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DETERMINATION OF CARBOHYDRATES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND OPTICAL ACTIVITY DETECTION

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SUMMARY

Improvements in a detector for liquid chromatography based on optical activity of the components have led to a detectability of 100 ng. This allows the simultaneous determination of six naturally occurring carbohydrates in 100-μl samples of human urine, which is injected directly except for a simple deionization step. The reproducibility and reliability of this method should allow better insight into the relation between urinary sugars and physiological conditions.

INTRODUCTION

The identification and the determination of the hundreds of compounds that are present in body fluids have been beneficial to establishing pathological conditions [1], understanding the molecular basis of diseases, monitoring the therapeutic effects of drugs [2], and maintaining proper mental health [3]. Except for the class of highly specific systems, such as enzyme—substrate or antigen—antibody reactions, the body fluids are in general too complex to be analyzed without some sort of a separation scheme. Very high resolution has been achieved in liquid chromatography for such samples [4, 5], but a combination of slow eluent gradient and small particle sizes for the packing material requires an analysis time of the order of many hours. For physiological profiling and for routine clinical usage, these analysis times are prohibitive. It is much more desirable to restrict the studies to individual classes of compounds, so that a simpler, more specific separation procedure can be performed. Or, in cases where sample manipulation needs to be reduced to a minimum, selective detection schemes for liquid chromatography can be employed to minimize interferences.

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The carbohydrates that are present in body fluids are an interesting class of compounds, and are related to metabolic processes in general. Much attention has been given to the routine clinical screening for glucose [6], because of the disease diabetes mellitus. The accepted method using hexokinase [7] is relatively free from interferences, but requires 500 μl protein-free filtrate. This can become impractical for fetal or pediatric applications. The other simple sugars are often neglected, primarily because of the lack of reliable quantitative methods at the low concentration levels typical of body fluids. Paper chromatography provides qualitative information in a reasonable time [8, 9], but even semi-quantitative results are difficult to obtain at these concentrations. Automated high-resolution analyzers with sensitivities in the μg range have been used to study carbohydrates in body fluids [10, 11], but again hours are required per analysis. More recently, cation-exchange resins have been successfully used for separating dextrose and fructose from the higher saccharides in food products [12], but the limitation on sensitivity makes the scheme unsuitable for studying body fluids. The use of a refractive index detector in these cases is dictated by the lack of convenient UV absorption bands for the carbohydrates, and is a particularly weak link since the columns must be operated at above-ambient temperatures. Post-column colorimetric methods have been used [13], but should be avoided if more reliable and faster methods can be found. Flame ionization detectors have been used [14], but again are not sensitive enough.

Several uses of carbohydrates other than glucose for physiological profiling are known. Excess fructose in urine can be a sign of an inherited metabolic defect [15]. Lactose is present in urine in late pregnancy and during lactation, but excess can indicate a rare metabolic disease [16]. The inherited disease galactosemia causes the presence of galactose in urine, but galactose can also be an indication of severe hepatitis or biliary atresia in neonatal infants [16]. The last condition can lead to liver damage, mental retardation, and cataracts. The presence of xylulose is related to yet another familial disorder [17]. The D-xylose absorption test can be used to diagnose either enterogenous steatorrhea [18] or kidney malfunction. Since the carbohydrates are directly involved in the metabolic cycles of the body, one would expect dietary and metabolic deficiencies to affect carbohydrate profiles in serum and urine. It is therefore important to have reliable methods for analysis, so that correlations can be studied.

A detector for high-performance liquid chromatography (HPLC) that is particularly suitable for carbohydrate analysis is one based on the optical activity of these compounds [19]. This detector eliminates most of the restrictions on the choice of eluents and gradients so that the chromatography can be optimized independently. Since the work published earlier [19], we have made several improvements on the system. These, and the separation of urinary sugars, will be described in this paper.

