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Sugar microanalysis by HPLC with benzoylation: Improvement via introduction of a C-8 cartridge and a high efficiency ODS column

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Abstract

An HPLC protocol for sugar microanalysis based on the formation of ultraviolet-absorbing benzoyl chloride derivatives was improved. Here, samples were prepared with a C-8 cartridge and analyzed with a high efficiency ODS column, in which porous spherical silica particles 3 μm in diameter were packed. These devices allowed us to simultaneously quantify multiple sugars and sugar alcohols up to 10 ng/ml and to provide satisfactory separations of some sugars, such as fructose and myo-inositol and sorbitol and mannitol. This protocol, which does not require special apparatuses, should become a powerful tool in sugar research.

Keywords: Sugar microanalysis; Benzoylation; Solid phase extraction

1. Introduction

As the view that sugars exert various effects to maintain the homeostasis of organisms strengthens [1], the demand for their accurate quantification increases. Although high pressure (or performance) liquid chromatography (HPLC) is one of the excellent tools for the quantification of various molecules, it was not applicable to sugars and sugar alcohols until recently since they do not absorb ultraviolet (UV) light; namely, they are not detectable by a UV detector that is equipped with a common HPLC system. Recent two major technical innovations to help overcome this situation are as follows: In the first one, sugars and sugar alcohols are ionized under a strong alkaline condition, separated by an ion-exchange column, and detected by an amperometric detector [2,3]; In the second one, sugars are modified into fluorescence derivatives in a pre- or post-column step, separated using an ODS or NH_2 column, and detected by a fluorophotometer [4–6]. However, since special tools and apparatuses are necessary to perform these procedures, they are only for limited laboratories.

The benzoyl group binds easily to the hydroxyl groups of various molecules including sugars. Since this group carries double bonds, benzoyl derivatives absorb UV regardless of the original molecule; namely, they can be detected by a UV detector. Using this principle, several groups were successful in detecting sugars in various specimens [7–9], even in clinical samples [10]. However, this method seems not to have become popular for sugar microanalysis so far. One of the reasons for this underestimation is supposed to be attributed to its sample preparation process. In this method, sugars must be collected after receiving benzoylation to be applied to HPLC analysis. In the previous protocols, organic solvents, such as ethyl acetate, which is later evaporated by nitrogen gas, were used for this purpose. However, this process leads to contamination by various organic compounds, including those existing in biological samples as well as the organic solvent used for the sample extraction per se, in the HPLC analytes. Such contaminants would lead to instabilities of the base-lines of chromatograms, resulting in inaccurate quantification even if their amounts are very small.

With this background, we established a new HPLC protocol for sugar microanalysis based on the sugar benzoylation technique, but in which benzoylated sugars are extracted by a solid phase extraction cartridge instead of organic solvent.

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2. Experimental

2.1. Standard sugars

Ribose, glucose, fructose, myo-inositol (Kanto Chemical Co., Inc., Tokyo, Japan), mannose (Wako Chemical Co., Inc., Osaka, Japan), mannitol, and sorbitol (Sigma–Aldrich Inc., MO, USA) were purchased and used as standards. They were dissolved in sterile distilled water at various concentrations to draw calibration curves. For the standard, a solution which contained fructose and the other sugars of which concentrations were adjusted to 1 and 500 ng/ml, respectively, were prepared (Standard A).

2.2. Human red blood cell samples

Blood samples were collected from authors of this manuscript (MM, HY). After blood was drawn from veins, each sample was rapidly put into a glass tube containing EDTA-2Na (Termo Co., Inc., Tokyo, Japan), fully mixed, and the serum fraction was removed after centrifugation at 3000 rpm for 5 min at 4 °C using a refrigeration centrifuge (EX-126, TOMY Co., Tokyo, Japan). Each sample was washed by sufficient amount of saline three times and stored at 4 °C until being applied for HPLC analyses which were performed within 48 h after sample collection.

