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SENSITIVE DETERMINATION OF DERIVATIZED CARBOHYDRATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

The separation and determination of the 4-nitrobenzoates of some monosaccharides, disaccharides and trisaccharides by high-performance liquid chromatography are described. The relatively apolar derivatives permit rapid isocratic separations on polar adsorbents such as silica gel and alumina. The sugar derivatives are detectable with high sensivity, detection limits being in the nanogram region for all sugars tested. The extinction maximum of 260 nm permits the use of low-cost UV detectors (254 nm). A complete analysis takes 70–75 min, 10–15 min of this time being needed for the chromatographic separation. The practical aspects of the method are demonstrated by some examples.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of trace amounts of sugars has previously been almost impossible owing to the low sensitivity of detection. Hitherto, HPLC separations of carbohydrates with various stationary phases have been described[1–5]. The absence of suitable chromophores demands the use of refractive index (RI) detectors, and the detection limit obtained by these detectors is about 20 μg of sugar[5].

Classical liquid chromatographic methods involve the use of cation exchangers[6] or sugar–borate complexes on anion exchangers with gradient elution for separation, which is the principle of commercial sugar analyzers[7–9]. The detection is effected by colour reactions after elution[7,8] and detection limits in the microgram region are achieved. Better results can be obtained by using an oxidative detector that relies on the reduction of cerium(IV) to fluorescent cerium(III) by the eluted carbohydrates[10]. Significant disadvantages of all classical liquid chromatographic methods are that they require several hours for one analysis and no anomer separations are effected.

The separation of perbenzoylated carbohydrates into their anomers by HPLC using gradient elution was described by Lehrfeld[11]. Another possibility for anomer separations is offered by gas chromatography (GC) following silylation. The detection

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limit of such procedures using a flame-ionization detector is about 10 ng of sugar[12].

Recently, the esterification of digitalis glycosides with 4-nitrobenzoyl chloride (4-NBCl) was described[13]. The derivatives showed excellent chromatographic characteristics and detection limits were in the nanogram region[14]. Nitrobenzoates are more suitable than the corresponding benzoates for trace determinations of carbohydrates. The incorporation of an additional chromophoric group into the aryl portion of the derivative results in a shift of the wavelength of maximum absorption from 230 to 260 nm, and the detection limit is lowered by a factor of ten[15].

In this paper, the possibilities of the determination of carbohydrates by HPLC are described.

EXPERIMENTAL

Reagents

Sugars and sugar alcohols were provided by Merck (Darmstadt, G.F.R.), Fluka (Buchs, Switzerland) and Serva (Heidelberg, G.F.R.). LiChrosorb SI 60 silica gel and Alox T alumina (Merck) of particle size 5 μm were used as stationary phases in HPLC. All solvents used were of analytical-reagent grade (Merck). The reagents used for derivatization were described elsewhere[13,14].

Instruments

For HPLC determinations, a Siemens S200 liquid chromatograph equipped with a Zeiss PM-4 spectrophotometric detector (260 nm) was used. The injection systems were a Siemens pneumatic syringe injector (10 μl) and a Siemens loop injector (50 μl).

Derivatization

The reaction is carried out in pyridine. The derivatization was studied with digitalis glycosides and can be used unchanged with all carbohydrates except fructose, for which room temperature (20–22°) must not be exceeded during all reaction and isolation steps. An exact description of the procedure was given elsewhere[13].

HPLC separations

Steel columns, 15 or 20 cm long and 3 mm I.D., were filled by means of the slurry technique using the mixture tetrabromoethane–dioxane–tetrachloromethane (1 : 1 : 1) as solvent for the slurry and *n*-hexane as the pressure solvent[16]. For Alox T, a suspension in methanol, saturated with *n*-hexane, was pressed into the column with *n*-hexane, saturated with methanol. This filling technique resulted in better columns than the "balanced slurry" technique. On applying a pressure of 300 atm, the packing of one column is completed within 10 min.

The wavelength of detection was 260 nm, which is the extinction maximum of 4-NB derivatives of sugars[17]. All separations were performed isocratically at room temperature (20–22°), thermostating being unnecessary.

RESULTS AND DISCUSSION

A problem in the determination of monosaccharides is the equilibrium of at least six compounds (the two pyranoses, the two furanoses, the aldehydo or keto

form and its hydrate) in solution. Hitherto, good separations were possible by the GC of silylated derivatives[12,15–20].

