Synthesis of Dabsylhydrazine and Its Use in the Chromatographic Determination of Monosaccharides by Thin-Layer and High-Performance Liquid Chromatography

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The chromophoric reagent dabsylhydrazine is synthesized from the reaction of dabsyl chloride with hydrazine. Dabsylhydrazine reacts readily with various monosaccharides including glucose, galactose, mannose, xylose, arabinose, ribose, deoxyribose, and glyceraldehyde to form corresponding dabsylhydrazones with strong absorbance at 425 nm. The application of this new reagent in the thin-layer chromatographic and high-performance liquid chromatographic analysis of monosaccharides is described. By use of a Nova-PAK C_{18} reverse-phase column and a concave gradient system of water and acetonitrile as eluent, detection limits in the range of 10 pmol (2 ng) have been reached. The optimum pH for the formation of monosaccharide dabsylhydrazones is around 2-3. The rate of hydrazone formation is different among the monosaccharides investigated. Generally, pentoses reacted more rapidly than hexoses. The present method has been successfully applied for the determination of glucose concentrations in human serum and various fruit juices. The contents of galactose and mannose released from serum glycoprotein on acid hydrolysis have been rapidly determined by the newly developed dabsylhydrazone method.

Monosaccharides are ubiquitous in the biosphere. Qualitative and quantitative analyses of these carbohydrates in biological specimens have become an exciting challenging subject for many biochemists. Recently, several new methods have been developed for the analysis of saccharides. Gas-liquid chromatographic methods have been improved for easier detection and higher sensitivity (*1*). High-performance liquid chromatography (HPLC) on anion-exchange resins (*2*, *3*) or bonded-phase silica columns (*4-6*) reduce sample preparation and give great flexibility. However, the sensitivities of refractive index and ultraviolet absorbance monitors are quite low for simple sugars. A previous procedure employed dansylhydrazine to label the reducing end of sugar with a fluorescent tag (*7*) for the determination of these monosaccharides as their dansylhydrazones by HPLC was developed (*8*). The somewhat instability of fluorophore emitted from dansyl derivatives hampered the accuracy and reproducibility of this method. It is apparent that a stable and chromophoric labeling reagent for sugar analysis is urgently needed.

In 1975, dabsyl chloride was first synthesized in this laboratory (*9*). During the last 10 years, in combination with HPLC this newly developed chromophoric reagent has been demonstrated to be very promising for the microdetermination of amino acids (*10-13*), aliphatic amines (*14*, *15*), and polyamines (*16*, *17*). Recently, dabsylhydrazine was synthesized from the reaction of dabsyl chloride with hydrazine. This new compound reacted readily with monosaccharides and gave corresponding chromophoric dabsylhydrazones. Herein is described a new procedure for the separation and quantitation of monosaccharide dabsylhydrazones by both thin-layer chromatography (TLC) and HPLC procedures.

EXPERIMENTAL SECTION

Instrumentation. Electronic spectra were recorded on a Shimadzu UV-200S double-beam spectrophotometer. IR spectra were measured in a Perkin-Elmer 983 IR spectrophotometer using a pressed pellet consisting of 1 mg of sample and 200 mg of KBr. NMR spectra were taken in a NMR spectrophotometer, JEOL, FX-100, Japan Electronic, ltd., Tokyo, and mass spectra were studied in a Finnigan 4510 quadrupole mass spectrometer, Palo Alto, CA.

HPLC separations were performed on a Waters Associates instrument with a two-pump Model 6000 solvent delivery system, a Model U6K manual injector, a Model 450 absorbance monitor at 425 nm, and a Model 660 solvent programmer. The recorder was a OmniScribe strip chart recorder, Model B 5000 (Houston Instruments, Austin, TX). The HPLC was performed on a Waters Associates Nova-PAK C_{18} (3.9 mm × 15 cm), 4-μm, or μBondapak C_{18} (4 mm × 30 cm), 10-μm, reverse-phase column. A concave gradient elution program (curve 7 or 8) was used: solvent A, water–acetonitrile (78:22 (v/v)); solvent B, acetonitrile (10–85%, 45 min or 15–85%, 45 min). A flow rate of 1.2 mL and an AUFS (absorbance unit at full scale) of 0.02–0.04 were normally employed.

TLC analysis on silica gel plates (E. Merck, Silica Gel 60 F254, catalog no. 5554) was performed in a rectangular or cylindrical glass chamber covered with a glass plate. The inner side of the chamber contained Whatman No. 1 filter paper to prompt and ensure the saturation of solvent vapor in the chamber. The following solvent systems were normally used: A, 1-butanol (water saturated)–triethylamine (30:1 v/v)); B, acetonitrile–1-butanol–hexane (20:2:1 (v/v)); C, acetonitrile–benzene–ethyl acetate (15:15:1 (v/v)); D, acetonitrile–1-butanol–ethyl acetate (20:2:1 (v/v)); and E, benzene–chloroform–ethanol (15:15:10 (v/v)).

Chemicals. D-Glucose, D-galactose, D-mannose, D-fructose, D-arabinose, D-xylose, D-ribose, D-deoxyribose, and D-glycerose (D-glyceraldehyde) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrazine hydrate, ammonia, hydrochloric acid, tetrahydrofuran, and acetonitrile were purchased from E. Merck Co. (Darmstadt, Germany). Dabsyl chloride (4-[[4-(dimethylamino)phenyl]azo]benzenesulfonyl chloride, mp 186–188 °C) was synthesized by reacting sodium 4-[[4-(dimethylamino)phenyl]-azo]benzenesulfonate with phosphorus pentachloride (*9*) or by chlorosulfonation of [[(dimethylamino)phenyl]azo]benzene (*18*).