2.3. Preparation of HPLC analytes

2.3.1. Protein elimination from the specimens

Four hundred microlitre of each red blood cell fraction or Standard A was put into a new glass tube, respectively. Each sample was combined with 1 ml of 1N of perchloric acid, mixed well by a Vortex Genie-2 (Scientific Industries Co., NY, USA). After centrifugation at 3000 rpm for 10 min at 4 °C, 0.8 ml of the supernatant was put into a new glass tube and then combined with 380 μl of 1.25 M K_2CO_3 (Sigma–Aldrich Inc.). It was centrifuged at 3000 rpm for 5 min at 4 °C and 200 μl of the supernatant was put into a 2.0 ml polypropylene tube (Safe-Lock Tubes, Eppendorf, Hamburg, Germany). Alternatively, 10% trichloroacetic acid (Sigma–Aldrich Inc.) was used for protein elimination instead of 1N of perchloric acid.

2.3.2. Sugar benzoylation

Each sample in the polypropylene tube that underwent protein elimination was combined with 60 μl of 1 M KH_2PO_4 (Sigma–Aldrich Inc.), mixed slightly, then combined with 10 μl of an undiluted benzoyl chloride solution (Kanto Chemical Co., Inc.), and mixed well. Just after 45 μl of 8N NaOH was added, each sample was mixed vigorously by a Vortex Genie-2 for exactly 50 s at mixing scale 2, left at room temperature for 10 min, and then combined with 45 μl of 16% phosphoric acid (Kanto Chemical Co., Inc.; Sample A). All processes were performed at 25 ± 2 °C.

2.3.3. Solid phase extraction

A Discovery DSC-C-8 cartridge 100 mg/1 ml (SUPELCO Inc., PA, USA), in which octyl groups are packed (C-8 cartridge), was equipped with a disposable bubble liner (SUPELCO Inc.), and 1 ml of 100% acetnitrile (Kanto Chemical Co., Inc.) was poured into the cartridge and drawn using a vacuum pump (ULVAC, Sinku Kiko Co., Tokyo, Japan) to remove bubbles and channels in the cartridge. Then, the cartridge was rinsed by dripping with 1 ml 100% acetnitrile three times and 1 ml of water twice. The whole quantity of each Sample A was poured into the conditioned cartridge. Each sample tube was rinsed twice with an adequate amount of distilled water that was also poured into the cartridge. Then, the cartridge was rinsed by dripping with distilled water twice, 0.8 ml of 60% acetnitrile once, and 0.3 ml of 100% acetnitrile once. Then, the sugar fraction was extracted by dripping with 0.5 ml of 100% acetnitrile (Sample B). Alternatively a Discovery DSC-18 cartridge (SUPELCO Inc.) was used for the solid phase extraction under appropriate conditions (details not shown) instead of a DSC-C-8 cartridge. All processes were performed at 25 ± 2 °C.

2.4. HPLC analysis

Fifty microlitre of Sample B was applied to the quantification using an HPLC system (Shimadzu Co., Kyoto, Japan) comprised of a system controller (SCL-10Avp), a UV detector (SPD-10Avp), a pump (LC-10ADvp), an auto sampler (Sil-10ADvp), an integrator (C-R8A), and a column oven (CO 631C, GL Science Inc., Tokyo, Japan). A Cadenza CD-C18 column in which porous spherical silica particles 3 μm in a diameter, modified with C18 groups, were packed (Imtakt Inc., Kyoto, Japan) was incorporated into the HPLC system. The mobile phase of HPLC was 77.5% acetnitrile that was flowed at a velocity of 1 ml/min. One cycle of HPLC, which was an isocratic performance, was set for 40 min. Benzoylated sugar groups were detected by UV of which the wavelength was 228 nm. The temperature of the column was kept at 25 °C by a column oven. Alternatively, an ODS column (anonymous) in which porous spherical silica particles 5 μm in diameter modified with C18 groups were packed was incorporated into the HPLC system instead of the Cadenza CD-C18 column.

