Determination of Reducing Sugars by High Performance Liquid Chromatography with Postcolumn Fluorescence Derivatization Using 1,2-Bis(4-methoxyphenyl)ethylenediamine

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A sensitive method for the determination of reducing sugars is described which employs high performance liquid chromatography with on-line postcolumn fluorescence derivatization. Reducing sugars are separated by borate complex anion-exchange chromatography on a trimethylammonium-bonded resin column (TSK gel Sugar AXG) and automatically converted into fluorescent derivatives by reaction with 1,2-bis(4-methoxyphenyl)ethylenediamine in an alkaline medium. The detection limits ($S/N=3$) for reducing sugars are 30–160 pmol in a 100- μ l injection volume. The quantification of reducing sugars in human urine and serum is also described.

Keywords Reducing sugar, postcolumn fluorescence derivatization, borate complex anion-exchange chromatography, 1,2-bis(4-methoxyphenyl)ethylenediamine, serum, urine

High performance liquid chromatography (HPLC) coupled with refractive index^{1,2}, ultraviolet absorption^{3,4} and fluorescence^{5–10} detection methods, has been introduced for the determination of reducing sugars. The refractive index and ultraviolet absorption detection methods are neither sufficiently sensitive nor selective to measure reducing sugars in biological samples. Post-column fluorescence derivatization HPLC methods for the determination of reducing sugars using taurine⁵, ethanolamine⁶, 2-cyanoacetamide⁷, 2-aminopropionitrile⁸ and arginine⁹ as fluorogenic reagents are fairly sensitive and selective. A less sensitive postcolumn derivatization HPLC using ethylenediamine has also been reported.¹⁰

We have recently reported that *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine (*p*-MOED) reacts with reducing sugars in an alkaline medium to give strongly fluorescent compounds.¹¹ This study was aimed at the establishment of a highly sensitive HPLC method for the determination of reducing sugars, based on the separation of reducing sugars by borate complex anion-exchange chromatography followed by an on-line postcolumn fluorescence derivatization with *p*-MOED. Reducing sugars in human urine and serum were also measured using α -melibiose as an internal standard.

Experimental

Reagents, solutions and apparatus

p-MOED was synthesized as described previously.¹²

Reducing sugar samples were obtained from Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). β -Galactosidase (β -D-galactoside galactohydrolase; EC 3.2.1.23, from *Escherichia coli*, 200 I.U./mg protein) was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade. Deionized and distilled water was used. Aqueous standard solutions of reducing sugars were stored at 4°C until use. Sera obtained from normal persons in the usual manner were kept frozen at –20°C. Normal human urine samples (24-h) were collected in the presence of 6 M hydrochloric acid (10 ml) and stored at 4°C until the time of the assay. A *p*-MOED (15 mM) solution prepared in aqueous 30% (v/v) ethanol and an aqueous sodium hydroxide solution (0.75 M) were degassed before use.

Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi MPF-4 spectrofluorometer in 10×10-mm quartz cells; spectral bandwidths of 10 nm were used for both excitation and emission monochromators.

Chromatographic system and its operation

A flow diagram of the HPLC system constructed for reducing sugars is shown in Fig. 1. Borate complexes of reducing sugars were separated on a strong anion-exchanger (a trimethylammonium-bonded styrene-divinylbenzene copolymer resin) column (TSK gel Sugar AXG, 150×4.6 mm i.d.; Tosoh, Tokyo, Japan); the column temperature was kept at 60°C in a Tosoh HLC-805 column oven. Borate buffer (0.5 M, pH 8.7) used

Fig. 1 Flow diagram of the HPLC system for reducing sugars.

as a mobile phase was delivered at a flow rate of 0.4 ml/min by means of a Tosoh CCPM pump equipped with a Rheodyne 7125 syringe loading sample injector valve (100- μ l loop).

The effluent from the column was mixed with a stream of a mixture of the *p*-MOED and sodium hydroxide solutions, both of which were delivered at 0.15 ml/min by an SSP PM-2M 1024 pump (Sanuki Kogyo, Tokyo, Japan). The mixture was then heated in a reaction coil (20 m $\times$ 0.5 mm i.d., stainless-steel tube) placed in a Shimadzu (Tokyo, Japan) CRB-6A reaction bath (140°C). The effluent from the reaction coil was passed through an air-cooling coil (1 m $\times$ 0.5 mm i.d., stainless-steel tube); finally, the fluorescence was monitored at 460 nm with excitation at 330 nm using a Tosoh FS-8000 spectrofluorometer equipped with a flow cell (15 μ l). The spectral bandwidths for both excitation and emission wavelengths were 20 nm.