4-Nitrobenzoates permit similar separations by HPLC. D-Glucose and D-mannose, which in aqueous solution exist only in the pyranose form at room temperature[21], result in the expected double peaks of the α- and β-pyranose. The same is true for D-maltose, maltotriose and D-lactose, while D-saccharose, D-raffinose and the sugar alcohols D-mannitol, D-xylitol and D-sorbitol result in a single peak. These findings, together with the results of NMR and UV investigations[17], indicate the absence of decomposition products originating from the esterification reaction and extraction of the derivatives.

Analogous to the silylation in pyridine[18], anomerizations that occur in pyridine during the preparation of the 4-nitrobenzoates for slowly mutorotating sugars such as glucose are minimal, but only if the sugar is not dissolved in pyridine before the derivatization is carried out. If the sugars are kept in pyridine, other anomerization equilibria occur. Similar results were found for the benzoylation reaction of carbohydrates[11]. The method described can be used for the determination of proportions of anomers in the nanogram range for slowly mutorotating sugars (see Fig. 3). However, these separations are disadvantageous for the determination of complex mixtures of monosaccharides as they result in increased peak overlapping between the anomers of different monosaccharides.

Polar adsorbents such as silica gel and alumina are the most suitable for effective separations. They have the advantages that solvents of low viscosity can be used as the mobile phase and it is possible to inject the derivatives, dissolved in chloroform, directly on to the column[14]. For alumina, a strictly reproducible adjustment of the water content is of great importance. This extreme dependence of the k' values on water content for a series of sugar derivatives is shown in Fig. 1. *n*-Hexane was saturated with water, ethyl acetate dried by molecular sieve 4A and a defined volume of water subsequently added. In general, a considerable decrease in the k' values with increasing water content is obvious, tending asymptotically towards a limit. For the derivative of α-D-mannose, this limit is reached in the range investigated. Disaccharides can be eluted from the column only after the addition of water. The peak resolution decreases continuously with increasing water content, except for the pair of derivatives of α-D-glucose and β-D-glucose. It is also interesting that the k' values do not conform with the number of 4-NB groups in the sugar molecules, *e.g.*, the trisaccharide raffinose (with eleven 4-NB groups) is eluted before the disaccharides maltose, saccharose and lactose (each with eight 4-NB groups).

As an example of a separation, Fig. 2 shows the mono-, di- and trisaccharide composition of malt extract (Oxoid, London, U.K.; Code No. L. 39). The malt extract can be derivatized directly, with no pre-treatment. The solvents for the mobile phase, a three-component mixture consisting of *n*-hexane–chloroform–acetonitrile (10:2.5:3) with the addition of 280 ppm of water, were pre-treated as described above: *n*-hexane was saturated with water and chloroform and acetonitrile dried with molecular sieve 4A. The first peaks, unmarked in Fig. 2, originate from chloroform, the solvent for the derivatives, and reagent contaminants.

Glucose, maltose, saccharose and maltotriose could be identified by comparing the retention values with those for reference substances. Whereas the peaks of α- and β-D-glucose were not separated, good separations of the anomers of maltose and

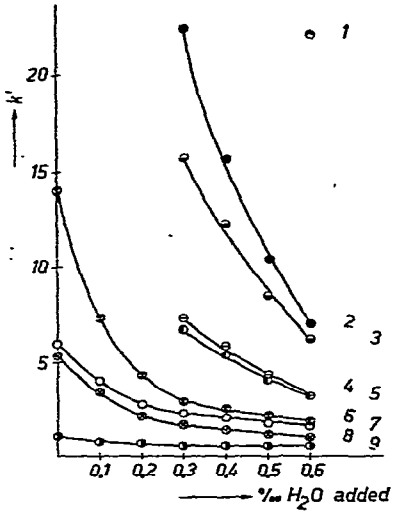


Fig. 1. k' values as a function of the water content of the mobile phase. 1 = α-D-Lactose (8); 2 = D-saccharose (8); 3 = β-D-maltose (8); 4 = α-D-maltose (8); 5 = D-raffinose (11); 6 = D-sorbitol (6); 7 = β-D-glucose (5); 8 = α-D-glucose (5); 9 = α-D-mannose (5). The number of OH groups esterified is given in parentheses. Solvent system: *n*-hexane–ethyl acetate (2:1), with different amounts of water added. Column: Alox T, 5 μm, 20 cm × 3 mm I.D. Flow-rate: 1.8 ml/min, p = 93 atm. Injection volume: 10 μl.

