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Note

Analysis of Palatinit® (isomalt) and its monomers in rat intestinal samples by high-performance liquid chromatography with ultraviolet detection

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The increasing importance of sugar substitutes in human food is based on their reduced cariogenic properties and on a postulated lower caloric value, suggesting possible applications in diets, sweets and drinks comparatively harmless in connection with obesity and diabetes [1].

In earlier investigations, we demonstrated some physiological effects of sugars and sugar substitutes on osmoregulation and glucose supply in the rat after oral administration [2]. Recently, to estimate the caloric utilization of sugar substitutes, intestinal recovery studies were performed with the rat model and revealed dose-dependent hydrolysis and absorption [3]. However, until now, systematic investigations of the caloric availability of these substances in a realistic low dose range of human consumption failed because of the limited sensitivity and selectivity of the analytical methods in physiological fluids.

Among various methods, such as enzyme assays and gas chromatography [4–10], high-performance liquid chromatography (HPLC) has become of increasing interest for the analysis of sugars and sugar substitutes. However, it has generally been used with pure substances or to assay these substances in food [11–15].

This paper describes a sensitive procedure with good reproducibility for the analysis of the disaccharide sugar alcohol Palatinit® and its monomers in a biological matrix (intestinal content of rats). The method, based on HPLC

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with UV detection after derivatization with benzoyl chloride, was shown to have a high sensitivity and low detection limit for carbohydrates [11,12].

EXPERIMENTAL

Animals and sample collection

Male juvenile Wistar rats weighing ca. 100 g were used. After a 16-h fasting period with prevention of coprophagy but with free access to drinking water, the rats were sacrificed after appropriate anaesthetization and the contents of definite intestinal segments were collected. To this matrix, different amounts of Palatinit were added for analysis.

Apparatus and HPLC conditions

The HPLC apparatus consisted of a Beckman chromatographic "Gold" system (Model 116 pump, Model 166 variable-wavelength UV–Vis detector, Beckman, Berkeley, CA, U.S.A.), linked to an injection valve with a 10-μl sample loop (Model 210 A, Beckman) and connected to an IBM PC 286 with Beckman "System Gold" software. The detector was set at 230 nm.

Benzoyl derivatives from disaccharide sugar alcohols and their monomeric compounds were separated on a LiChrosorb Si 60 column (particle size 5 μm, 300 mm $\times$ 40 mm I.D.) coupled to a LiChroprep Si 60 precolumn (particle size 5–20 μm, 12 mm $\times$ 4 mm I.D., both Säulentechnik Dr. Knauer, Berlin, F.R.G.).

Sugar solutions were eluted with a mobile phase of isooctane–diethyl ether–acetonitrile (150:80:20, v/v); the flow-rate was 0.7 μl/min and the column temperature 21 °C.

Reagents

Methanol, isooctane, diethyl ether and acetonitrile (all HPLC grade) and pyridine (dried) were purchased from Merck (Darmstadt, F.R.G.). Benzoyl chloride was obtained from Fluka (Buchs, Switzerland).

Palatinit (isomalt), an equimolar mixture of α-D-glucopyranosido-1,6-sorbitol (GPS) and α-D-glucopyranosido-1,6-mannitol (GPM), was purchased from Bayer (Leverkusen, F.R.G.), glucose from Merck and sorbitol and mannitol from Fluka.

Sample pretreatment and derivatization

Sample preparation was performed as described by Galensa [11], with modifications (i.e. deproteinization and filtration). After centrifugation for 30 min (4500 *g*, 4 °C), 0.75 ml of the intestinal specimen were taken from the supernatant and 1 ml containing 250 μg of ribitol was added as internal standard. After deproteinization with a four-fold volume of methanol for 30 min at −27 °C, a second centrifugation (4500 *g*, 4 °C, 30 min) was carried out, fol-

lowed by filtration of the deproteinized solution through a 0.22-μm membrane filter (Sartorius, Göttingen, F.R.G.).