Synthesis of Dabsylhydrazine (Figure 1). Dabsyl chloride (100 mg) was dissolved in 25 mL of tetrahydrofuran and mixed with 0.2 mL of hydrazine hydrate. The resulted mixture was allowed to stand at room temperature for 30 min. After concentration, orange-yellow crude products (44 mg, yield 44.6%) were obtained. The crude products were further purified by recrystallizing with 30–40 mL of boiling ethanol to give orange crystals (30 mg), mp 163–164 °C. Anal. Calcd for $C_{14}H_{17}N_5O_2S$: C, 51.79; H, 5.07; N, 20.14. Found: C, 51.85; H, 5.06; N, 20.26.

Formation of Monosaccharide Dabsylhydrazones (Figure 1). A 1.0-mL sample containing 1–20 nmol of monosaccharide in ethanol containing 0.1% acetic acid is mixed with 1 mL of 0.1% dabsylhydrazine in ethanol. The mixture is heated at 60 °C for 60 min and then cooled to room temperature. The same procedure can also be performed with smaller samples (5–20 μL) of test solution, keeping the same proportions of reagents as described

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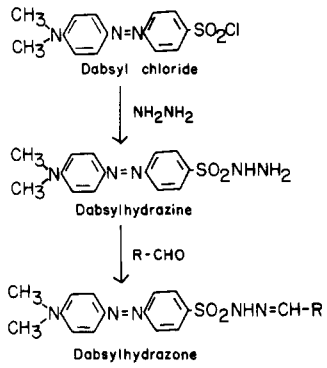

Figure 1. Synthesis of dabsylhydrazine and its reaction with a carbonyl compound.

above. A control tube containing no monosaccharide is used as a reference system to aid in the detection of dabsylhydrazine and its degradation products. The reagents have been stored for weeks in the dark at 4 °C with no loss of potency.

TLC of Monosaccharide Dabsylhydrazones. Samples of the reaction mixture (2–10 μL containing 2–10 pmol of monosaccharides) are applied to starting points of silica gel plates and then developed by the described TLC procedure. The monosaccharide dabsylhydrazones appear as bright yellow spots and dabsylhydrazine also appears as a yellow spot which is usually well separated from the hydrazones. All these yellow compounds appear as bright pink spots when the developed TLC plates were briefly exposed to hydrogen chloride vapor in a closing chamber. The sensitivity of monosaccharide detection was enhanced dramatically after this treatment. The yellow spots may be scraped off the plate into a conical centrifuge tube and the hydrazones are then eluted with ethanol for quantitative analysis.

HPLC of Monosaccharide Dabsylhydrazones. Samples of the reaction mixture obtained as described are passed through a Millipore filter (0.45 μm, HAWP catalog. no. 02500, Millipore Corp., Bedford, MA) and the filtrates (1–10 μL) were used directly for HPLC. For the separation of neutral sugars including glucose, galactose, mannose, xylose, arabinose, ribose, deoxyribose, and glycerose, a concave elution program was employed. The details of the chromatographic conditions are described in the instrumentation section.

HPLC Procedure for the Determination of Glucose in Grape Juice. The original juice of Californian green grapes (seedless, sweet) is obtained by gentle pressing. Four-tenth (0.4)-milliliter aliquots of the clear juice is placed in a vial and lyophilized to dryness. The residue is extracted with 2 mL of warm absolute ethanol (50 °C) and passed through a Millipore filter (0.45 μm). A 1-mL aliquot of the filtrate is taken and reacted with 1 mg of dabsylhydrazine in 1 mL of ethanol at 60 °C for 1 h. The reaction mixture is cooled to room temperature and passed through the Millipore filter. One- to ten-microliter aliquots of the filtrates were used for HPLC. A glucose standard solution (0.1 mg/mL) in ethanol is run simultaneously and used as control.

Other fruit juices such as pineapple, pear, watermelon, banana, and plum were obtained by homogenization and analyzed by the same procedure.

HPLC Procedure for the Determination of Glucose in Human Serum. Human serum (0.2 mL) is deproteinized by homogenizing with 5 mL of absolute ethanol. The mixture is allowed to stand at room temperature for 30 min and then passed through a Millipore filter (0.45 μm). A 1-mL aliquot of the filtrate is mixed with 5 mL of absolute ethanol and again passed through a Millipore filter. A 1-mL aliquot of the filtrate is reacted with 1 mg of dabsylhydrazine in 1 mL of ethanol at 60 °C for 1 h. The reaction is remarkably accelerated when 0.1% of acetic acid is added to the reaction mixture. The reaction mixture is brought to room temperature and passed through a Millipore filter. Finally, 1–5-μL aliquots of the filtrate are taken for HPLC. Authentic glucose (0.1–1 mg/mL) solution in ethanol is reacted with dabsylhydrazine in a similar way and used as standard for HPLC determination.

HPLC Procedure for the Determination of Galactose and Mannose in Serum Glycoprotein. Serum glycoproteins are isolated by the procedure of Mrochek et al. (2) with some modifications. Human serum (0.05 mL) is thoroughly mixed with 0.55 mL of 95% ethanol in an Eppendorf tube. After centrifugation at 5000 rpm for 15 min, the precipitate in the bottom of the tube is washed with 0.6 mL of ethanol and dissolved in 0.1 mL of 0.1 N NaOH. The resulting solution is mixed with 0.2 mL of doubly distilled water and 30 μL of concentrated hydrochloric acid (approximately 12 N). The mixture is deaerated with nitrogen for 5 min and sealed tightly with a septum-sealed cap. The hydrolysis is carried out at 100 °C in an electric oven for 4 h; 0.1 mL of the hydrolysate is removed and freeze-dried. The residue is reacted with 1 mg of dabsylhydrazine in 1 mL of ethanol at 60 °C for 1 h. The reaction mixture is brought to room temperature and passed through a Millipore filter (0.45 μm, HAWP). Finally, 1-μL aliquots of the filtrate are taken for HPLC analysis.

