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Liquid chromatographic analysis of monosaccharides with phenylisocyanate derivatization

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Abstract: Because of the lack of sensitivity in carbohydrate analysis, HPLC pre-column derivatization techniques which give strongly UV absorbing compounds have been reported. These techniques have the disadvantage of leading to several chromatographic peaks from each reducing sugar. To enhance both the sensitivity and the selectivity of such specific separation problems, a simplex procedure was applied to optimize the phenylisocyanate derivatization of monosaccharides: a major and stable derivative was formed under the optimal conditions established. The method was extended to deoxysugars and methylglycosides. The limit of detection was 0.2–1 ng for all sugars tested.

Keywords: *Monosaccharides; reversed-phase LC; phenylisocyanate derivatization; optimization; limit of detection.*

Introduction

The sensitivity of detection of carbohydrates by LC may be increased by pre-column conversion to light-absorbing or fluorescent derivatives [1]. Many such derivatization processes lead, however, to multiple products.

Phenylisocyanate reacts quantitatively with the free hydroxyl groups of sugars by nucleophilic addition to form phenylcarbamate derivatives (phenylurethanes or esters of phenylcarbamic acid):

$$C_6H_5NCO + ROH \rightarrow C_6H_5NHCOOR.$$

This reaction has been applied to the LC detection of hydroxylated compounds [2–4] because phenylurethanes are very stable and strongly absorbing compounds (230–240 nm). The major drawback to the derivatization of sugars with phenylisocyanate is that a chromatographic peak is obtained from each isomeric form (pyranose/furanose, α and β anomers) of the sugar present in the reaction mixture. Overlapping of such peaks was not observed in a previous study [4].

The present work was undertaken to investigate the influence of the derivatization reaction conditions on the chromatogram quality. The modified simplex procedure [5] was applied to determine the optimal conditions of derivatization. The purpose of this investigation was achievement of improved sensitivity and selectivity in respect to the major chromatographic peak from each sugar.

Experimental

Apparatus and chromatographic conditions

Eluent was delivered by a Spectra Physics model SP 8700XR pump (Les Ulis, France) equipped with a Rheodyne injector model 7125 (a 10 μl loop) from Touzart et Matignon (Vitry sur Seine, France). The Shimadzu SPD-2A LC variable-wavelength detector (Touzart et Matignon, Vitry sur Seine, France) was set at 240 nm and the chromatograms were recorded with a Spectra Physics SP 4200 integrator.

A Brownlee Labs ODS 224 RP18 (5 μm, 220 × 4.6 mm i.d.) column from Touzart et Matignon was employed for reversed-phase chromatography, the eluent being acetonitrile–water (60:40, v/v). The mobile phase was pumped through the column isocratically at a rate of 2 ml min^{-1}.

Reagents

The eluent was a mixture of acetonitrile HPLC grade (Carlo-Erba, Paris La Défense, France) and twice-distilled water. The HPLC solvents were continuously degassed with a moderate stream of helium. All sugars were biochemical grade and obtained from Fluka (Buchs, Switzerland) and Merck (Nogent sur

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Marne, France). For the derivatization procedure, phenylisocyanate (PHI) was purchased from Merck; *N*,*N*-dimethylformamide (DMF) and methanol from Prolabo (Paris, France). (Caution: use hood for handling PHI because it is an eye and respiratory irritant and avoid reaction with water.)

Derivatization procedure

Samples were dissolved in DMF to obtain 1.0 mg ml^{-1} concentration of each monosaccharide. One millilitre of each solution was transferred to a screw-capped tube, and a volume (X) ml of phenylisocyanate was added (corresponding to the carbohydrate/PHI molar ratio $1/R$); the mixture was heated at a temperature (T)°C for a duration (D) min in a thermostated block (code 92617, Bioblock Scientific, Illkirch, France). (R, T and D values were determined in the simplex procedure and modified in each experiment.)

In order to destroy the excess of phenylisocyanate, methanol (0.5 ml) was added to the cooled tube. After 5 min, the final volume was adjusted to 6 ml by addition of DMF. This solution could be stored at 4°C for several days with no noticeable change in the chromatograms. A 10 μl volume of this solution was diluted 10-fold with DMF and injected onto the chromatograph. The chromatographic peaks of derivatized sugars were located by comparison with a blank for each assay in the same derivatization conditions as the sample.