EXPERIMENTAL

Chromatography

Separations were performed on a heavy metal cation-exchange column that is commercially available (Bio-Rad Labs., Richmond, CA, U.S.A., HPX-85 Heavy Metal). This column has been optimized for the monosaccharides. The operating conditions were as recommended by the manufacturer, i.e., water at a flow-rate of 0.64 ml/min was used as the eluent and the column was maintained at 85°C with a home-built water jacket. All injections were through a 100-μl loop at a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010). To reduce any pressure fluctuations caused by the pump (Milton Roy, Riviera Beach, FL, U.S.A., Model 196-0066-001) at the detector, we used a commercial pulse-dampener (Alltech, Deerfield, IL, U.S.A., Model 9404) in conjunction with a pressure gauge (Alltech, Arlington Heights, IL, U.S.A., Model 9228). Pressure fluctuations were further reduced as a result of having a UV absorbance detector (Spectra Physics Chromatronix, Santa Clara, CA, U.S.A., Model 210) in series and before the optical activity detector. Since the flow-cell was essentially at room temperature, the eluent must be cooled to some extent from 85°C so that turbulence would not exist in the cell. It was found that having the UV detector in series was sufficient for cooling. Alternately, a 50-cm length of standard chromatographic stainless-steel tubing was also satisfactory. Cooling or temperature control, however, was not as critical as in a refractive index detector. Test solutions were all reagent grade material (Fisher Scientific, Fair Lawn, NJ, U.S.A.) dissolved in deionized water. To protect the chromatographic column, urine samples were passed through a mixed-bed (Mallinckrodt, Paris, KY, U.S.A., Amberlite MB-3) ion-exchange column, but otherwise untreated. Identical protection can be achieved using a commercial guard column, so that pretreatment is eliminated from the procedure.

Optical activity detector

The basic arrangement for an optical activity detector for HPLC has been reported earlier [19]. In this work, the laser was operated at 488 nm to better match the spectral response of the photomultiplier tube and to take advantage of the slightly larger specific rotations of these compounds, although these differences are minimal. Since it was found previously [19] that a major source of noise is shot noise from the incompletely extinguished laser beam in the absence of a sample, we introduced intensity stabilization in the laser. This was accomplished by passing the laser light through a Pockels cell (Lasermetrics, Teaneck, NJ, U.S.A., Model 1058-FV) and then a Glan prism (Karl Lambrecht, Chicago, IL, U.S.A., Model MGLS-DW-8) aligned slightly off-axis from the polarization direction of the laser. A photodiode was used to monitor the intensity after this arrangement. After proper amplification, the signal was compared with an adjustable reference level, so that an error signal could be generated. A high voltage operation amplifier (Burleigh Instruments, E. Rochester, NY, U.S.A., Model PZ-70) then provided feedback

to the Pockels cell to control the intensity. The intensity stabilization improved the signal-to-noise ratio and avoided drifting in the baseline in the chromatograms.

The internal volume of the flow-cell has been reduced to 80 μl using a smaller drill-bit without any adverse effects, as predicted in the earlier work [19]. It was found that the cell windows were useful for months unless they were contaminated by the chromatographic effluent, as evidenced by visible deposits. The position of the cell in the optical path was such that the laser beam cleared the cell walls, since scattering caused depolarization. The cell windows were installed for a given position of the cell, but only occasional, minor adjustment by the cell positioner was needed over the period of a week. The entire assembly was mounted on a 2-in. thick optical breadboard (Newport, Fountain Valley, CA, U.S.A., Model LS-48) on a conventional laboratory bench, since we found that sophisticated vibration isolation was not needed. In our effort to reduce stray light, we found that reflections off the window surfaces could be a serious problem, so the cell windows were placed slightly off-normal to the laser beam.

We have improved the Faraday rotators from the previous design [19]. The modulating Faraday cell was placed after the flow-cell. This is an improvement since birefringence in the cell windows depends on the polarization direction, and must be decoupled from the applied modulation for the best signal-to-noise ratio. To increase the efficiency of the modulation driver, we used two matched Faraday rotators with windings in opposite directions. The pair was thus driven during alternate half-cycles of the square wave. This also guaranteed that electronic zero was always maintained in the lock-in amplifier and that the point of maximum extinction with the modulators off was the best setting for the analyzing polarizer. The modulating field was effectively four times that used previously, in view of eqn. 2 in ref. 19.