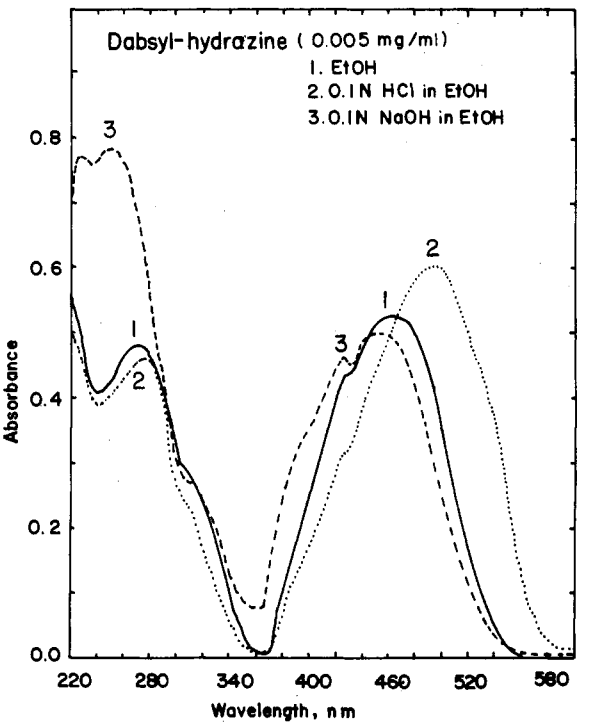

Figure 2. Electronic spectra of dabsylhydrazine.

Table I. Molar Absorptivities of Dabsylhydrazine and Its Glucose Derivative

	dabsylhydrazine		glucosedabsyl-hydrazone	
solvent	absorption max, nm	molar absorptivity ($\times 10^{-4}$)	absorption max, nm	molar absorptivity ($\times 10^{-4}$)
ethanol	463	3.3	460	5.4
	425	2.7	440	5.3
	272	3.0	422	5.0
	220	3.5		
0.1 N HCl in ethanol	494	3.8	495	5.1
	425	2.9	425	2.7
	275	2.9		
	220	3.0		
0.1 N NaOH in ethanol	452	3.1	445	3.8
	425	2.0	424	3.9
	250	4.9		
	229	4.8		

RESULTS AND DISCUSSION

Characterization of Dabsylhydrazine. Dabsylhydrazine was synthesized as described in the Experimental Section. The new compound was fully characterized by its elemental analysis, IR, UV, visible (Figure 2), NMR, and mass spectra.

Table II. TLC Data of Dabsylhydrazones Derived from Monosaccharides

monosaccharide	R_f[a] A	B	C	D	E
dabsylhydrazine	0.88	0.92	0.90	0.83	0.80
glucose	0.64	0.52	0.09	0.51	0.59
galactose	0.56	0.42	0.09	0.42	0.50
mannose	0.64	0.39	0.09	0.42	0.52
fructose	0.48	0.77, 0.71	0.19, 0.10	0.81, 0.73	0.70
ribose	0.63	0.80	0.59	0.78	0.75
deoxyribose	0.60	0.80	0.55	0.78	0.75
xylose	0.69	0.75	0.42	0.75	0.72
arabinose	0.50	0.68	0.35	0.68	0.65
glyceraldehyde	0.52	0.92	0.56	0.88	0.47

[a] Solvent systems: A, 1-butanol (water saturated)–triethylamine (30:1 (v/v)); B, acetonitrile–1-butanol–hexane (20:2:1, (v/v)); C, acetonitrile–benzene–ethyl acetate (15:15:1 (v/v)); D, acetonitrile–1-butanol–ethyl acetate (20:2:1 (v/v)); E, benzene–chloroform–ethanol (15:15:10 (v/v)). The R_f values are the averages of three determinations.

The molar absorptivities of dabsylhydrazine and its glucose derivative in neutral, acidic, and alkaline media are given in Table I.

Dabsylhydrazine showed a typical azo dye peak at 463 nm which was shifted to a longer wavelength, 494 nm, in acidic medium. A similar shift was also observed in its glucosehydrazone derivatives. IR spectra (KBr pellet, cm^{-1}): 3350 ($-NH_2$), 3220 ($-SO_2-NH-$), 1602 (-N=N-), 1515, 1420, 1361, 1334, 1162, 1134 ($-SO_2-NH-$), 849, 821, and 600. NMR spectra ($CdCl_3$, Me_4Si as internal reference, ppm): 3.08 (singlet, ^6H, $-N(CH_3)_2$), 3.62 (doublet, ^2H, $-NH_2$), 5.56 (triplet, ^1H, -NH-), 6.72 (quadruplet, ^2H, two aromatic hydrogens ortho to the dimethylamino group), 7.86 (quadruplet, ^2H, two aromatic hydrogens meta to the dimethylamino group), and 7.92 (singlet, ^4H, four aromatic hydrogens on the benzene ring linked to the sulfonamide group). Mass spectra (m/e, relative intensity, %): 319 (4, molecular ion, M^+), 304 (0.3, $M - CH_3$), 303 (0.2, $M - NH_2$), 224 (3, $M - SO_2NHNH_2$), 136 (100), and 150 (56).

On the basis of these spectrometric features, the structure of dabsylhydrazine was characterized as 4-[[4-(dimethylamino)phenyl]azo]benzenesulfonylhydrazine as depicted in Figure 1.

Reaction of Dabsylhydrazine with Monosaccharides. Monosaccharides were readily reacted with dabsylhydrazine. Formation of the dabsylhydrazones of individual monosaccharides or their mixtures was found to be proportional to their initial concentrations (Figure 6). The rate of hydrazone formation at less acidic solutions was appreciably slower. Hydrazone formation was found to be complete within less than 60 min when the system was heated up to 60 °C. The sugar dabsylhydrazones were quite stable in the acidic solution and could be chromophorically analyzed by HPLC and TLC.