maltotriose were achieved. This considerable improvement in separation with increasing size of the sugar molecules (higher degree of substitution) was also observed on the 4-NB derivatives of digitalis glycosides[14]. LiChrosorb SI 60 has a better selectivity than Alox T for anomer separations. Fig. 3 shows an anomer separation of the

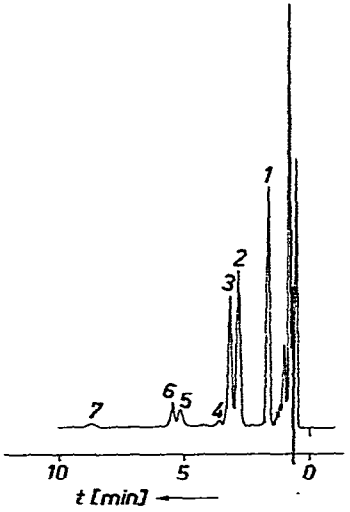
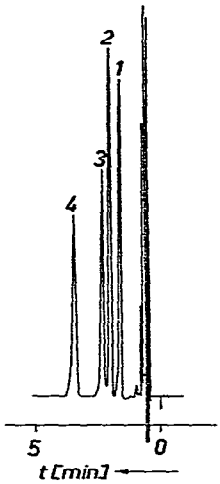


Fig. 2. HPLC of sugars (4-NB derivatives) in malt extract. 1 = D-Glucose (α- + β-); 2 = α-D-maltose; 3 = β-D-maltose; 4 = D-saccharose; 5 = α-maltotriose; 6 = β-maltotriose; 7 = unknown. Solvent system: *n*-hexane–acetonitrile–chloroform (10:3:2.5), 280 ppm of water added. Column: Alox T, 5 μm, 20 cm × 3 mm I.D. Flow-rate: 1.64 ml/min, p = 120 atm. Injection volume: 50 μl.



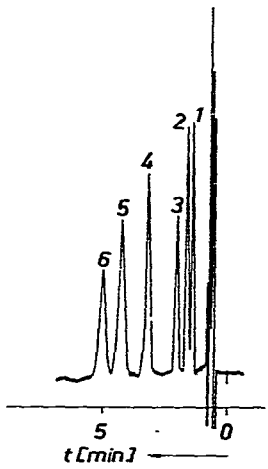


Fig. 3. HPLC of the anomers of D-glucopyranose and 1-methyl-D-glucopyranose (4-NB derivatives). 1 = α-D-Glucopyranose; 2 = 1-methyl-α-D-glucopyranose; 3 = β-D-glucopyranose; 4 = 1-methyl-β-D-glucopyranose. Solvent system: *n*-hexane–ethyl acetate (3:1), with 5% dioxane. Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 2.1 ml/min, p = 155 atm. Injection volume: 10 μl.

Fig. 4. HPLC of four carbohydrates (4-NB derivatives). 1 = α-D-Glucose; 2 = β-D-glucose; 3 = D-sorbitol; 4 = D-saccharose; 5 = α-D-lactose; 6 = β-D-lactose. Solvent system: *n*-hexane–chloroform–acetonitrile–tetrahydrofuran (10:5:1:0.5). Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 1.46 ml/min, p = 116 atm. Injection volume: 10 μl.

4-NB derivatives of D-glucopyranose and 1-methyl-D-glucopyranose. The methyl groups are not split off during the derivatization. On the chromatogram it can also be seen that the sequence of peaks does not necessarily correspond with the number of 4-NB groups in the molecule.

Quantitation

The different anomer ratios of some sugars in the sample and standard in most instances prevent quantitation by comparison of peak heights. It is possible, as previously reported[17], to define a specific extinction coefficient (ε_{OH}): the extinction coefficient of one esterified hydroxy group for the 4-NB derivatives of carbohydrates. This ε_{OH} value is valid for all aldoses tested, but not for fructose. Quantitation by applying the ε_{OH} value after calculation of the peak areas gives good results. For the determination of the peak areas, the peak height was multiplied by the peak width at half-height. For standardization, all carbohydrates that do not form isomers are suitable, *e.g.*, sugar alcohols.