Urine and serum sample preparation for HPLC

Deproteinization of urine and serum with acetonitrile (recommended procedure for sample preparation). To 250 μ l of urine or serum, 10 μ l of 10 μ mol/ml α -melibiose (internal standard) and 250 μ l of acetonitrile were successively added. The mixture was centrifuged at 1500g for 10 min. The supernatant (100 μ l) was mixed with 200 μ l of 0.5 M borate buffer (pH 8.7) to form borate complexes of sugars. A 100 μ l aliquot of the mixture was injected into the chromatograph.

Deproteinization of urine and serum with trichloroacetic acid or perchloric acid. To 250 μ l of urine or serum, 10 μ l of 10 μ mol/ml α -melibiose and 250 μ l of 0.7 M

trichloroacetic acid or 1.0 M perchloric acid were successively added. The mixture was centrifuged at 1500g for 10 min. To 100 μ l of the supernatant was added 200 μ l of 1.0 M borate buffer (pH 9.0). A 100 μ l aliquot of the mixture was injected into the chromatograph.

Hydrolysis of lactose with β -galactosidase. A portion (500 μ l) of urine was mixed with 200 μ l of 10 μ mol/ml α -melibiose, 500 μ l of 0.1 M phosphate buffer (pH 7.3) and 100 μ l of 0.2 ng/ml β -galactosidase. The mixture was incubated at 37°C for 12 h. To 500 μ l of the mixture was added 500 μ l of acetonitrile. The mixture was centrifuged at 1500g for 10 min. To 500 μ l of the supernatant was mixed with 500 μ l of 0.5 M borate buffer (pH 8.7). A 100 μ l portion of the mixture was injected into the chromatograph.

Results and Discussion

Separation and postcolumn fluorescence derivatization of reducing sugars

The conditions of separation and postcolumn fluorescence derivatization were investigated mainly by using eight reducing sugars (cellobiose, maltose, D-ribose, D-mannose, D-arabinose, D-galactose, D-xylose and D-glucose).

Reducing sugars have been separated on an amino-bonded silica column with aqueous acetonitrile as the mobile phase^{5,6,16,17} or on an anion-exchanger column with borate buffer of alkaline pH.^{5-8,10,13-15} Since aqueous acetonitrile at a concentration of 50% (v/v) or higher

Fig. 2 Chromatogram of a standard mixture of 14 reducing sugars. Peaks and concentrations (nmol/100 μ l injection volume) in parentheses: 1, *N*-acetyl-D-glucosamine (20); 2, cellobiose (2.5); 3, maltose (7.5); 4, lactose and L-rhamnose (5 each); 5, D-ribose (10); 6, D-mannose (10); 7, D-fructose (20); 8, D-arabinose (20); 9, D-galactose (20); 10, D-xylose (20); 11, D-mannoheptulose (20); 12, D-glucose (20); 13, α -melibiose (20).

interfered with the postcolumn derivatization reaction, the separation on an amino-bonded column could not be used. An alkaline borate buffer, on the other hand, was usable for the derivatization reaction. Thus, anion-exchange HPLC on a trimethylammonium-bonded column with a borate buffer as the mobile phase was adopted.

The retention times for reducing sugars decreased with increasing concentration and pH of the borate buffer; 0.5 M borate buffer of pH 8.7, which was recommended in the HPLC system, could afford a rapid and good separation of the reducing sugars. A higher temperature (up to 80°C) of the column resulted in a better resolution of the sugars, though a temperature higher than 70°C decomposed D-fructose; the column was kept at 60°C in the HPLC system.

Figure 2 depicts a chromatogram obtained with a standard mixture of fourteen reducing sugars under the HPLC conditions; the retention times and relative peak heights for eighteen reducing sugars are listed in Table 1. Although most of the reducing sugars were well separated, lactose and L-rhamnose (Fig. 2, peak 4 and Table 1), and D-arabinose and D- and L-fucoses (Table 1) were inseparable under the HPLC conditions.

The fluorescence excitation and emission maxima of the final reaction mixtures of all the sugars were at approximately 330 and 460 nm, respectively, and the spectra were almost identical with those obtained in the manual spectrofluorometric method.¹¹

The concentration of *p*-MOED, ranging from 15 to 20 mM in the reagent solution, provided almost maximum peak heights for the reducing sugars; 15 mM was selected as the optimum. Though *p*-MOED was

Table 1 Retention times and relative peak heights for reducing sugars

Reducing sugar ^a	Retention time/min	Relative peak height ^b
2-Deoxy-D-ribose	12.2	52
<i>N</i> -Acetyl-D-glucosamine	12.8	49
<i>N</i> -Acetyl-D-galactosamine	13.6	31
2-Deoxy-D-glucose	14.0	42
Cellobiose	14.9	248
Maltose	17.9	118
Lactose	19.5	85
L-Rhamnose	19.5	82
D-Ribose	22.6	100
D-Mannose	30.3	84
D-Fructose	35.9	44
D-Arabinose	40.0	41
D-Fucose	40.0	37
L-Fucose	40.0	37
D-Galactose	46.4	30
D-Xylose	56.4	36
D-Glucose	72.6	40
α -Melibiose	93.3	23

a. Concentrations of reducing sugars, 10 nmol/100 μ l injection volume.

b. The peak height for D-ribose was taken as 100.