For the feasibility studies using the He—Ne laser (Spectra Physics, Mountain View, CA, U.S.A., Model 134), a 2X beam expander based on a Galilian telescope was used before the focusing lens so that the proper beam-waist can be achieved at the cell. In that case, an Amperex 56TVP phototube (North American Philips, Hicksville, NY, U.S.A.) was used to provide a better spectral response.

RESULTS AND DISCUSSION

The modifications in the detection system have led to a limit of detection of 100 ng of fructose injected (signal-to-noise ratio = 3) when two standard 10-μm C_{18} columns were used in series to increase the chromatographic efficiency. The limit in detectability seems to be a combination of (1) residual depolarized light through the crossed polarizers, (2) residual pumping noise in the eluent which causes the windows to distort minutely, (3) the remaining ±1% noise in the laser, and (4) dust particles causing flickering in the optical path. It appears that further improvements can be made, perhaps to a detectability of 20 ng.

In the course of identifying sources of noise, we used a 2-mW He—Ne laser as the light source in place of the argon ion laser. There is much more short-

TABLE I

CONCENTRATIONS OF SUGARS IN URINE

Sugar	Concentration (μg/ml)*		
	This work	Ref. 10	Normal range
Sucrose	22	12	0—50
Lactose	10	49	0—100
Glucose	16	24	10—120
Xylose	10	10	0—30
Arabinose	7	26	0—30
Fructose	7	2.7	0—50

*All concentrations are ±10%.

term fluctuation in the He—Ne laser, so that the intensity cannot be stabilized as well. The lower light level also makes it more difficult to identify sources of stray light and to eliminate them. Even with these problems, we were able to obtain a detectability of 1 μg (signal-to-noise ratio = 3) for fructose under the same conditions. This demonstrates that photon statistics was not the limiting factor in detectability in the case of the argon ion laser. For the He—Ne laser, however, photon statistics was a major contribution. We estimate that a laser with a power of 10—50 mW can be used instead of the expensive argon ion laser, without sacrificing performance. We also found that when the lower power laser was used, the system took less time to warm up. This is because heating causes changes in birefringence in the polarizing crystals as well as the cell windows, and lower laser powers are desirable, up to the photon statistics limit.

Test solutions of individual sugars were run to determine the retention times under our experimental conditions. In general the order of elution and chromatographic efficiency were as specified by the manufacturer. No effort to further optimize was made. The results of the analysis of human urine are shown in Fig. 1. We have identified six urinary carbohydrates, based on the specificity of this column, retention times as correlated to injection of test solutions of sugars, the lack of UV absorption at the corresponding locations, the sign and magnitude of the individual specific rotation, and the estimated concentration levels in normal human urine [10]. The last comparison is shown in Table I, with the method for calculation given below. It is clear that determination of these carbohydrates as outlined here presents no problems. The peaks that appear prior to sucrose represent the unretained components and the higher saccharides, in that order. The major peak at 27 min does not seem to correlate with any of the carbohydrates tested, although one would expect species similar to mannitol and sorbitol to elute about then. It is interesting to note that the three major peaks in the UV detector correspond to a shoulder, a minor peak, and nothing at all in the optical activity detector. This emphasizes the substantial differences in the two detectors for studies in biological fluids. The UV peaks also indicate that even if a refractive index detector can approach this level of sensitivity,