A quantitative determination was carried out with a synthetic mixture of D-glucose, D-sorbitol, D-saccharose and D-lactose, using D-sorbitol as the internal standard. Eight test mixtures were derivatized under identical conditions and 10 μl (syringe injector) or 50 μl (loop injector) of the derivatized solutions injected on to the column. A chromatogram is shown in Fig. 4, and corresponds to the second largest

sugar composition tested (see also Table II, $E = 8$). For the calculation of the sugar content, the following equation was used:

$$C_{sugar} = \frac{A_{sugar}}{A_{standard}} \cdot \frac{A_{standard}}{f} \quad (1)$$

the conversion factor, f, being

$$f = \frac{MW_{standard}}{MW_{sugar}} \cdot \frac{n \cdot OH_{sugar}}{n \cdot OH_{standard}} \quad (2)$$

where

C (ng) = amount (concentration) reacted;
A (mm²) = peak area;
MW = molecular weight;
$n \cdot OH$ = number of hydroxy groups reacted.

The conversion factors for the components of the test mixture are presented in Table I and quantitative results are given in Table II. For a mixture containing C ng of sugar, one calculates C_s ng after HPLC determination and standardization with sorbitol. The given values are averages of eight determinations. For glucose and lactose, the sum of the concentrations of the α- and β-forms are given. An increase in the injection volume from 10 to 50 μl results in an increase in the height of the signal by the same factor. Only the derivatives of α-D-glucose ($k' = 2.20$) and β-D-glucose ($k' = 2.62$) show a small degree of peak broadening. A comparison of the standard deviations of the means at the smallest sugar content ($E = 32$) for injection volumes of 10 and 50 μl shows the advantage of using the greater injection volume. The detection limit is between 0.75 ng (α-D-glucose) and 2.5 ng (β-D-lactose) for an injection volume of 50 μl. The criterion for the calculation of the detection limit is a signal-to-noise ratio of 3. Applying a filter photometer (DuPont 842 UV detector, 254 nm), the detection limit can be decreased by a factor of 4.

Quantitation by external standardization is also possible. This is shown for the determination of D-sorbitol in a confectionery for diabetic patients (Fig. 5). A 1-mg amount of the pulverized confectionery was dissolved in 1 ml of pyridine and 50 μl of this solution was used for derivatization, as described earlier[13]. D-Sorbitol exists in only one isomeric form and results in one peak in the chromatogram. Therefore, quantitation by calculating the peak height is possible. In addition to D-sorbitol,

TABLE I

CONVERSION FACTORS

$n \cdot OH$ = number of substituted OH groups in the molecule; f = conversion factor.

Derivative	$n \cdot OH$	f
Glucose monohydrate	5	0.7661
Sorbitol	6	1.000
Saccharose	8	0.7099
Lactose monohydrate	8	0.6741

TABLE II

QUANTITATIVE DETERMINATION OF SACCHARIDES BY INTERNAL STANDARDIZATION WITH SORBITOL

C = Actual concentration; C_s = concentration found using sorbitol as internal standard; s = standard deviation (8 determinations); V = volume injected; E = amplification factor.

Saccharide	*C (ng)*	*C_s (ng)*	*s (%)*	*V (µl)*	*E*
Glucose (α- + β-)	199.0	195.3	1.7	10	2
	39.8	40.4	1.7	10	8
	49.8	52.0	0.6	50	16
	10.0	9.6	4.5	10	32
	10.0	12.0	4.0	50	32
Saccharose	209.0	218.6	2.6	10	2
	41.8	43.4	2.3	10	8
	52.3	55.4	1.3	50	16
	10.5	11.2	7.4	10	32
	10.5	11.2	2.1	50	32
Lactose (α- + β-)	200.0	190.0	1.1	10	2
	40.0	35.7	2.3	10	8
	50.0	43.9	2.0	50	16
	10.0	9.6	9.1	10	32
	10.0	10.2	2.5	50	32

a significantly smaller amount of some unidentified monosaccharides is detectable in the chromatogram (small peaks before sorbitol). The amount of sorbitol found was 90.3% with a relative standard deviation of 2.3% (seven determinations), compared with a value of 91% stated by the manufacturer. A reference analysis was carried out by another laboratory using polarimetry, and 86.6% of sorbitol was found.