Many reducing sugars such as glucose, galactose, mannose, arabinose, xylose, ribose, deoxyribose, and glycerose yielded hydrazones with different mobilities on silica gel plates. The rates and yields of hydrazones formed with free hexoses were similar to that observed for glucose. Glycerose and pentoses including xylose, arabinose, ribose, and deoxyribose reacted very rapidly with dabsylhydrazine and yielded high levels of hydrazones under the conditions described. Prolonged heating or even standing at room temperature seemed to increase the yields of hydrazones. It was noted that if hydrazone formation was conducted at pH 4 or higher, ketose (fructose) interacted very slowly with dabsylhydrazine. The reaction of fructose was promoted by lowering the pH to 2 with 0.1 M trichloroacetic acid.

Preparation and Purification of D-Glucosedabsylhydrazone. L-Glucose (0.5 mg) in 1 mL of ethanol was mixed with 0.5 mg of dabsylhydrazine in 1 mL of ethanol and 0.01 mL of acetic acid. The mixture was heated at 60 °C for 1 h. The D-glucosedabsylhydrazone thus formed was isolated by TLC on a silica gel plate (0.2 mm, E. Merck, catalog no. 5554) with a solvent mixture of hexane–1-butanol–acetic acid (50:50:1 (v/v)). Two major spots, R_f = 0.49 (hydrazone) and R_f = 0.60 (dabsylhydrazine) were obtained. The hydrazone spots were scraped off the plate into a standard conical centrifuge tube and eluted with ethanol. After removal of the solvent, an orange-yellow crystalline powders of D-glucosedabsylhydrazone (mp 308–309 °C) was obtained. The hydrazone gave the following spectrometric properties. IR spectra (KBr pellet, cm^{-1}): 3600–3300 (OH group of sugar), 3000–2900 (=N-NH-), 1600–1500 (-N=N-), and 1415 ($-SO_2-NH-$). Mass spectra did not show up the molecular ion 481 (M^+) but gave a significant peak at 466 ($M - CH_3$).$^+$ The electronic spectra of the product were analyzed and are summarized in Table I. With these spectrometric features, the product was tentatively identified as D-glucosedabsylhydrazone.

TLC Separation of Monosaccharide Dabsylhydrazones. The monosaccharide dabsylhydrazones were prepared from the reaction of individual sugar with dabsylhydrazine as described. A sample of the reaction mixture (2–10 μL containing 0.5–100 nmol of sugar) was applied to starting points on silica gel plates and then developed by the standard TLC procedure as described in the Experimental Section. The R_f values of these monosaccharide dabsylhydrazones are summarized in Table II. The chromophoric properties of these hydrazones provided a reliable marker for rapid detection of these monosaccharides on TLC plate. The lowest limit of visual detection of the chromophoric spot on the plate was found to be in the range of 0.1–1.0 nmol of glucosedabsylhydrazone.

HPLC Separation of Monosaccharide Hydrazones. Monosaccharides were readily labeled with dabsylhydrazine by the described derivation procedure. The monosaccharide dabsylhydrazones were found to be stable for at least 6 days at 4 °C (Table III). The C_{18} reverse-phase column (Nova-PAK C_{18}) with a gradient water–acetonitrile mobile phase (solvent A, water–acetonitrile (78:22 (v/v)); solvent B, acetonitrile, curve 7, 15–85%, 45 min) gave sharp peaks for the neutral monosaccharides commonly found in the biosphere (Figure 3). All peaks were sufficiently resolved to permit quantitative estimation. With organic solvents such as methanol, ethanol, tetrahydrofuran, acetone, and ethyl acetate, either alone or in combination, the selectivity or resolution power was much less than that for acetonitrile alone. The following reverse-phase columns were tested: Nova-PAK C_{18}, 4 μm, 3.9 mm × 15 cm; μBondapak C_{18}, 10 μm, 4 mm × 30 cm; Zorbax ODS, 5 μm, 4.6 mm × 25 cm; MN Nucleosil $_{10}C_{18}$, 10 μm, 4 mm × 25 cm. Better resolution was obtained with

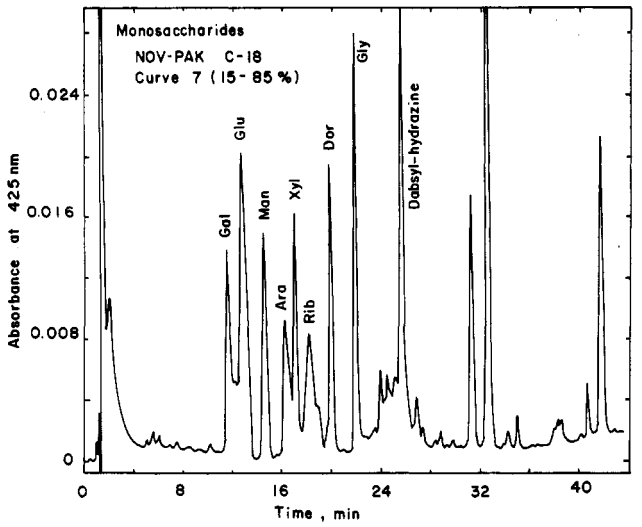

Figure 3. HPLC separation of monosaccharide dabsylhydrazones: chromatographic conditions, Nova-PAK C_{18} reverse-phase column; mobile phase, concave elution program, curve 7, solvent A water–acetonitrile (78:22 (v/v)), solvent B acetonitrile (15–85%, 45 min); flow rate, 1.2 mL/min; AUFS, 0.04. The abbreviations and concentrations (pmol) of monosaccharides are as follows: Glu, glucose (102); Gal, galactose (68); Man, mannose (48); Ara, arabinose (41); Xyl, xylose (33); Rib, ribose (82); Dor, deoxyribose (28) and Gly, glycerose (41).

