Short communication

Determination of sucrose in equine serum using liquid chromatography–mass spectrometry (LC/MS)

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ABSTRACT

Mucosal integrity may be objectively assessed by determination of the absorption of exogenous substances such as sucrose. Gas chromatography–mass spectrometry (GC/MS) and liquid chromatography–mass spectrometry (LC/MS) have been reported for the accurate quantification of low concentrations of sucrose in serum. LC/MS offered the advantage of high sensitivity and mass selectivity without the need for extensive sample derivatization required for GC/MS methods. However, the high polarity and non-volatile nature of the sucrose molecule renders LC/MS techniques challenging. Previously published reports lacked sufficient detail to permit replication of methodology. Problems encountered with existing protocols included poor peak resolution and weak fragmentation of the parent molecule. This communication describes a LC/MS protocol developed to provide improved resolution and product detection.

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1. Introduction

Gastric absorption of complex carbohydrates such as sucrose has been used to demonstrate increased mucosal permeability associated with gastric ulceration or the administration of non-steroidal anti-inflammatory drugs in a variety of species [1–4]. Gas chromatography/mass spectrometry (GC/MS), high performance liquid chromatography/mass spectrometry (LC/MS), enzymatic assays and colourimetric analysis may be used to quantify carbohydrates in complex matrices [5–10], including serum and urine. Serum samples are readily obtained and, by collection of serial samples, can provide a dynamic picture of sucrose absorption kinetics, whereas urine samples are less readily collected and can accumulate in the bladder for a variable time before collection.

Although several techniques are available for sucrose analysis, the quantitation of sucrose in serum is challenging. Serum is a complex matrix which potentially includes a number of other saccharides such as glucose, fructose and maltose, as well as proteins, amino acids, electrolytes, lipids and other biological molecules. Sucrose is a highly polar, non-volatile substance that exhibits poor absorbance in the ultraviolet (UV) range, thereby limiting the use of UV and photodiode array (PDA) detection.

LC/MS offers the advantage of high sensitivity and mass selectivity without the need for extensive sample derivatization required for GC/MS methods for detection of polar constituents [9,11]. Conversion of target compounds to volatile derivatives for analysis and quantitation using GC/MS is time consuming and potentially damaging to heat labile compounds [11]. Other problems include excess labelling and subsequent problems with separation or mass spectrometry techniques, as well as derivative reagents reacting with target compounds [12]. Enzymatic assays and colourimetric analyses are simple to perform, but lack the accuracy and precision of LC/MS and GC/MS [8]. Both the detection limit and separation of saccharides in these assays may be inconsistent, rendering the techniques unsuitable for applications where precision and sensitivity are required.

To date there are only two reported sucrose permeability studies involving horses, both of which used LC/MS analysis for determination of sucrose in serum and urine respectively [2,13]. Despite descriptions of the methodology used to quantify sucrose levels in serum of other species, published accounts contained insufficient detail to permit replication of methodology. This communication reports refinements to LC/MS techniques to optimise the objective quantification of sucrose in equine serum.

2. Experimental

2.1. Reagents

Analytical grade sucrose, HPLC grade acetonitrile and trichlormethiazide were purchased from Sigma–Aldrich Pty

Ltd (Castle Hill, Australia). LC grade Milli-Q water was obtained by purification (Millipore Pty Ltd, Kilsyth, Australia).

2.2. Standard preparation

Analyses of sucrose in both aqueous solution and equine serum were performed following preparation of standards in purified water and equine serum. Analytical grade sucrose was added to LC grade water or serum and vortexed for 10 s. Standards were prepared at concentrations of 8000, 4000, 2000, 1000, 500, 250, 125 and 62.5 pg sucrose/µL. Equine serum was obtained from horses that had been fasted for a minimum of 12 h to ensure low background levels of blood sugars and other metabolites [14].

2.3. Sample preparation

Standard/sample dilution and deproteinization of equine serum was achieved by adding 0.01 mL of serum or water to a 2 mL microcentrifuge tube containing 0.9 mL of diluent consisting of 90% acetonitrile and 10% milli-Q water spiked with 5000 pg/µL of internal standard (trichlormethiazide, TCM). TCM has been utilised as an internal standard in similar studies [2] and was retained in the current study because it is easily detected using both UV and MS techniques. Diluted samples were vortexed and centrifuged (10,000 × g for 10 min), and supernatant was transferred to 2 mL vials for LC/MS analysis.

2.4. Instrumentation and acquisition parameters

Analysis was performed using an Agilent 1200 ultra high performance liquid chromatograph coupled with an Agilent 6410QQQ triple quad mass spectrometer (Agilent Technologies, Forest Hill, Victoria). Separation of saccharides and the internal standard (TCM) was achieved using an Alltech C18 (4.6 mm × 0.5 µm × 15 cm) reverse phase column (Grace Davison, Rowville, Victoria). The column was operated at 30 °C with a flow rate of 0.5 mL/min. Sample injection was 20 µL and achieved by use of an autosampler maintained at 4 °C. Conditions for the mobile phases were modified from previous publications [4,11] as follows: A – aqueous solution containing 0.1% acetic acid; B – acetonitrile containing 0.1% acetic acid. Elution began with 90:10 (A:B) for 2 min, increasing linearly to 0:100 over 8 min, isocratic for 3 min at 0:100, decreasing linearly to 90:10 over 3 min and maintained isocratically at 90:10 for 3 min, for a total run time of 17 min.