2.5. Recovery rate of solid phase extraction using the C-8 cartridge

One microgram of each sugar, dissolved in sterile distilled water, underwent protein extraction, benzoylation, and solid phase extraction according to the present protocol (Sample C). Each eluate provided at each step of the solid phase extraction; namely, during the cartridge washing process with 0.8 ml of 60% acetnitrite (fraction 1) and at that with 0.3 ml of 100% acetnitrite (fraction 2) and during the sugar extraction process with 0.5 ml of 100% acetnitrile (fraction 3), was collected. After the sugar extraction process, the cartridge was washed with 0.3 ml of 100% acetnitrile three times and eluates from these steps were also collected (fractions 4–6). The concentrations of sugars in these eluates (fractions 1–6) were examined by the present HPLC protocol. The ratio of the amount of each benzoylated sugar in fraction 3 and the total amount in the whole fractions

was tentatively designated as its recovery rate of the present solid phase extraction.

2.6. Stabilities of benzoylated sugars in the solvents

Sample C was left for 5 h at room temperature, and concentrations of benzoylated sugars were quantified by the present HPLC protocol before and after leaving it. Examinations with the same conditions but in which the concentration of acetnitrile was diluted to 77.5 or 60% were also performed. Moreover, stabilities of these samples at 4 °C were also examined.

3. Results

Fig. 1 is a chromatogram of HPLC analysis with a Cadenza CD-C18 column for Standard A which underwent protein elimination, benzoylation and solid phase extraction according to the present protocol. The base-line of the chromatogram was almost flat from the beginning of analysis. Ribose, mannose, glucose, fructose, myo-inositol, sorbitol, and mannitol were clearly separated. However, in addition to major peaks, multiple small peaks were observed.

The reproducibility of the retention time (RT) of each major peak, its area under the curve (AUC), and the range of reliable quantification are summarized for each sugar or sugar alcohol (Table 1). The limits of quantification were 10 ng/ml for mannitol and sorbitol, 50 ng/ml for fructose, and 25 ng/ml for the others examined at this time.

The resolution power of the Cadenza CD-C18 column was stronger than that of a common ODS column, and the former provided satisfactory separations of fructose and myo-inositol as well as mannitol and sorbitol for their quantification (Table 2).

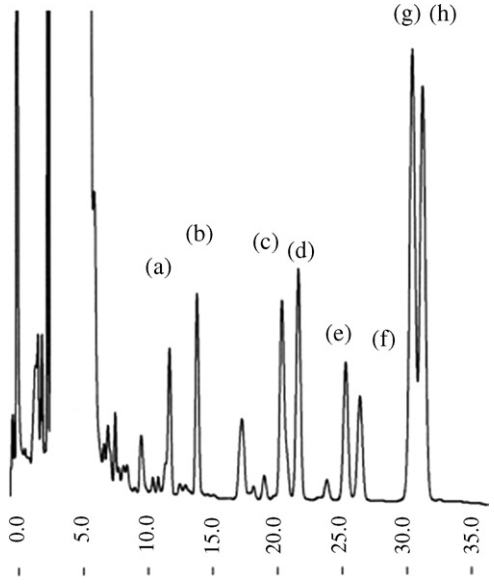

Fig. 1. A chromatogram of sugar microanalysis for standard sugars using the present HPLC protocol. Mixtures of various sugars and sugar alcohols, of which the concentrations were 500 ng/ml (fructose was 1000 ng/ml), underwent protein elimination, solid phase extraction using a C-8 cartridge, and HPLC analysis with a Cadenza CD-C18 column. Ribose (a, b), mannose (c), glucose (d), fructose (e), myo-inositol (f), mannitol (g), and sorbitol (h) were clearly separated.

