C_3$) and sorbitiol-$^{13}C_6$ (D_2) spiked in post-extracted human red blood cell blanks were suppressed in ESI whereas approximately 50% of the responses were suppressed in APCI. Thus, APCI method was further investigated.

In order to reduce the ionization suppression, solid-phase extraction (SPE) method was used to purify the PPE extracted samples that went through polymer anion exchange (PAX) to retain anionic interferences and then through cation exchange (PCX) columns successively to retain canionic interferences. Because the above procedure was time-consuming and less efficient, multi-mode mixed mode (MM)-SPE column was used instead of PAX and PCX. MM-SPE columns contain non-polar (C18), strong cation exchange ($–SO_3^-$) and strong anion exchange ($–NR^{3+}$) functional groups with three different primary mechanisms. The monosaccharides were eluted with 50% MeOH whereas non-polar, cationic and anionic components were all retained on MM-SPE columns. The PPE followed by MM-SPE procedures reduced ionization suppression of fructose-$^{13}C_6$ ($^{13}C_3$) and sorbitiol-$^{13}C_6$ (D_2) in human red blood cells from approximately 50–15%.

Table 3
Calibration curve parameters for sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$

$N = 3$	Slope	Intercept	R-Squared
Sorbitiol-$^{13}C_6$			
Mean	0.003015	−0.041105	0.9986
S.D.	0.000097	0.010382	0.0002
%CV	3.2	−25.3	0.0
Fructose-$^{13}C_6$			
Mean	0.000186	−0.008574	0.9990
S.D.	0.0000006	0.002722	0.0008
%CV	3.2	−31.7	0.1

Linear weighted 1/x. The two curves were prepared and run on 3 different days.

3.5. Quantitative determination of endogenous sorbitol and fructose in human red blood cells by LC/APCI-MS/MS

Sorbitol and fructose are endogenous compounds in humans and thus we are unable to use sorbitol and fructose as calibration standards and QCs to quantify these compounds in the study samples. Our experimental results showed that the peak area ratio of sorbitol-D_0 and sorbitol-$^{13}C_6$ to the internal standard sorbitol-D_2 was equal; likewise the peak area ratio of fructose-D_0 and fructose-$^{13}C_6$ to internal standard frucose-$^{13}C_3$. Therefore, it is reliable to quantify the concentrations of sorbitol-D_0 and fructose-D_0 using the calibration curves of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$. We determined the concentrations of endogenous sorbitol and fructose using sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ as calibration standards. Sorbitol-D_2 and fructose-$^{13}C_3$ were used as the internal standards of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$, respectively.

Six different lots of extracted blank human red blood cells were analyzed under optimized LC conditions as blanks (no internal standard) and as QC0s (red blood cells blank with internal standards, Fig. 2). The results showed that there were no measurable peak areas detected in the blanks and QC0s for the analyte or internal standard that could affect quantitation, indicating the method was specific and selective. The linearity, intra and inter-assay precision

Table 4
Back-calculated concentrations of calibrators for sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$

	Nominal conc. (ng/mL)							
	50.0	100	500	1000	2500	5000	8000	10,000
Sorbitiol-$^{13}C_6$								
Mean ($n = 6$)	53.8	97.3	484	999	2470	4910	8170	9980
%CV	5.5	6.5	4.2	4.8	4.8	3.6	1.8	4.1
%Bias	7.6	−2.7	−3.2	−0.1	−1.2	−1.8	2.1	−0.2
	Nominal conc. (ng/mL)							
	250	500	2500	5000	125,00	25,000	40,000	50,000
Fructose-$^{13}C_6$								
Mean ($n = 6$)	257	499	2490	4910	12,300	25,800	39,200	50,300
%CV	4.5	6.6	2.6	3.1	2.6	2.8	2.4	2.6
%Bias	2.8	−0.2	−0.4	−1.8	−1.6	3.2	−2.0	0.6

Table 5
Intra-assay precision and accuracy for sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$ quality control (QC)