The sample was then lyophilized in a tapered flask and dissolved in 4 ml of dried pyridine for 30 min at 60°C by sonication. The derivatization was performed for 1 h at 60°C in a supersonic bath (Sonorex RK 102H, Bandelin, Berlin, F.R.G.) by adding 0.5 ml of benzoyl chloride; the benzoyl derivatives were precipitated with 40 ml of distilled water, and the flask was held for 30 min at room temperature. With a Luer Lock syringe the solution was pressed through a Sep-Pak C_{18} cartridge (Millipore/Waters, Göttingen, F.R.G.), which was then washed with 20 ml of distilled water to remove the pyridine. Subsequently, the benzoates were eluted with 50 ml of the mobile phase; 10 μl of this solution were injected onto the column.

RESULTS AND DISCUSSION

The free hydroxyl groups of sugars and sugar substitutes that react with benzoyl chloride form very stable products; accordingly, our derivatized samples and standards could be stored at room temperature for several days with no noticeable chromatographic changes.

Between-day reproducibility and recovery were established for six different amounts of the internal standard ribitol as well as for Palatinit (GPS and GPM) and its monomeric compounds (glucose, sorbitol and mannitol) after adding these substances to blank samples of rat intestine. The recovery and the coefficients of variation (C.V.) were within acceptable limits (Table I).

A typical chromatogram of a derivatized blank intestinal fluid is shown in Fig. 1. An unidentified peak after a retention time of 35 min ("unknown" in Fig. 1) had no influence on the separation of the Palatinit components (Fig. 2). The corresponding retention times were: ribitol, 13.62 min; glucose, 16.41 min; sorbitol, 19.43 min; mannitol, 20.38 min; GPS, 46.53 min; GPM, 48.36 min.

A linear relationship was observed between the ratio of the corresponding peak areas and the internal standard and the amounts of the carbohydrate compounds.

The linear regression equations in the range 10–500 μg (absolute amount added) were: $y=0.003x-0.028$ for glucose; $y=0.004x-0.028$ for sorbitol; $y=0.003x-0.0012$ for mannitol; $y=0.003x-0.022$ for GPS; and $y=0.002x-0.011$ for GPM. The intercepts were not significantly different from zero. The correlation coefficients for the regression lines were better than 0.998 for all substances investigated.

The detection limits were 0.5 ng for glucose, sorbitol and mannitol and 1.0 ng for Palatinit, respectively, at a signal-to-noise ratio of 3 in an injection volume of 10 μl. It is evident that with an increased injection loop volume the detection of even lower concentrations would be feasible. UV detection of ben-

TABLE I

BETWEEN-DAY REPRODUCIBILITY AND RECOVERY OF RIBITOL (INTERNAL STANDARD) AND PALATINIT (GPS, GPM) AND ITS MONOMERIC COMPONENTS (GLUCOSE, SORBITOL, MANNITOL) IN RAT SMALL INTESTINE SAMPLES

Compound	n	Absolute amount (μg)		C.V. (%)	Recovery (mean ± S.D.) (%)
		Added	Found		
Ribitol	4	10	9.0 ± 0.2	2.5	90.5 ± 2.3
		25	22.3 ± 0.3	1.3	89.3 ± 1.2
		50	46.0 ± 0.5	1.1	92.0 ± 1.0
		125	105.1 ± 1.3	1.2	84.1 ± 1.0
		250	239.0 ± 2.6	1.1	95.6 ± 1.1
		500	477.5 ± 14.8	3.1	95.5 ± 3.0
Glucose	4	10	9.5 ± 0.5	5.3	95.2 ± 5.0
		25	22.5 ± 0.5	2.2	89.9 ± 2.0
		50	42.9 ± 1.3	3.0	85.9 ± 2.6
		125	101.5 ± 2.1	2.1	81.2 ± 1.7
		250	237.7 ± 3.2	1.3	95.1 ± 1.2
		500	473.3 ± 20.5	4.3	94.7 ± 4.1
Sorbitol	4	10	9.9 ± 0.2	2.0	98.8 ± 2.0
		25	21.2 ± 0.3	1.4	84.9 ± 1.2
		50	47.7 ± 0.6	1.3	95.3 ± 1.2
		125	105.9 ± 1.5	1.4	84.7 ± 1.2
		250	231.1 ± 1.6	0.7	92.4 ± 0.6
		500	465.7 ± 22.0	4.7	93.1 ± 4.4
Mannitol	4	10	9.7 ± 0.1	1.0	96.6 ± 1.0
		25	21.6 ± 0.3	1.4	86.3 ± 1.2
		50	49.5 ± 0.9	1.8	98.9 ± 1.8
		125	109.0 ± 2.1	1.9	87.2 ± 1.7
		250	229.3 ± 1.8	0.8	91.7 ± 0.7
		500	449.0 ± 17.3	3.9	89.8 ± 3.5
GPS	4	10	8.2 ± 0.4	4.9	82.2 ± 4.0
		25	22.3 ± 0.9	4.0	89.0 ± 3.6
		50	45.5 ± 2.6	5.7	91.0 ± 5.2
		125	101.3 ± 2.3	2.3	81.0 ± 1.9
		250	231.3 ± 6.9	3.0	92.6 ± 2.8
		500	469.5 ± 16.0	3.4	93.9 ± 3.2
GPM	4	10	7.7 ± 0.4	5.2	76.8 ± 4.0
		25	19.0 ± 0.3	1.6	76.0 ± 1.2
		50	40.7 ± 0.4	1.0	81.5 ± 0.8
		125	93.7 ± 2.5	2.7	75.0 ± 2.0
		250	206.1 ± 6.5	3.1	82.5 ± 2.6
		500	411.5 ± 8.0	1.9	82.3 ± 1.6