Fig. 3 Effect of the sodium hydroxide concentration on the peak heights of reducing sugars. Concentrations (nmol/100- μ l injection volume): cellobiose, D-mannose and D-xylose, 10; others, 20. Curves: 1, cellobiose; 2, maltose; 3, D-ribose; 4, D-mannose; 5, D-arabinose; 6, D-galactose; 7, D-xylose; 8, D-glucose.

hardly soluble in water, it was soluble in water-miscible organic solvents such as methanol, ethanol and acetonitrile. These solvents also served to accelerate the fluorescence reaction at concentrations lower than 50% in the reagent solution. Of these solvents, ethanol was most effective in the concentration range 30–40% (v/v); 30% (v/v) was used. The highest peaks were

attained at sodium hydroxide concentrations of 0.7–0.75 M (Fig. 3); 0.75 M was selected.

The peak heights increased with increasing reaction temperature up to approximately 150°C. A temperature higher than 150°C caused background noise in the chromatogram, due to air-bubble formation in the reaction coil; 140°C was employed in the HPLC system. Almost maximum and constant peak heights were achieved when a reaction coil longer than 20 m was used; a 20-m reaction coil was adopted in the system (passage period of the reaction mixture, 5.6 min).

The calibration graphs obtained by plotting the peak heights *versus* the amounts of the eight reducing sugars (0.1–10 nmol/injection volume) were linear and passed through the origin. The detection limits (pmol/injection volume of 100 µl; $S/N=3$) were 30 (cellobiose), 40 (maltose), 40 (D-ribose), 50 (D-mannose), 80 (D-arabinose), 130 (D-galactose), 120 (D-xylose) and 160 (D-glucose). The precision was established by repeated determinations ($n=10$) of a standard mixture of the eight reducing sugars (10 nmol/ml each). The relative standard variations (%) were 3.5 (cellobiose), 3.5 (maltose), 3.1 (D-ribose), 2.9 (D-mannose), 2.5 (D-arabinose), 2.8 (D-galactose), 3.1 (D-xylose) and 1.9 (D-glucose).

The determination of reducing sugars in human urine and serum

α -Melibiose does not occur in human physiological fluids. It could therefore be used as an internal standard. Three deproteinization procedures were compared for HPLC sample preparations (Table 2). Deproteinization with acetonitrile generally provided the highest recoveries of the sugars in both urine and serum samples; it was therefore recommended in the present method.

Figures 4 and 5 show typical chromatograms obtained with urine and serum from a normal person according to the above-mentioned procedure. The peak components were identified on the basis of the retention times in comparison with the standard solutions and

Fig. 4 Chromatogram obtained with a temporary urine from a healthy person (male, 24 years old). Peaks and concentrations (nmol/ml urine) in parentheses: 1, *N*-acetyl-D-glucosamine (170); 2, cellobiose (130); 3, maltose (180); 4, lactose and/or L-rhamnose; 5, D-fructose (200); 6, D-arabinose and/or L-fucose; 7, D-galactose (280); 8, D-xylose (230); 9, D-glucose (370); 10, α -melibiose (400, internal standard); 11, endogenous fluorescent compounds; 12, unidentified, probably reducing sugars.

Fig. 5 Chromatograms obtained with a normal human serum (male, 24 years old; 2 h after lunch). Peaks and concentrations (nmol/ml serum) in parentheses: 1, D-mannose (40); 2, D-fructose (40); 3, D-glucose (5010); 4, α -melibiose (400, internal standard); 5, endogenous fluorescent compounds. Detector sensitivities: a, 8; b, 1.