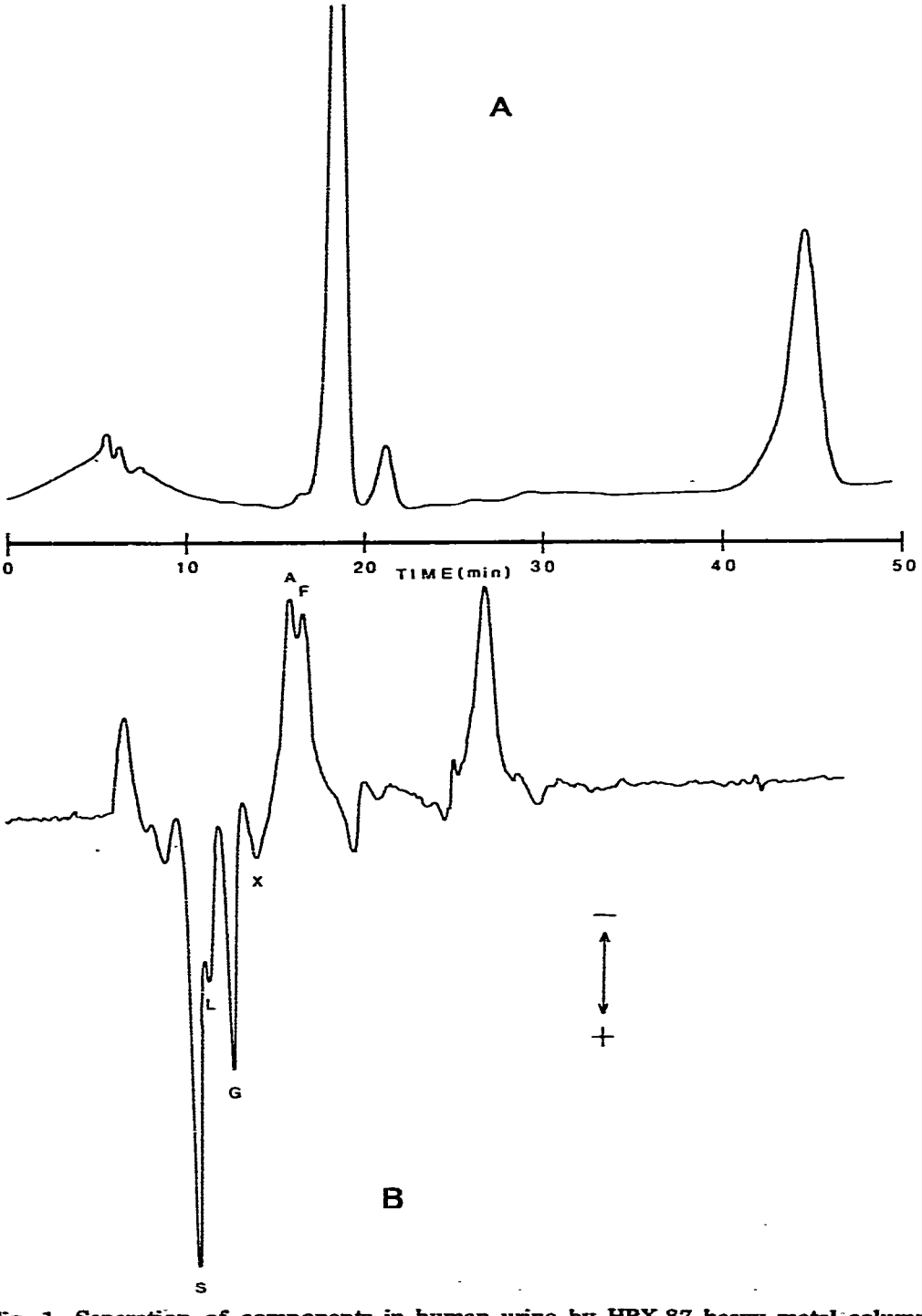

Fig. 1. Separation of components in human urine by HPX-87 heavy metal column. (A) UV detector, (B) optical activity detector. Peaks: S, sucrose; L, lactose; G, glucose; X, xylose; A, arabinose; F, fructose.