Sucrose and the internal standard were detected by use of selected ion monitoring with mass spectrometry. Product ion detection and selection for sucrose quantification was performed under full (SCAN) acquisition mode within the range of m/z 50–500. Sucrose, glucose and fructose analysis and quantification were then performed in selective ion monitoring (SIM) mode. An m/z value of 365.4 was used for the parent ion of sucrose (sodiated). A product ion with an m/z value of 203 was generated in positive ionization mode and was utilised for sucrose quantification. TCM was detected in the negative mode, because of the electronegative chlorine in the molecule under our conditions, using m/z values of 379 for the parent ion and 305 for the product ion.

2.5. Validation

Assay validation was performed according to the guidelines outlined by Hewetson et al. [2]. Analytical recovery was determined by adding known amounts of sucrose to serum from fasted horses and comparing measured and actual concentrations. Repeatability was assessed by measuring sucrose concentration a minimum of 3 times, in serum from 6 fasted horses, to which known amounts of sucrose (125, 500, 1000 pg/µL) were added. Assay

Table 1

Assessment of assay repeatability by measurement of recovery of known concentrations of sucrose (215, 500 and 1000 pg/µL) in serum from 6 fasted horses on three occasions, and replicate injections demonstrating the percentage recovery of sucrose in equine serum (1000 pg/µL), after various storage conditions.

Serum sample	Sucrose concentration (pg/µL)	Percentage recovery			SD	Variance
		Test 1	Test 2	Test 3		
1	125	97	95	98	2.3	5.5
2	125	93	98	93		
3	500	93	95	96	1.6	2.7
4	500	97	94	97		
5	1000	98	96	93	2.1	4.6
6	1000	93	97	94		
Storage conditions						
24 h at 21 °C		95	95	96		
7 days at 4 °C		89	90	89	3.5	12.4
28 days at −20 °C		97	97	97		

reproducibility after storage over time was determined by freezing 3 portions of serum containing known amounts of sucrose (125, 500, 1000 pg/µL), then thawing and assaying each portion on separate days. The stability of sucrose in serum was further evaluated by repeated assay of aliquots of serum samples containing a sucrose concentration of 1000 pg/µL after storage for 24 h at room temperature, 7 days at 4 °C, and 28 days at −20 °C. Specificity of the assay for target sugars was validated by spiking fasted equine serum with sucrose and associated sugars, glucose and fructose, to determine whether sucrose could be distinguished from its component monosaccharides. The standard deviation (SD) and variance of the percentage recovery for each analysis were calculated using statistical software (PASW statistics 17.0, SPSS Inc., New York).

3. Results

3.1. Chromatography

In both water and serum, sucrose exhibited a sodiated parent molecule with a m/z of 365.4, with major product ions at m/z 181 and m/z 203. A typical chromatogram of sucrose in equine serum is shown in Fig. 1.

3.2. Standard curve and sensitivity

Standard curves were developed for the major product ions of sucrose, 203 and 181. The standard curve obtained for product ion 203 demonstrated increased signal strength and greater linearity in dose response relative to that obtained for product ion 181. Sucrose was detected in serum at levels as low as 11 pg/µL in preliminary experimentation. However replicate injections indicated that quantitation below 50 pg/µL was unreliable. Therefore 50 pg/µL was considered the limit of detection for this assay.