Number of runs: 3, nominal conc. (ng/mL)	Low QC 150	Medium QC 6000	High QC 8000	Dilution QC 60000, DF = 10 (n = 1)	LLOQ 50.0 (n = 1)
Sorbitiol-$^{13}C_6$					
Mean observed conc.	152	6050	7950	64100	44.3
%CV	5.7	6.4	4.9	3.3	12.5
%Bias	1.3	0.8	−0.6	6.8	−11.4

Number of runs: 3, nominal conc. (ng/mL)	Low QC 750	Medium QC 30000	High QC 40000	Dilution QC 300000, DF = 10	LLOQ 250
Fructose-$^{13}C_6$					
Mean observed conc.	767	30300	40300	324000	231
%CV	11.3	4.9	3.3	1.4	16.9
%Bias	2.3	1.0	0.8	8.0	−7.6

Sample numbers of each run: 6.

Table 6
Inter-assay precision and accuracy for sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$ quality control (QC)

Number of runs: 3 nominal conc. (ng/mL)	Low QC 150	Medium QC 6000	High QC 8000	Dilution QC 480, DF = 10
Sorbitiol-$^{13}C_6$				
Mean observed conc.	157	6010	7800	457
%CV	3.5	1.0	1.9	5.4
%Bias	4.7	0.2	−2.5	4.8

Nominal conc. (ng/mL)	Low QC 750	Medium QC 30000	High QC 40000	Dilution QC 2400, DF = 10
Fructose-$^{13}C_6$				
Mean observed conc.	776	30500	39300	2330
%CV	1.2	3.6	6.0	1.6
%Bias	3.5	1.7	−1.7	−2.9

Sample numbers of each run: 6.

and accuracy were fully evaluated. The method exhibited linearity over the range 50.0–10,000 ng/mL for sorbitol-$^{13}C_6$ for sorbitol-$^{13}C_6$ and 250–50,000 ng/mL for fructose-$^{13}C_6$ (Tables 3 and 4). The accuracy and precision of the LC/MS/MS method for sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ in human red blood cells were determined by analyzing quality control samples in replicates of six on three separate days. Dilution QCs were analyzed in replicates of six on a single day. The method showed excellent reproducibility (overall CV < 6%) and accuracy (overall Bias < 5%) with

Table 7
Freeze-thaw, benchtop storage, autosampler and processed extract run stability for sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$ QC

Stability (n = 3) nominal Conc. (ng/mL)	Sorbitiol-$^{13}C_6$		Fructose-$^{13}C_6$	
	Low QC 150	High QC 8000	Low QC 750	High QC 40000
Three cycles F/T stability				
Mean	148	7130	693	35,400
%CV	6.8	14.2	10.0	14.2
%Bias	−1.3	−10.9	−7.6	−11.5
RT Benchtop storage stability				
Mean	144	7200	793	38,600
%CV	3.2	3.7	1.1	0.7
%Bias	−4.0	−10	5.7	−3.5
Autosampler stability				
Mean	142	7500	751	38,200
%CV	4.7	1.3	1.3	2.9
%Theoretical	94.7	93.8	100.1	95.5
%Bias	−5.3	−6.3	0.1	−4.5
RT processed extract stability				
Mean	158	7880	775	40,000
%CV	4.5	8.8	5.1	2.1
%Bias	5.3	−3.8	3.3	0.0

Table 8
Sorbitiol-$^{13}C_6$ and fructose-$^{13}C_6$ extraction efficiency

$N = 3$	Sorbitiol-$^{13}C_6$			Fructose-$^{13}C_6$		
	Low QC	Med QC	High QC	Low QC	Med QC	High QC
%Mean recovery	69.3	60.3	62.3	57.5	64.1	61.8
Overall mean CV (%)		7.39			5.48	
Overall mean recovery (%)		64.0			61.1	

Table 9
Quantitative determination of fructose and sorbitol in human RBC

	Conc. (ng/ml)	Ave.	S.D.	%CV
Fructose $N = 3$				
QC0-1	676	638		
QC0-2	628		34.5	
QC0-3	609			5.41
Sorbitol $N = 3$				
QC0-1	49.7	67.7		
QC0-2	85.5		17.9	
QC0-3	68.1			26.4