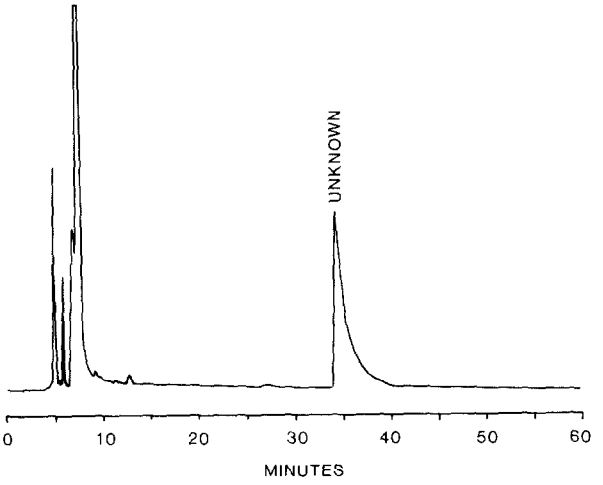

Fig. 1. Typical chromatogram of the derivatized blank small intestinal content of a fasted rat.

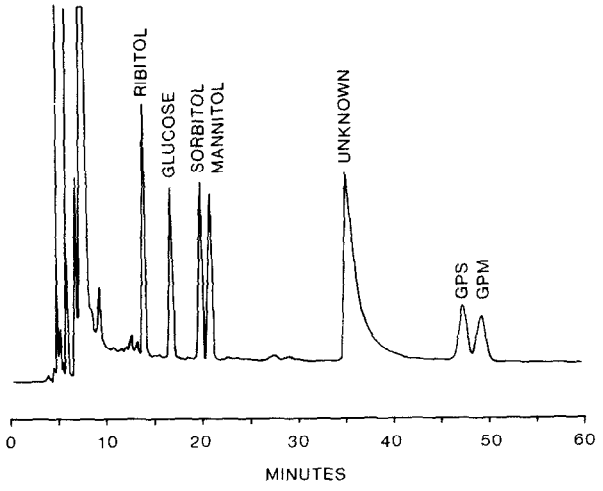

Fig. 2. The same matrix as in Fig. 1, but spiked with 125 μg each of ribitol (internal standard), glucose, sorbitol, mannitol and Palatinit (GPS, GPM).

zoates has the potential to exceed up to 1000-fold the sensitivity of usual HPLC procedures performed with differential refractometers [16]. Unfortunately, it was not possible to shorten the total separation time with the column used; experiments with higher flow-rates led to overlapping peaks (sorbitol/mannitol and GPS/GPM).

In summary, an HPLC assay for the analysis of the disaccharide sugar alcohol Palatinit and its monomeric constituents in a biological matrix is described. The method requires deproteinization, filtration and derivatization by

benzoylation. Separation can be performed with good recovery and reproducibility on a LiChrosorb Si 60 column with subsequent UV detection at 230 nm. Since we were unable to find any other publication on this special problem, we suppose that this is the first attempt to analyse Palatinit in a physiological fluid by HPLC.

It can be concluded, that this method is suitable for recovery and absorption studies of low dosages of sugars and sugar substitutes in biological matrices.

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