Simplex procedure

The modified sequential simplex method [5] was applied to find the optimal derivatization conditions of different monosaccharides.

The derivatization parameters to be optimized were the temperature T, the reaction time D and the carbohydrate/PHI molar ratio: $1/R$.

The parametric space is defined as: T: 20–100°C; D: 15–180 min; $1/R$: 1/50–1/2600.

The quality of each chromatogram was evaluated by an optimization criterion F which is a function of the relative response coefficient and the number of chromatographic peaks:

$$F = \frac{H_i^2}{\Sigma H} \times \frac{1}{N},$$

where H_i = height of the major chromatographic peak; ΣH = sum of the height of all the peaks from the derivatized compounds from each aldose; and N = number of chromatographic peaks from the derivatized compounds from each aldose.

Each following step was determined on the basis of the previous experiments. The worst vertex was discarded and replaced by the reflected point or the reflected contracted point [5].

Results and Discussion

Chromatographic separation: selectivity and sensitivity

The modified simplex procedure was applied to obtain a major derivative from each monosaccharide. The optimal conditions of derivatization were based on an increase in the criterion F value of D-ribose and D-glucose selected as model compounds. Initial conditions of derivatization were chosen to cover all the parametric space. The movement of the simplex was the same for the two monosaccharides and the optimization process was stopped when no noticeable change was observed after nine experimental steps.

The optimal conditions were: 55°C for temperature (T), 1 h 35 min for duration (D), 1/92 for molar ratio ($1/R$) corresponding to 55 μl of PHI.

The chromatograms of D-ribose and D-glucose are illustrated in Fig. 1(A). The first unmarked peaks originate from DMF and artifacts. The peak R, corresponding to sym-triphenylbiuret, was a secondary product from the reaction of PHI with traces of water [6]. This peak constantly eluted at about 6 min under the optimal conditions, and thus was chosen as the reference compound for the relative elution time of sugar derivatives.

The optimal conditions were applied to different hexoses, pentoses, deoxysugars, and methylglycosides. The chromatographic analysis of each derivatized sugar corresponded to satisfactory F values in all cases ($F > 40$). Results are presented in Table 1 with the number of derivatives from each sugar, their relative retention referred to sym-triphenylbiuret and their relative percentage.

The methylglycosides and D-allose gave rise to a single chromatographic peak. In most cases a pair of derivatives was obtained (a major peak with a percentage area up to 84% and a minor peak), although D-ribose and D-arabinose were exceptional (four peaks).

The limit of detection (LOD), defined as a signal-to-noise ratio of 3:1, ranged between 0.2