Table 1
Reproducibility of retention time and quantification, and reliable range of quantification of each sugar in HPLC analysis

	Retention time (min) (CV)	Quantification of 1 μg/ml of sugars (CV)	Reliable range of quantification (ng/ml)[a]
Ribose 1	12.08 (0.24)	3.3	25–5000
Ribose 2	14.15 (0.33)	3.2	25–5000
Mannose	20.44 (0.24)	1.8	25–5000
Glucose	21.65 (0.26)	1.9	25–5000
Fructose	25.19 (0.26)	1.6	50–10000
Myo-inositol	26.24 (0.29)	2.2	25–5000
Mannitol	30.17 (0.29)	1.9	10–5000
Sorbitol	30.90 (0.26)	1.5	10–5000

CV: coefficient of variation (%), $n = 5$ each.
[a] Ranges in which calibrations are linear.

Table 2
Comparison of resolution of fructose and myo-inositol and mannitol and sorbitol by a regular ODS column and Cadenza CD-C18 column

	A common ODS column (anonymous)	Cadenza CD-C18 column
Fructose and myo-inositol	Less than 0.5	0.92
Mannitol and sorbitol	0.96	1.69

Degree of resolution.

The effect of the reaction time of benzoylation on analysis was studied. One microgram per millilitre of sorbitol was applied to the benzoylation with various reaction times. As shown in Fig. 2, the area under the curve of the peak reflecting benzoylated sorbitol increased as the reaction time became longer, but reached a plateau at 45 s.

Fig. 3 shows an HPLC chromatogram of sugar microanalysis of a human blood specimen prepared using the present protocol. Peaks of glucose, fructose, myo-inositol, mannitol, and sorbitol were identified. However, several major peaks that could not be identified were also observed.

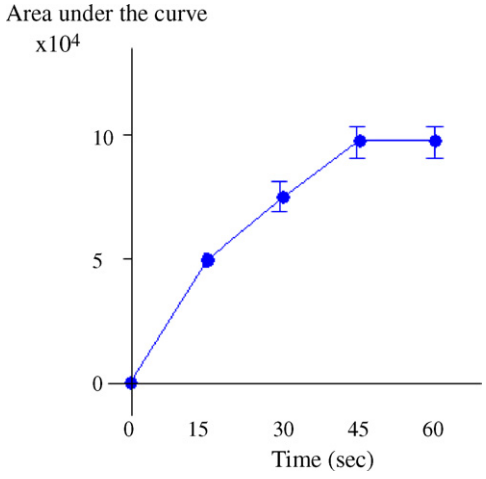

Fig. 2. Effect of reaction time of benzoylation on sugar microanalysis performance using the present HPLC protocol. One microgram per millilitre of sorbitol was applied to the present HPLC protocol but the reaction time of benzoylation varied. The area under the curve of the peak reflecting sorbitol became larger as the mixing time increased, but reached a plateau at 45 s ($n = 3$ in each spot).

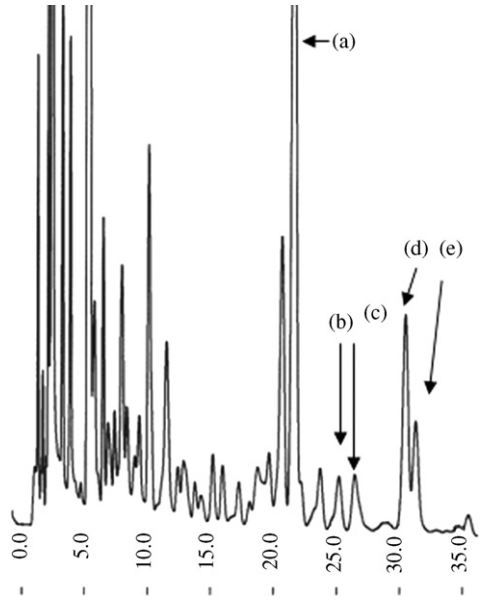

Fig. 3. A chromatogram of sugar microanalysis for a specimen of human red blood cells using the present HPLC protocol. Human red blood cell fractions were applied to HPLC analysis using the present protocol. Glucose (a), fructose (b), myo-inositol (c), manitol (d), and sorbitol (e) were detectable. In addition, several peaks that were not identified at this time were also observed.