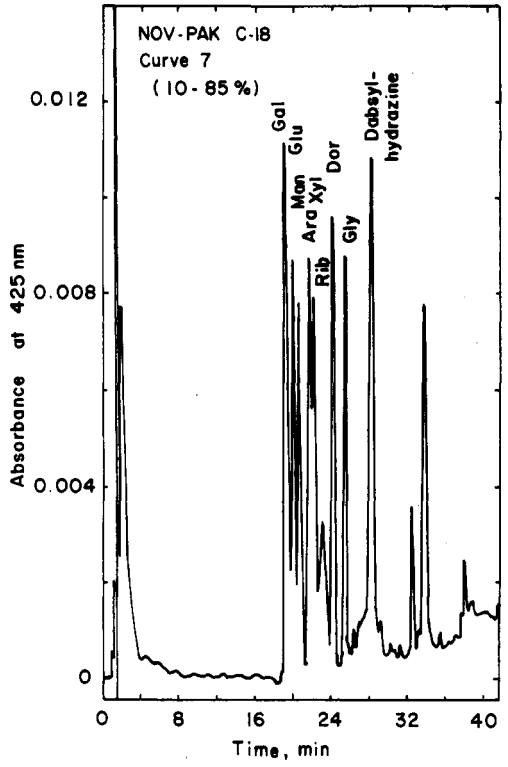

Figure 4. Rapid concave gradient elution of monosaccharide dabsylhydrazones. Chromatographic conditions and abbreviations are as described in Figure 3, except that solvent B is acetonitrile (10–85%, 45 min) and the concentrations of monosaccharides (pmol) are as follows: Glu, 37; Gal, 48; Man, 44; Xyl, 35; Ara, 66; Rib, 66; Dor, 29 and Gly, 12.

the Nova-PAK C_{18} column under these experimental conditions.

Several isocratic elution systems prepared from various combinations of acetonitrile and water were evaluated; none was good enough to resolve all the monosaccharides tested. Gradient elution systems provided better separation of these sugars. It appeared that a concave gradient program (curve 7 or 8) gave better resolution as compared to the linear gradient program (curve 6). The rate of increment of acetonitrile in the mobile phase has a profound effect on the resolution of hydrazones. If the programmer is set at curve 7, the increment of acetonitrile is rather rapid and the first hydrazone (galactose) comes out earlier at 19 min (Figure 4). The chromatogram in Figure 4 was run with B ranging from 10 to 85%. If the programmer is set at curve 8, then the increment of acetonitrile is rather slow and the first hydrazone comes out later at 24 min (Figure 5). In the latter case, the resolution is remarkably improved.

Table III. Stability of Dabsylhydrazones Derived from Monosaccharides

storage period, day	absorbance of dabsylhydrazone at 425 nm[a]		
	glucose	galactose	mannose
1	0.0150	0.0128	0.0410
2	0.0144	0.0130	0.0402
3	0.0146	0.0131	0.0420
5	0.0144	0.0128	0.0396
7	0.0141	0.0122	0.0400
8	0.0138	0.0122	0.0380

[a] An ethanolic solution (1 mL) containing 16 nmol of glucose, galactose, or mannose was reacted with 1 mg of dabsylhydrazine in 1 mL of ethanol at 60 °C for 60 min and the resulting hydrazone solutions were incubated at 4 °C for the indicated intervals. At each interval, 20-μL aliquots were removed and the absorbances of dabsylhydrazones at 425 nm were determined by HPLC as described in the Experimental Section.

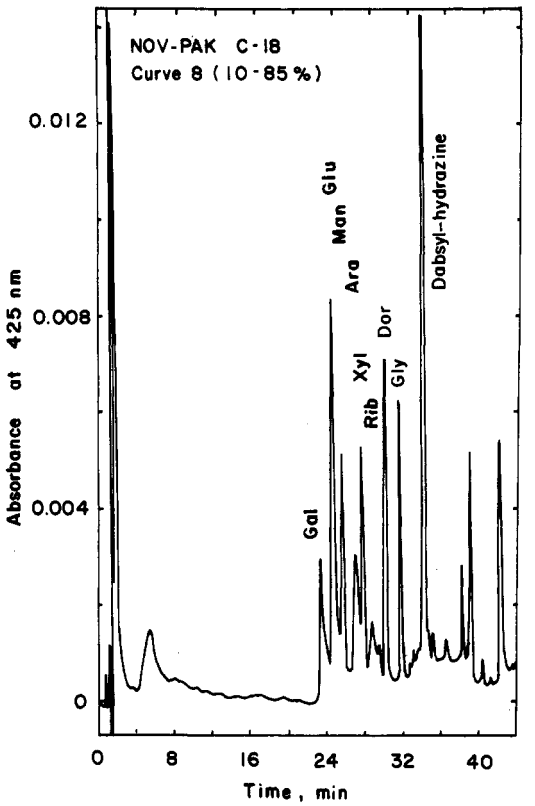

Figure 5. Slow concave gradient elution of monosaccharide dabsylhydrazones. Chromatographic conditions and abbreviations are as described in Figure 3 except that the elution program is set at curve 8, solvent B is acetonitrile (10–85%, 45 min), and the concentrations of monosaccharides (pmol) are as follows: Glu, 50; Gal, 20; Man, 30; Xyl, 35; Ara, 20; Rib, 60; Dor, 20; Gly, 15.