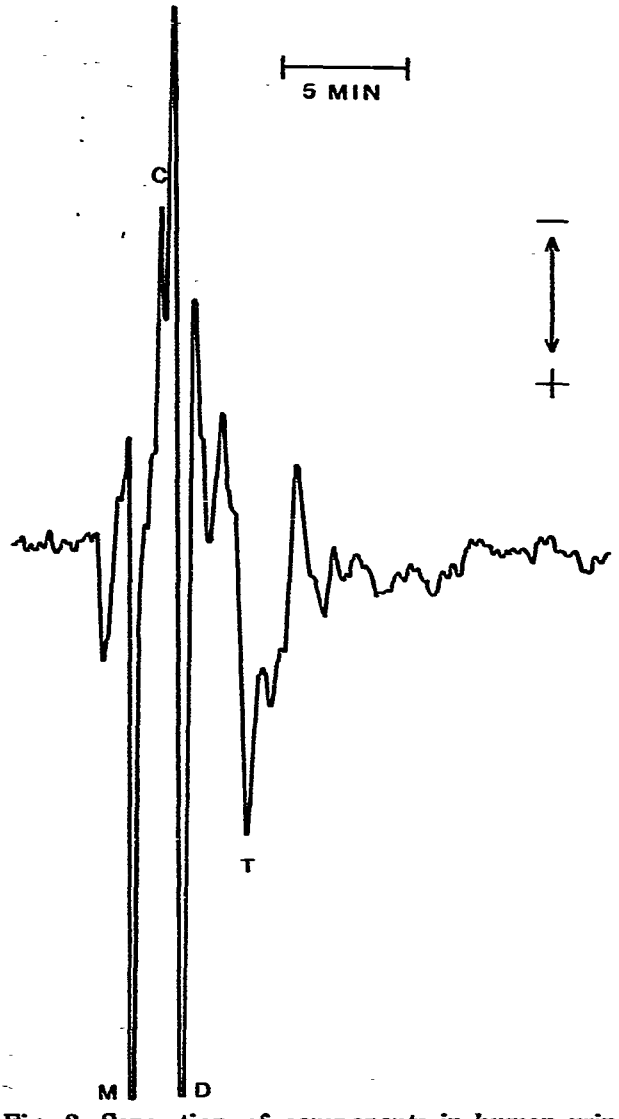

Fig. 2. Separation of components in human urine by 10-μm C_{18} reversed-phase column and optical activity detector. Peaks: C, cystine; M, monosaccharides; D, disaccharides; T, trisaccharides.

additional interferences will be present and limit its usefulness. The chromatographic efficiency can probably be further increased by lowering the flow-rate or by using a second column in series, but the simplicity of the chromatogram makes such a compromise with the analysis time not necessary. We have also studied the same deionized urine sample using two C_{18} reversed-phase columns in series, and the chromatogram shown in Fig. 2 is a substantial improvement over our earlier work [19]. We note that most of the sugars identified in Fig. 1B showed up as the second major positive peak, which was off-scale. This was accompanied by turbulence in the flow-cell caused by the large refractive index change, a condition that never oc-

curred in Fig. 1B. Even though the sugars and cystine are the only components positively identified, one can see that potentially useful information is present, particularly since very few of these peaks also show up in the UV detector.

Calculations of the concentrations are straightforward. Although naturally standards must be used for checking, one can use the absolute standard given by a d.c. Faraday cell of known properties at a known current. In this work we used a current of 0.25 A to produce a field of 250 G. With air, this corresponds to a net rotation of 0.28 millidegrees. Using the specific rotations $[\alpha]$ for the Na D line, which are not too different for this wavelength, one has:

$$c = \frac{\alpha}{[\alpha]} \tag{1}$$

where c is the concentration in g/ml at the detector, and α is determined by the ratio of the peak height and the effective height of the d.c. Faraday rotation, multiplied by 0.28 millidegrees, since our cell is 1 dm in length. The $[\alpha]_D$ values were used taking into account mutarotation. The peaks in Fig. 1 are typically in an eluent volume much larger than that of the flow-cell, so that a triangular approximation can be used for the area. Conversion to total quantity injected is then simple, knowing that the flow-rate is 0.64 ml/min. Since the chromatography was highly reproducible, one could deconvolute the overlapping peaks using a Gaussian shape if desired. However, the fluctuations in human urine did not warrant such an attempt at high accuracy in this work, and the simple triangular approximation was used. The results of this particular urine sample are presented in Table I, together with some other reference values [10]. We find that the determined values are reasonable. For routine applications, one would probably rely on peak height measurements established by standard solutions, rather than use the absolute standard method described here.

In summary, we have refined our method of detection of optically active components in HPLC. Sensitivity is sufficient for the study of urinary carbohydrates, particularly the monosaccharides. We report a straightforward procedure for the determination of six sugars simultaneously in human urine by direct injection, a scheme which should allow more clinical studies correlating these with various physiological conditions.

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