A Discovery DSC-18 cartridge was also applicable for the preparation of biological samples. However, since a larger amount of acetnitrile was required to extract benzoylated sugars from the cartridge, samples were more diluted compared to when the Discovery DSC-C-8 cartridge was used.

We used trichloroacetic acid instead of perchoric acid for protein elimination from the human red blood cell samples, and found that there were no significant differences in the results of HPLC analysis between the two protocols.

As shown in Table 3, regarding all benzoylated sugars and sugar alcohols, most extracts appeared in fraction 3 during the solid phase extraction process under the present conditions. The recovery rate was from 97.6 to 98.8% for each benzoylated sugar or sugar alcohol, respectively.

All benzoylated sugars examined at this time were stable in 100, 77.5, or 60% acetnitrile solution. We confirmed that their breakdowns hardly occurred up to 5 h when they were left at room temperature (25 ± 2 °C) and up to 120 h when they were stored at 4 °C.

4. Discussion

So far, the HPLC protocol combined with the benzoylation technique seems not to have been widely used for sugar microanalysis, in spite of its big advantage of being able to be performed without special apparatuses. This underestimation may, at least in part, be due to the fact that the contamination of organic solvents, such as ethyl acetates, is inevitable during the sample preparation process as far as it is performed following the previous protocols [7,10]. Such contamination of the HPLC analytes causes instability of the base-line of a chromatogram, hampering accurate microanalysis. To solve this problem, we established a new protocol for sugar microanalysis using the benzoylation technique, but in which benzoylated sugars were collected using a solid phase extraction technique with a C-8 cartridge instead of extraction using ethyl acetate. This change allowed us to achieve a chromatogram with a stable base-line.

The reduction of contaminants in the HPLC analytes enables us to equip the present HPLC system with a Cadenza CD-C18 column instead of a common ODS column. The number of theoretical plates of the former is 53400 per column, whereas that of the latter is just 22000 per column. The resolution of analysis with the former was greater than that with the latter, resulting in the marked separation of fructose and myo-inositol and that of mannitol and sorbitol. Since the diameter of porous spherical silica that is packed in the former (3 μm) is smaller than that in the latter (5 μm), the former is more susceptible to impurities causing column plugging. Thus, the former might have not been applicable for an analysis using the previous protocol.

With these innovations, the present protocol allowed us to simultaneously quantify multiple sugars and sugar alcohols, including ribose, mannose, glucose, fructose, myo-inositol, sorbitol, and mannitol, up to ng/ml levels by a common HPLC system without any special apparatuses, even in biological samples. The limit of identification of each sugar was equal or rather superior compared to the other sugar microanalysis techniques available at this time.

In addition, the present protocol reduced the time needed for analysis compared to that of the previous protocol using the benzoylation technique. The average time for sample preparation was reduced from 60 to 30 min. Moreover, the present HPLC analysis is isocratic, whereas previous protocol requires a gradient analysis to wash out contaminants. Thus,

Table 3
Determination of recovery rate of benzoylated sugars by solid phase extraction using the C-8 cartridge

Fraction[a]	1	2	3	4	5	6	Recovery rate[b]
Ribose 1	ND	819 (434)	33568 (546)	ND	ND	ND	97.6 (1.3)
Ribose 2	ND	704 (357)	43030 (704)	ND	ND	ND	98.4 (0.8)
Mannose	ND	310 (174)	68170 (1154)	531 (76)	ND	ND	98.8 (0.3)
Glucose	ND	310 (171)	65128 (1001)	766 (136)	ND	ND	98.4 (0.2)
Fructose	ND	ND	41259 (983)	490 (98)	ND	ND	98.8 (0.2)
Myo-inositol	ND	ND	35512 (740)	554 (120)	ND	ND	98.5 (0.3)
Mannitol	ND	ND	158703 (2534)	2394 (423)	384 (51)	ND	98.3 (0.3)
Sorbitol	ND	417 (269)	148380 (2336)	2264 (386)	363 (31)	ND	98.0 (0.2)

Area under the curve (unit) × volume (ml), mean (standard error). ND: not detectable. $n = 3$ for each value.