Linearity and Sensitivity. Visible absorbance detection of monosaccharide dabsylhydrazones provided simplicity and reproducibility with good sensitivity. Mixtures containing various amounts of eight standard sugars were derived with

Table IV. Sensitivities of Recent Published Methods for Sugar Determinations

author	year	method	sensitivity	ref
Mawhinney et al.	1980	GLC, silylation	2 μg	1
Binder	1980	HPLC, UV, and RI	12 μg	6
Vratny et al.	1984	HPLC, UV	18 μg	*a*
Honda et al.	1981	automated analyzer, UV with 2-cyanoacetamide	5 nmol	20
Kesler	1967	anion exchange chromatography	1 μg	3
Mrochek et al.	1975	anion exchange chromatography, cerate oxidimetric	1 nmol	2
Verhaar and Kuster	1981	cation exchange chromatography	1 μg	19
Alpenfels	1981	HPLC with dansylhydrazine, UV 254 nm	1 nmol	8
Grimble et al.	1983	HPLC, postcolumn, cuprammonium	450 ng	*b*
Rabel et al.	1976	HPLC, normal-phase partition, RI	84 μg	5
Mopper and Johnson	1983	HPLC with dansylhydrazine, fluorescence detector	5–15 pmol	*c*
Rosenfelder et al.	1985	HPLC with 4′-(*N*,*N*-dimethylamino)-4-aminoazobenzene	5–80 pmol	22
Hull and Turco	1985	HPLC with dansylhydrazine, fluorescence detector	25–50 pmol	23
Lin and Wu	1986	HPLC, precolumn dabsylhydrazine, 425 nm	2 ng (10 pmol)	*d*

[a] Vratny, P.; Frein, R. W.; Brinkman, U. A. Th; Nielen, M. W. F. *J. Chromatogr.* **1984**, *275*, 355–366. [b] Grimble, G. K.; Barker, H. M.; Taylor, R. H. *Anal. Biochem.* **1983**, *128*, 422–428. [c] Mopper, K.; Johnson, L. *J. Chromatogr.* **1983**, 27–38. [d] Present work.

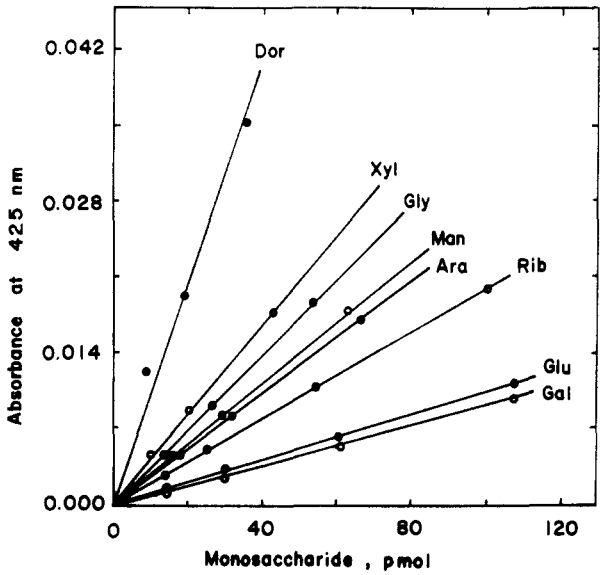

Figure 6. Dose-response curve of monosaccharide dabsylhydrazones. The abbreviations are as described in Figure 3. A mixture containing various concentrations of monosaccharides is reacted with dabsylhydrazine and the resulting hydrazones are determined by HPLC as described in the Experimental Section.

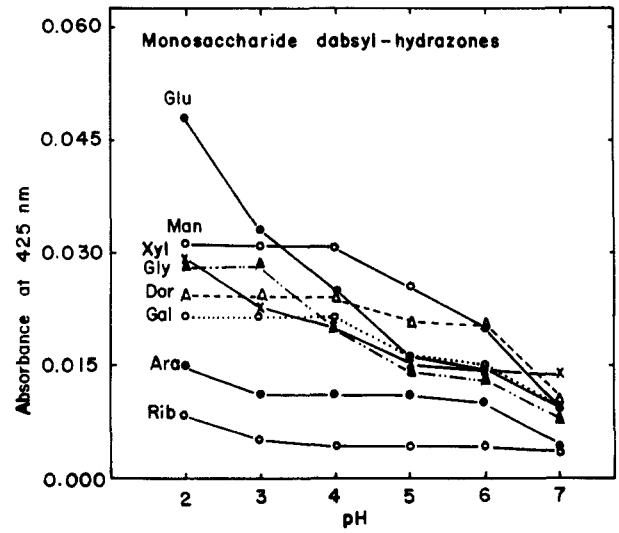

Figure 7. Effect of pH on the formation of monosaccharide dabsylhydrazones.

dabsylhydrazine. The HPLC data demonstrated the linear relationships between the absorbances and the concentrations of the individual monosaccharides (Figure 6). The varying responses as illustrated by different slopes of the regression lines may reflect varying equilibrium constants for the derivative reactions for the different sugars. However, the excellent fit and linearity indicate that this procedure is suitable for quantitative determination.

The pH of the reaction mixture was found to affect the rate of hydrazone formation. The optimum pH range for the reaction was around pH 2 as illustrated in Figure 7. It is worthy to note that the formation of glucosedabsylhydrazone at pH 2 is approximately 4 times higher than that at pH 7. The formations of ribose- and arabinosedabsylhydrazones are less affected by pH variation in the 3 to 6 range.

The absolute sensitivity of the method was measured by deriving 100 pmol of monosaccharides by the described procedure. One-tenth of the sample was injected, giving a peak height response within the linear range of dose–response curve and a signal-to-noise ratio of 10. Therefore, the minimum practical detection limit for glucose or other sugars is about 10 pmol (2 ng). This picomole detection limit for any sugar is comparable and even better than gas–liquid chromatographic methods (*1*). A comparison of detection sensitivity among the published methods for reducing sugars is given in Table IV. Several early methods developed before 1980 employed a refractive index (RI) monitor for sugar detection; the limit of detection was in the microgram range (*3*, *5*, *6*, *19*). The sensitivity of detection was significantly improved by using the appropriate labeling reagent and detector (*8*, *20–23*).