Quantitative determinations of more complex sugar mixtures by external standardization are also possible, as shown by the analysis of a pharmaceutical syrup

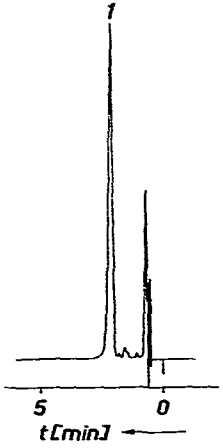


Fig. 5. HPLC of diabetic confectionery after derivatization with 4-NBCl. 1 = D-Sorbitol. Solvent system: *n*-hexane–chloroform–acetonitrile (10:3:2.5). Column: Alox T, 5 µm, 20 cm × 3 mm I.D. Flow-rate: 1.7 ml/min, p = 130 atm. Injection volume: 50 µl.

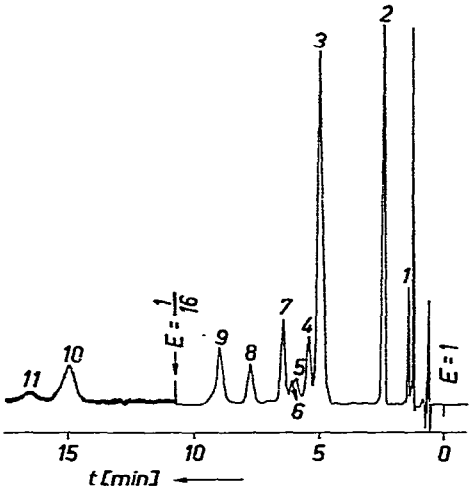


Fig. 6. HPLC determination of mono- and disaccharides (4-NB derivatives) in a pharmaceutical syrup. 1 = Propylene glycol; 2 = glycerine; 3 = D-glucose (α- + β-); 4–7, 9 = isomers of D-fructose; 8 = D-sorbitol; 10 = D-saccharose; 11 = unknown. Solvent system: *n*-hexane–chloroform–acetonitrile (10:3:1.9), 0.1% of water added. Column: LiChrosorb SI 60, 5 μm, 15 cm × 3 mm I.D. Flow-rate: 1.4 ml/min, p = 135 atm. Injection volume: 50 μl.

in Fig. 6. A 10-μl volume of the syrup was pipetted into a test-tube and the water removed by a water suction vacuum within 10 min; the residue was dissolved in 2 ml of pyridine and 25 μl of the solution were derivatized as described above. If fructose is present, all operations must be carried out at room temperature (20–22°); the use of a temperature of 50° for isolation of the derivatives[13] results in artefacts of the fructose derivatives. The derivatives of α- and β-D-glucose are eluted in one peak with the chosen separation system, but D-fructose-4-NB produces five peaks. NMR investigations showed that these five peaks are not artefacts but isomers, which is in agreement with the work of Curtius *et al.*[20], who were able to separate persilylated D-fructose into five components by means of GC; two compounds represent the pyranoside, two the furanoside and one the open-chain form.

The quantitation is made more difficult by the presence of five peaks. It is essential to calculate the peak areas, because the isomer ratio can hardly be kept constant. The peak areas were calculated by cutting the peaks from copies of the chromatograms and weighing the cuttings. This explains the relatively high standard deviation (Table III) in relation to the determination of glucose, which was quantified by cal-

TABLE III

DETERMINATION OF MONO- AND DISACCHARIDES IN A PHARMACEUTICAL SYRUP

s = Relative standard deviation (7 determinations).

Sugar	*Content (%)*	*s (%)*	*Method of calculation*	*Content (%) by reference analysis*
Glucose	21.3	2.4	Peak height	19.9
Fructose	21.5	6.8	Peak area	
Sorbitol	2.7	4.0	Peak height	
Saccharose	0.36	7.2	Peak height	0.36

culating the peak height. As already mentioned, the ε_{OH} value is not valid for fructose-4-NB and external standardization with a fructose standard is therefore necessary. The results of the quantitation are summarized in Table III. The agreement with a reference analysis, made by another laboratory using classical chemical procedures[22], is satisfactory. The finding of the same amounts of fructose and glucose in the syrup permits the conclusion that the monosaccharides were formed during storage by the hydrolysis of saccharose.

CONCLUSIONS

Nitrobenzoates of sugar and sugar alcohols are suitable for the determination of small amounts of these substances by HPLC. Separations on LiChrosorb SI 60 and Alox T are possible within a few minutes. The increase in sensivity by this method, compared with RI detectors, is four orders of magnitude.

Excellent separations of anomers can be carried out. On the other hand, these anomer separations can cause difficulties, especially in the determination of monosaccharides, because of the overlapping of peaks originating from the isomers of different saccharides. Quantitation can be effected by internal and external standardization; for complex mixtures, the latter is more favourable.

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