[a] The designation of each fraction is described in the text.

[b] Amount of fraction 3/total amount × 100 (expressed as %).

the HPLC running time per sample was also reduced from 70 to 40 min. This also contributes to a reduction of the mobile phase; namely acetnitrile, resulting in economical and ecological benefits.

The validities of conditions were confirmed in each step of the present protocol. Although Kyang-Hyok mentioned that protein elimination using perchloric acid was not sufficient and recommended the use of trichloroacetic acid for sugar microanalysis using HPLC based on the benzoylation technique [9], we did not find a significant difference between these two methods, at least when sugars were quantified by the present protocol. In terms of the reaction time of benzoylation, we believe that 50 s was sufficient for the present analysis since its level reached a plateau at 45 s. The conditions of the present solid phase extraction were adjusted to the optimum with reference to the data of preparatory experiments (details not shown). The suitability was warranted by the present data regarding the recovery rates of the solid phase extraction for each benzoylated sugar. Not only a C-8 cartridge but also a C-18 cartridge was applicable for the extraction of benzoylated sugars. However, since the latter required a larger amount of acetnitrile for eluting the benzoylated sugars from the cartridge than the former, resulting in the dilution of samples, the latter was thought to be inferior to the former for the present purpose.

Although sugars carry plural hydroxyl groups, not all of them undergo benzoylation and sugars carrying benzoylated hydroxyl groups with different numbers are produced during the benzoylation process. This causes the appearance of multiple peaks assigned to a single sugar in HPLC analysis [7]. Since this incomplete benzoylation may be attributed to the three-dimensional configurations of sugars and benzoyl groups, changes in the conditions of benzoylation, such as the buffer and reaction time, cannot be solution to prevent their formation [7]. Although such features were reproduced in the present study, the present calibration curves indicate that these extra peaks never hamper microanalysis of each sugar under the present protocol as long as the major peak is selectively examined. In terms of ribose, two major peaks were observed when it was examined under the present protocol. However, the present calibration curves also indicated that both peaks were applicable for its quantification. The identification limits of sugars were not constant, namely 10 ng/ml for sorbitol and mannitol, 50 ng/ml for fructose, and 25 ng/ml for the others. The difference may, at least in part, be due to variation in the efficiency of benzoylation among various sugars.

In the present study, the stability of benzoylated sugars was examined in various solvents that are used for sample preparation and analysis in the present protocol. Since all benzoylated sugars and sugar alcohols examined in the present study were confirmed to be stable in 60–100% acetnitrile, even when they were at 25 °C, their breakdowns should hardly occur during the present sample preparation and HPLC analysis. Furthermore, the benzoylated derivatives were shown to be stable up to 120 h at 4 °C as far as they were kept in acetnitrile solution. Some sugars are susceptible to degradation caused by bacteria, leading to errors in quantification. In that bacteria cannot utilize benzoylated sugars as nutrients, the present protocol may have a merit of avoiding such bacterial effects.

In conclusion, the previous HPLC protocol for sugar microanalysis based on sugar benzoylation was improved. Applications of a C-8 cartridge for the preparation of HPLC analytes and an ODS column packed with porous spherical silica 3 μm in diameter for HPLC analysis increased the efficiency of sugar microanalysis. Since the analysis using the present protocol could be performed with a common HPLC system without any special apparatuses, it may become a powerful tool in sugar research.

Appendix A

Column	Cadenza CD-C18 column
Supplier	Imtakt Inc., Kyoto, Japan
Length	25 cm
Internal diameter	4.6 mm
Packing	Porous spherical silica
Diameter of packed particle	3 μm
Mobile phase (isocratic)	77.5% acetnitrile
Flow-rate	1 ml/min
The wavelength of UV for detection	228 nm
Injection volume	50 μl
Column temperature	25 °C
Autosampler temperature	5 °C

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