(Dimethylamino)-4-aminoazobenzene has been used to reductively aminate sugars and provide a chromophoric quantitative method at the 5 pmol level (*22*), but the method including amination and purification steps is rather tedious and time-consuming. In the present study, dabsylhydrazine was found to react readily with monosaccharides to form the highly chromophoric dabsylhydrazones, which were easily monitored spectrophotometrically at 425 nm and provided a straightforward method for sugar detection in the picomole range.

Precision and Recovery. The within-run precision of the assay was measured by processing aliquots of glucosedabsylhydrazone solution through the procedure during a single day. The coefficient of variation (CV) was 4.7% at 100 pmol per injection (n = 6). The percentage of analytical recovery of glucose spiked in grape juice samples (20 ng added to 0.2 mL of grape juice) was 97–102% (n = 7).

Determination of Glucose Content in Fruits and Human Serum. The aforementioned procedure was applied for the determination of glucose content in various fruit juices. The glucose concentrations of grape, pineapple, banana, pear, plum, and watermelon were found to be 5.7, 10.5, 10.9, 5.5,

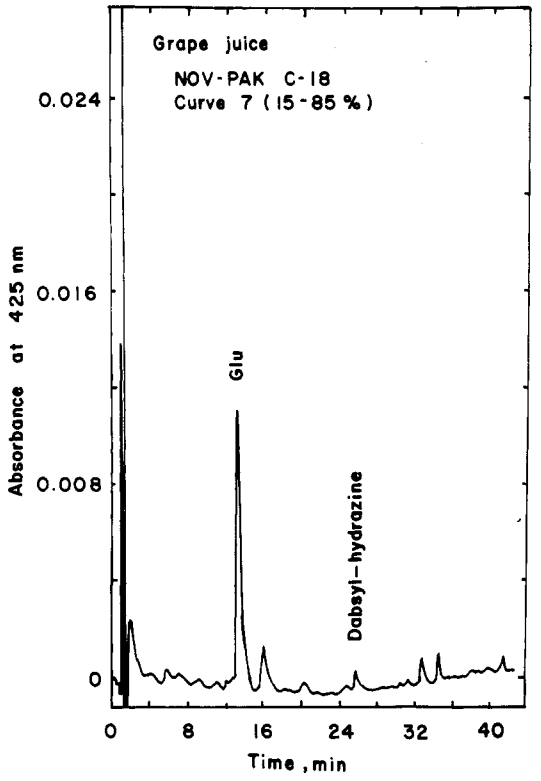

Figure 8. HPLC profile of glucosedabsylhydrazone in grape juice. The chromatographic conditions are as described in Figure 3.

Table V. Comparison of Present Method with Automated Analysis of Serum Glucose

serum sample	automated analysis,[a] mg/dL	present method,[b] mg/dL	
		1	2
1	94	87	88
2	109	107	106
3	151	141	134
4	256	250	256
5	314	319	315
6	419	407	406
7	343	339	334
8	174	177	166
9	345	321	332

[a] The autoanalysis was performed with Olympus AU-550 based on the glucose oxidase colorimetric method. [b] HPLC–dabsylhydrazine method, the determination was performed twice (1 and 2).

4.3 and 2.9 mg/g, respectively. A representative HPLC profile of grape juice was given in Figure 8. Glucosedabsylhydrazone was resolved as a single sharp peak under the experimental conditions.

The present procedure was also employed for the determination of glucose concentration in human serum. A typical chromatogram of glucose analysis in human serum is illustrated in Figure 9. Glucosedabsylhydrazone was separated completely from other serum components and estimated easily from its peak height. A comparative study on the present method and automated analysis in determining the glucose content of human sera was performed and the results are summarized in Table V. The correlation of the data obtained from these two methods is quite acceptable. It seems that the values obtained from the present method are somewhat lower than those obtained by automated analysis. The autoanalysis of serum glucose was performed with an Olympus AU-550 based on glucose oxidase reaction. It is conceivable

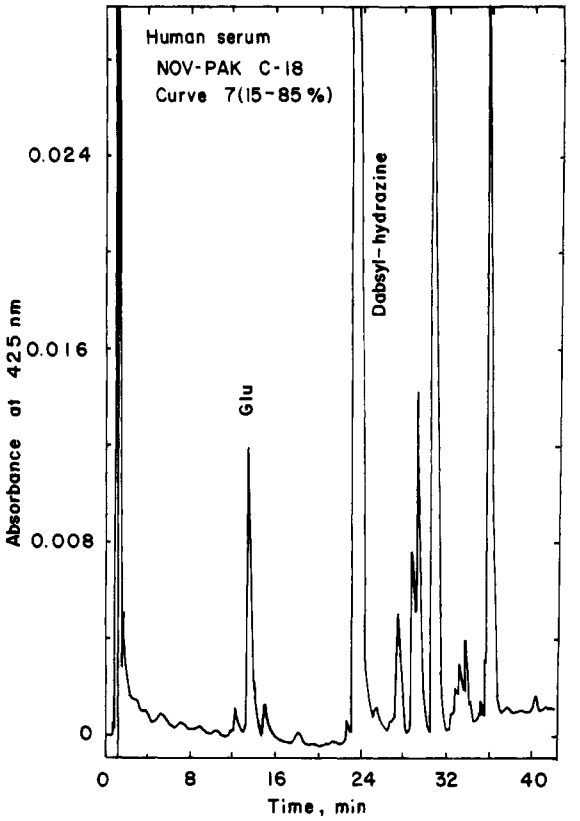

Figure 9. HPLC profile of glucosedabsylhydrazone in human serum. The chromatographic conditions are as described in Figure 3.