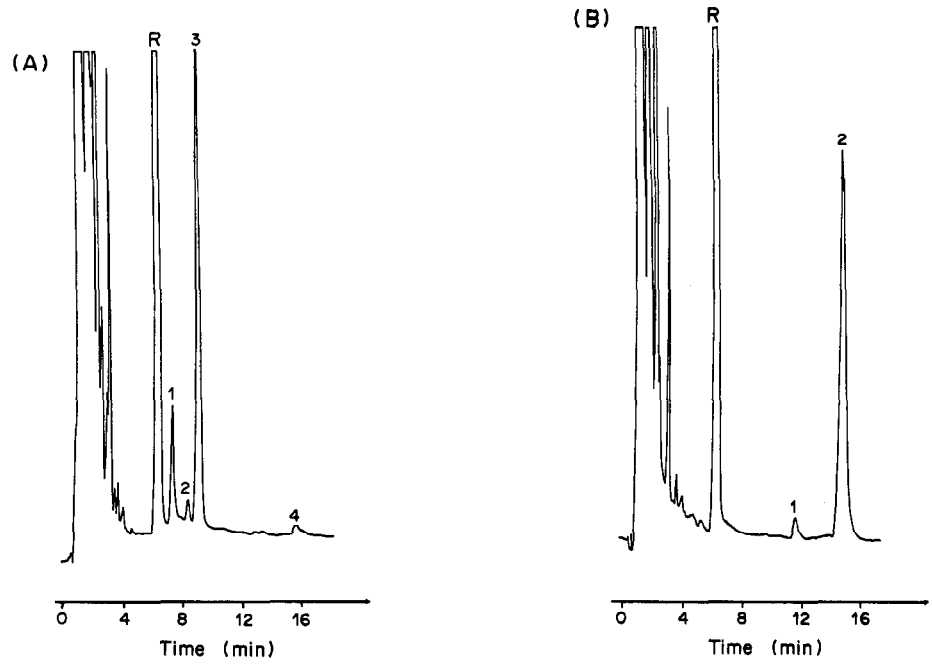

Figure 1
RP-HPLC of phenylcarbamate derivatives of D-ribose and D-glucose. (A) D-ribose (0.22 μg injected). R = reference compound, sym-triphenylbiuret; 3 = major peak; 1,2,4 = minor peaks. (B) D-glucose (0.22 μg injected). R = reference compound; 2 = major peak; 1 = minor peak.

Table 1
Application of the optimal conditions of derivatization to different sugars

Sugars tested	N	Relative retention k'/k'_R	Relative percentage	Limit of detection (ng)
D-ribose	4	1.16	12	
		1.34	3	
		1.49	84	0.2
		2.79	1	
D-xylose	2	1.25	16	
		1.57	84	0.2
D-arabinose	4	1.30	14	
		1.41	5	
		1.66	66	0.2
		1.86	15	
D-lyxose	2	1.51	16	
		2.00	84	0.2
D-glucose	2	2.02	8	
		2.61	92	0.2
D-galactose	2	2.65	6	
		3.29	94	0.2
D-mannose	2	2.43	23	
		3.18	77	0.2
D-allose	1	3.09	100	0.5
2-deoxy-D-ribose	2	0.83	99	
		1.96	0.7	0.5
2-deoxy-D-glucose	2	1.32	86	
		1.57	14	0.5
L-fucose	2	1.52	3	
		2.03	97	0.5
L-rhamnose	2	1.67	8	
		2.26	92	0.5
α-methyl-D-glucoside	1	1.47	100	0.5
α-methyl-D-galactoside	1	1.80	100	0.5
α-methyl-D-mannoside	1	1.79	100	1

N is the number of derivatives formed and k'/k'_R is the relative retention of the derivative with R the reference compound mentioned in the text. The relative percentage of the derivative is calculated on the basis of the sum of peak areas of derivatives formed.

and 1 ng for all sugars tested. These results were compared with the detection limits of HPLC methods using pre-column addition of other strong UV absorbing functional groups. The use of benzoate [7] and nitrobenzoate [8] derivatives permits detection limits of 10 and 1 ng, respectively. 2,4 Dinitrophenylhydrazine, recommended by Karamanos [9] allows detection to 4 ng for fucose and 10 ng for galactose. Golik *et al.* [10] described a method that permits characterization of 100 pg to 1 ng quantities of methylglycosides by perbromobenzoylation or pernaphthoylation. Although this latter technique is sensitive, derivatization overnight was required. Dansylhydrazone derivatives were separated by Alpenfels [11], the limit of detection being estimated to be about 180 ng for galactose. Björqvist [4], using PHI reported a detection down to the 0.5–10 ng level but no details concerning the LOD of each monosaccharide were mentioned.

It is difficult to compare these techniques reliably: as regards the LOD defined above, no comparison of HPLC pre-column derivatization of carbohydrates under standardized instrumental conditions has been presented in the literature. An overall view of these data shows an improvement of the sensitivity by the use of PHI under the optimal conditions described. Golik *et al.* [10] obtained a similar sensitivity but their procedure was more time-consuming.

The stability of derivatives may be mentioned: samples were stored for a week at 4°C with no significant change in the peak ratios and areas, except for methylglycosides whose derivatization and chromatographic analysis must be performed on the same day.