Table 2 Recoveries of reducing sugars added to urine and serum^a

Deproteinizing agent	Recovery (%; mean $\pm$ standard deviation, $n=5$)								
	<i>N</i> -Acetyl-D-glucosamine	Cellobiose	Maltose	D-Mannose	D-Fructose	D-Galactose	D-Xylose	D-Glucose	α -Melibiose
Urine									
Acetonitrile	95 $\pm$ 1	94 $\pm$ 2	91 $\pm$ 2	93 $\pm$ 1	92 $\pm$ 1	94 $\pm$ 2	94 $\pm$ 2	90 $\pm$ 1	88 $\pm$ 1
Trichloroacetic acid	88 $\pm$ 1	86 $\pm$ 1	88 $\pm$ 2	90 $\pm$ 1	89 $\pm$ 2	90 $\pm$ 2	87 $\pm$ 2	86 $\pm$ 2	85 $\pm$ 1
Perchloric acid	91 $\pm$ 2	85 $\pm$ 2	87 $\pm$ 1	87 $\pm$ 2	92 $\pm$ 1	87 $\pm$ 3	91 $\pm$ 2	93 $\pm$ 3	85 $\pm$ 2
Serum									
Acetonitrile	95 $\pm$ 1	94 $\pm$ 1	94 $\pm$ 2	95 $\pm$ 1	93 $\pm$ 1	93 $\pm$ 2	90 $\pm$ 2	89 $\pm$ 1	89 $\pm$ 1
Trichloroacetic acid	90 $\pm$ 1	90 $\pm$ 2	91 $\pm$ 2	90 $\pm$ 1	93 $\pm$ 1	88 $\pm$ 2	89 $\pm$ 2	89 $\pm$ 2	88 $\pm$ 2
Perchloric acid	91 $\pm$ 1	90 $\pm$ 2	88 $\pm$ 2	87 $\pm$ 2	90 $\pm$ 2	86 $\pm$ 2	88 $\pm$ 2	87 $\pm$ 2	88 $\pm$ 2

a. The amounts added to 250 µl of urine and serum, 0.1 µmol each.

Table 3 Urinary 24-h excretion of reducing sugars from healthy persons

Sex ^a	Age	Concentration (μmol/day)							
		N-Acetyl-D-glucosamine	Cellobiose	Maltose	D-Mannose	D-Fructose	D-Galactose	D-Xylose	D-Glucose
M	37	96	9.4	71	N.D.	84.3	73.4	55.3	249
M	30	193	27.1	217	N.D.	283	196	131	357
M	27	145	20.8	141	N.D.	22.4	90.1	95.6	297
M	27	454	45.5	107	N.D.	63.4	99.9	84.9	397
M	24	163	14.0	275	35.6	25.7	108	68.7	297
M	24	175	16.6	107	N.D.	50.7	73.5	85.7	325
M	24	164	14.9	122	N.D.	143	76.9	71.0	361
M	24	193	18.9	141	N.D.	85.6	107	77.0	339
F	24	155	14.6	114	N.D.	45.3	47.2	66.3	283
F	23	147	20.3	107	N.D.	106	81.0	73.9	262
F	23	137	30.1	86	N.D.	45.7	78.0	104	231
F	23	160	14.4	95	14.6	276	79.5	43.3	184
F	21	124	15.0	79	N.D.	124	59.7	80.4	175
F	21	140	19.7	92	N.D.	117	71.6	81.9	189
Mean±SD		175±81.3	20.10±8.77	125±54.2	25.1±10.5	105±79.5	88.7±33.9	79.9±20.5	282±67.8

a. M, male; F, female. N.D., not detected. SD, standard deviation.

also by co-chromatography of the standards and the samples. A peak ascribable to lactose and/or L-rhamnose was observed in the chromatogram for urine (peak 4 in Fig. 4). When the urine sample was subjected to a hydrolysis with β -galactosidase, which cleaves lactose into D-galactose and D-glucose, the height of peak 4 decreased only by 6.5%. This indicates that peak 4 is mostly attributable to L-rhamnose. A peak for D-arabinose and/or L-fucose was seen in the chromatogram (peak 6 in Fig. 4), though it could not be fully characterized. The sugar species identified in the urine and serum were the same as those found by other workers^{14,18-20}, except for cellobiose which was found for the first time in the present study.

Unidentified peaks 12 in Fig. 4 appeared to be due to reducing sugars for the following reasons. Firstly, their fluorescence excitation and emission maxima of their elutes were at 330 and 460 nm, respectively, wavelengths characteristic of reducing sugars.¹¹ Secondly, the peaks all disappeared when *p*-MOED was omitted in the postcolumn derivatization. Finally, catechol compounds (such as epinephrine, norepinephrine, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethyleneglycol and 3,4-dihydroxymandelic acid) were not eluted from the HPLC column under the present conditions, though they could also fluoresce when treated with *p*-MOED.¹¹

The concentrations of reducing sugars in urines and sera from healthy persons were determined by the present method (Tables 3 and 4). The concentrations of reducing sugars measured by the present method agreed with data obtained by other workers.^{14,18-20}

This method should be useful for biological investigations of reducing sugars.

Table 4 Concentrations of reducing sugars in sera from healthy persons^a

Sex ^b	Age	Concentration (nmol/ml)		
		D-Mannose	D-Fructose	D-Glucose
M	27	41.2	43.3	4770
M	27	16.8	55.1	5750
M	24	37.8	39.4	5010
F	23	46.3	23.6	4140
F	23	33.6	39.4	5330
Mean±SD		35.1±10.1	40.2±10.1	5000±540

a. Blood samples were collected at 9:00–10:00 a.m. from healthy volunteers who did not have a breakfast.

b. M, male; F, female.

SD, standard deviation.

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