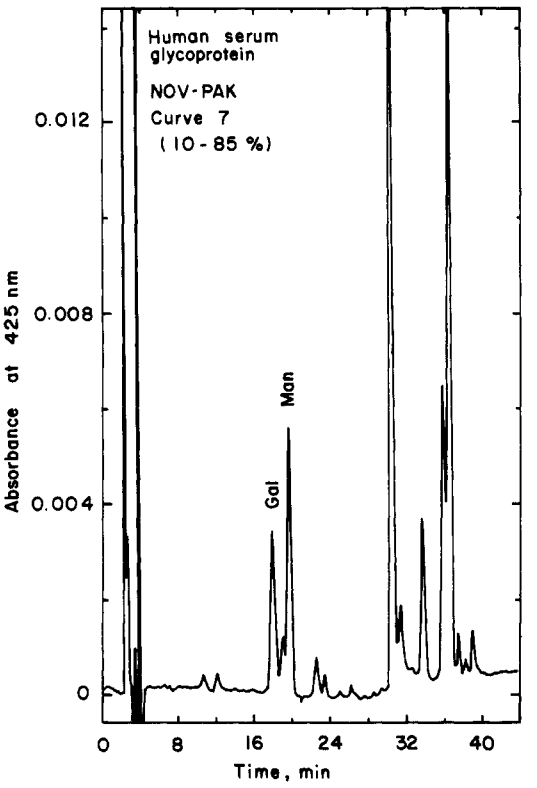

Figure 10. HPLC profiles of galactose- and mannosedabsylhydrazones from human serum glycoprotein. The chromatographic conditions are as described in Figure 4. The experimental procedures for the isolation and hydrolysis of serum glycoprotein are described in the Experimental Section.

that any serum inhibitor or activator of glucose oxidase will certainly affect the accuracy of the routine method. We believe that the present method is a straightforward procedure for glucose determination in various biological samples. The

HPLC separation and chromophoric detection will stay away from most visible and UV absorbing impurities that preexisted in the samples.

Galactose and Mannose Released from Human Serum Glycoprotein. The present procedure was also applied for the determination of monosaccharides released from serum glycoprotein on acid hydrolysis. A representative HPLC profile of serum glycoprotein hydrolysates is given in Figure 10. The contents of galactose and mannose in the serum glycoprotein were found to be 360 and 260 mg/L, respectively. A previous ion exchange chromatography method gave the values of 414 (range, 302–575) and 392 (range, 345–626) mg/L for galactose and mannose, respectively (2). It seems that both methods give the comparable values for the levels of these two monosaccharides in serum glycoprotein. The present HPLC analysis was accomplished in 40 min; whereas the previous method required 18–19 h. Furthermore, the dabsylhydrazone method used far less serum sample (0.05 mL instead of 0.5 mL in the previous method). Theoretically, the volume of serum sample could be reduced down to 0.005 mL if the final aliquot for HPLC analysis were increased from 1 to 10 μL. It is worthy to mention that several unknown peaks appeared on the HPLC chromatogram (Figure 10), further characterization of these unknown sugars derived from serum glycoprotein is in progress.

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Comparison of Extraction Techniques for Munitions Residues in Soil

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Extraction of trinitrotoluene (TNT), trinitrobenzene (TNB), hexahydrotrinitrotriazine (RDX), and octahydrotetranitrotetrazocine (HMX) from two soils was studied in terms of process kinetics and recovery. Two solvents, acetonitrile and methanol, and four extraction techniques, Soxhlet, ultrasonic bath, mechanical shaker, and homogenizer–sonicator, were compared. The results were complex in that several interactions were found among analyte, method, and solvent. Acetonitrile was superior to methanol for RDX and HMX from the perspectives of kinetics and recovery, due in part to a much higher solubility. The Soxhlet and ultrasonic bath generally recovered more than the homogenizer or shaker, although a complicating factor was that all techniques were not necessarily at equilibrium. In terms of sample throughput, the ultrasonic bath and shaker offer advantages over the Soxhlet and homogenizer–sonicator. The ultrasonic bath generally approached equilibrium more rapidly than the shaker, so it appears to be the best overall choice. A spike-recovery study using fortified soil and the sonic bath method yielded complete recoveries of TNT and RDX at 1.6 and 2.3 μg/g, respectively.

The Soxhlet extractor is probably the most widely used method for extraction of organic residues from soils and other solids. Solvents used have included methylene chloride (*1–8*), benzene (*1, 3, 6, 7,* 9), toluene (*1, 6*), chloroform (*1, 6, 10*), acetone/hexane (*1, 8, 11*), methanol (*2, 5, 6, 10*), acetone (*6, 12–14*), pyridine (*3, 6*), cyclohexane (*3, 6*), dimethyl sulfoxide (*3, 6*), dimethylformamide (*3, 6*), *N*-methylpyrollidone (*3*), petroleum ether (*13*), acetonitrile (*10, 14*), hexane (*5–7, 14*), diethyl ether (*5, 6*) and various other binary and ternary mixtures (*6, 7, 10, 14*). The Soxhlet procedure has been compared to methods based on equilibration with solvent using either shaking (*1, 10, 13*), an ultrasonic probe (*3, 4, 11, 12*), or an ultrasonic bath (*1, 2*). Depending on the analyte and matrix studied, the preferred solvent or method varies. In general, though, a relatively polar solvent or binary mixture containing a polar solvent is often recommended with either a Soxhlet or ultrasonic method.

Recently two methods were outlined for the determination of six polynitro compounds in soil (*15*) and furnace ash (*16*). The first method involves extraction of the soil with aceto-

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