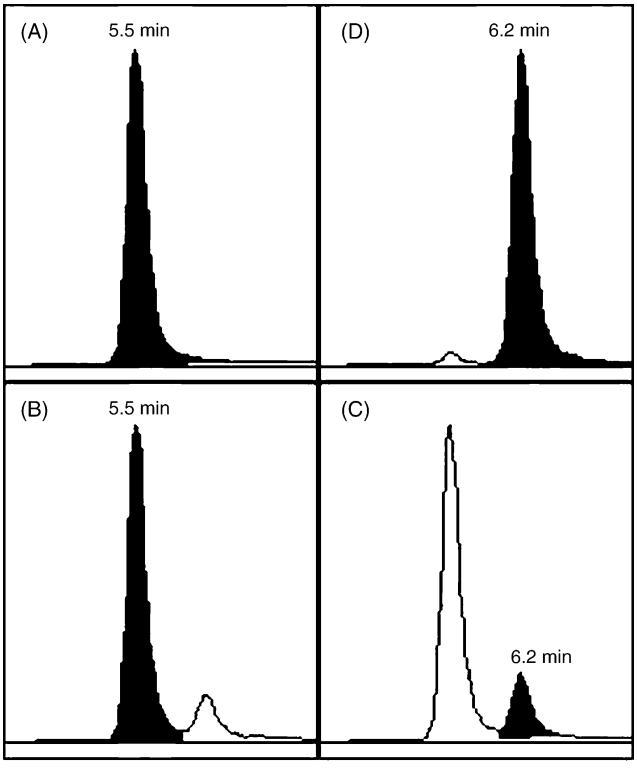

Fig. 3. Extracted ion chromatograms of a typical clinical sample from human red blood cells by LC/APCI-MS/MS. Experimental conditions: mobile phase (A): acetonitrile with 0.1% dichloromethane, (B): methanol:water (50:50); flow rate: 800 μL/min; LC column: Luna 5 μm NH_2 100A column (150 mm × 4.6 mm); injection volume: 20 μL. Extraction: 200 μL of clinical sample from human red blood cells spiked with internal standards was extracted by PPE and followed by SPE. (A): fructose (MRM transition 215 → 179); (B): fructose-$^{13}C_3$ (MRM transition 218 → 182); (C): sorbitol (MRM transition 217 → 181); (D): sorbitol-D_2 (MRM transition 219 → 183).

a linear range and 50.0–10000 ng/mL ng/mL for sorbitol-$^{13}C_6$ and 250–50,000 ng/mL for fructose-$^{13}C_6$ in human red blood cells (Tables 5 and 6). Sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ were stable in human red blood cells after three freeze-thaw cycles, under bench-top storage at room temperature (RT) for 6 h, in the reconstitution solution at RT for more than 2 days, or in processed-extracts stored at RT for more than 3 days (Table 7). The extraction recovery of sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$ in human red blood cells was 64.0 and 61.1%, respectively (Table 8).

The concentrations of unlabeled sorbitol and fructose in human red blood cells were calculated using calibration curves employing the surrogate calibrators (sorbitol-$^{13}C_6$ and fructose-$^{13}C_6$) and using sorbitol-D_2 and fructose-$^{13}C_3$ as internal standards. The concentrations of unlabeled sorbitol and fructose in normal human red blood cells were shown in Table 9, which were the same as reported [33–35], indicating that the method is reliable and accurate. This validated assay has been successful in analyzing the clinical human red blood cell samples. Fig. 3 shows chromatograms of a typical clinical sample.

4. Conclusions

A novel, reliable and reproducible negative ion APCI LC/MS/MS method has been developed for the quantitative determination of endogenous sorbitol and fructose along with their isomers in human red blood cells. This fully validated assay offers improved selectivity, accuracy, sensitivity, linear range and ruggedness over previously published methods. This assay has been successfully applied to the analysis of human red blood cell samples from clinical studies.

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