3.3. Assay validation and specificity

Sucrose recovery, determined by comparison of measured and actual concentrations in spiked samples, ranged from 94 to 99%. Assessment of repeatability of the assay produced a relative SD ranging from 1.6 to 2.3% for repeated (intra-assay) measurements and from 2.1 to 4.1% for the repeated (interassay) measurements. Sucrose was generally stable in serum after storage under various conditions; storage resulted in sample variation of only 3.5% (Table 1). Sucrose was readily differentiated from component monosaccharides present in equine serum, namely fructose and glucose (Fig. 2).
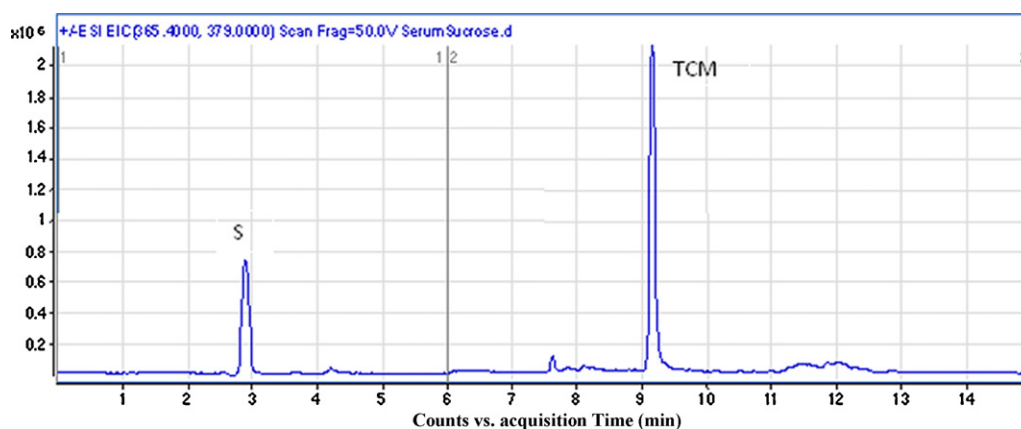
Fig. 1. Sucrose in water (1000 pg/μL). Sucrose (S) eluted at 2.8 min and the internal standard (TCM) at 8.6 min.
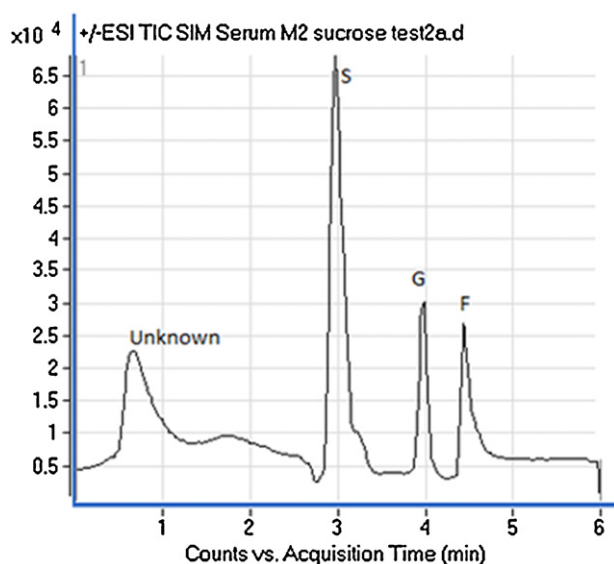

Fig. 2. Chromatogram from an injection of fasted equine serum which contained sucrose, glucose and fructose at a concentration of 500 pg/μL. Sucrose (S) had a retention time (r/t) of 3.0 min, glucose (G) had a r/t of 3.9 min and fructose (F) had a r/t of 4.5 min, demonstrating the specificity of the developed assay to distinguish sucrose from other saccharides.

4. Discussion

There are few reports involving LC/MS quantification of sucrose in mammalian blood or serum. We successfully quantified sucrose in equine serum or water using LC/MS with modifications to published methodology, specifically adjusting the mobile phase constituents and gradient, injection volume, and optimisation of mass spectrometry protocols.

Initial analyses resulted in poor peak resolution and weak fragmentation of the parent molecule. Carbohydrate peak resolution is commonly problematic [11,15]. Our initial assays employed the mobile phase ACN and water with a gradient as described previously [4]. However, we modified the mobile phase to improve resolution by addition of acetic acid to the mobile phase to enhance potential ionization, and thus resulting signal intensity of sucrose, glucose and fructose under similar chromatographic conditions. This resulted in considerable improvement in peak resolution and product detection. In previous reports, sucrose was identified as the sodiated molecule in both prepared serum and aqueous samples. The addition of sodium chloride to the mobile phase may therefore be helpful in improving resolution of carbohydrate peaks, as has been reported by Sims [15]. We found, however, that the addition of sodium, or further modifications to effect additional improvement in peak resolution was not necessary in our studies as the changes to the mobile phase outlined above produced desired results.

Problems associated with the analysis of equine serum in published studies [5,16] included column saturation and a masking effect of unknown serum constituents upon sucrose and other simple sugars in this complex matrix. In the current study, sample overloading of the LC column was overcome by reduction of the injection volume from 100 μL, as suggested by Buddington et al. [4], to 20 μL. Additionally, we noted that samples obtained from horses that had not undergone a fasting period routinely contained high levels of saccharides, resulting in potential column overloading and subsequently poor peak resolution and inability to detect treatment differences. It was therefore critical for equine subjects to undergo fasting, in order to reduce background levels of saccharides, and ensure observable treatment effects.

The validation of our methodology was performed using sucrose dissolved in both aqueous and equine serum solutions to ensure the integrity of measurements derived from the more complex serum matrix. We also included an internal standard (TCM) to validate our results [2,4]. The modification to mobile phase, sample size and ionization parameters resulted in improvements in sensitivity of the assay, and reduced variation among sample replicates, in comparison to published reports with equine subjects as well as other species [2,4]. We also demonstrated that sucrose concentrations are stable after freezing ($-20\,°C$) for up to 28 days, or following storage for 24 h at $21\,°C$ or 7 days at $4\,°C$. The methodology described successfully quantified sucrose in serum containing glucose and fructose, was repeatable and was reliable at concentrations as low as 50 pg/μl, a significant improvement in sensitivity in comparison to reported results from GC/MS, enzyme and colourimetric assays which detect sucrose at levels ranging from μM to nM concentrations.

Conflicts of interest

The authors have no conflicts of interest pertaining to this paper.

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