Simultaneous analysis of different carbohydrates

Carbohydrate samples such as those of biological origin, pharmaceutical formulations, food, etc. contain frequently more than one sugar, consequently mixtures of carbohydrates tested above were derivatized under the optimum conditions with a view to facilitate the identification of derivatives and to improve specificity. The chromatograms shown in Figs 2 (pentoses and hexoses), 3 (deoxysugars) and 4 (methylglycosides) give suitable information and complete the data listed in Table 1 in which the retention of every derivatized anomeric form is detailed. The results show the

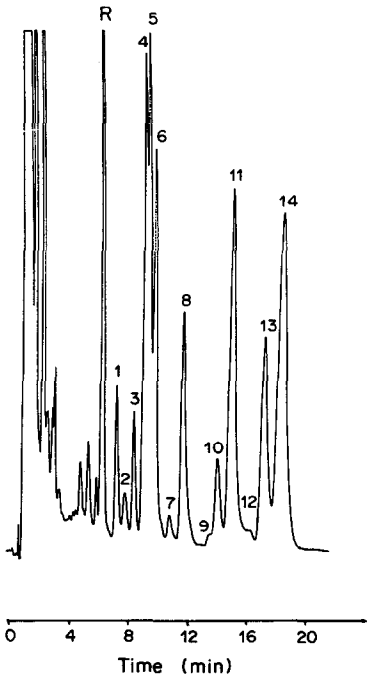

Figure 2
RP-HPLC of pentoses and hexoses phenylcarbamate derivatives (0.11 μg injected). *Major peak; 1, D-ribose; 2, D-lyxose + D-arabinose; 3, D-ribose + D-arabinose; 4, D-lyxose + D-ribose*; 5, D-xylose*; 6, D-arabinose*; 7, arabinose; 8, D-lyxose*; 9, D-glucose; 10, D-mannose; 11, D-glucose* + D-galactose; 12, D-ribose; 13, D-allose*; 14, D-mannose* + D-galactose*.

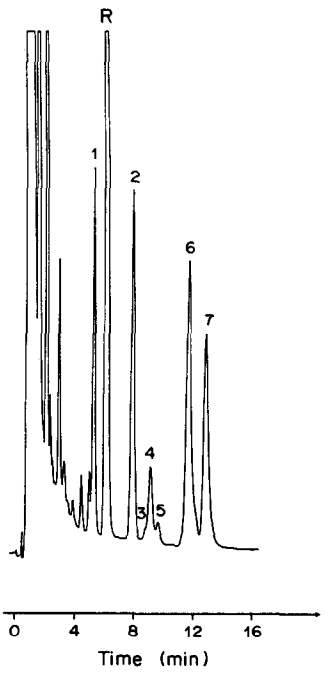

Figure 3
RP-HPLC of deoxysugar derivatives (0.11 μg injected). *Major peak; 1, 2-deoxy-D-ribose*; 2, 2-deoxy-D-glucose*; 3, L-fucose; 4, 2-deoxy-D-glucose; 5, L-rhamnose; 6, L-fucose*; 7, L-rhamnose*.

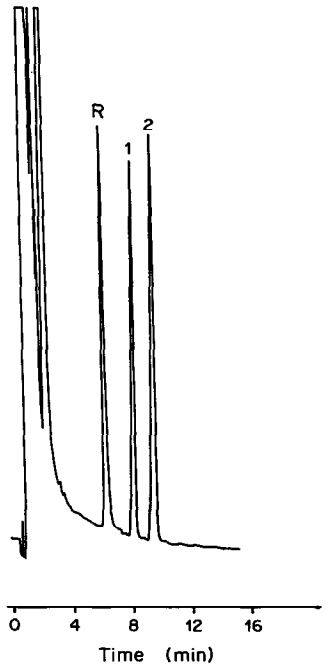

Figure 4
RP-HPLC of methylglycoside derivatives (0.22 μg injected). 1, α-methyl-D-glucoside; 2, α-methyl-D-galactoside + α-methyl-D-mannoside.

separation potential of this analytical technique in a given situation.

Conclusions

The sensitivity and the selectivity of chromatographic analysis of sugars were improved using optimal conditions of phenylisocyanate derivatization. A major derivative was obtained with rapid isocratic elution for each sample on reversed phase. Satisfactory results were observed with configurational isomers of all classes such as pentoses, hexoses, methylglycosides, deoxysugars and detection limits in the nanogram region were achieved (0.2–1 ng). Because of the quick and easy sample preparation, the stability of derivatized samples, and the high sensitivity of the readings, this method is suitable for trace sugar and specific sugar analysis.

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