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QUANTITATIVE GAS—LIQUID CHROMATOGRAPHY OF NEUTRAL SUGARS IN HUMAN SERUM GLYCOPROTEINS

FUCOSE, MANNOSE, AND GALACTOSE AS PREDICTORS IN OVARIAN AND SMALL CELL LUNG CARCINOMA*

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SUMMARY

A precise and accurate gas—liquid chromatographic (GLC) method has been developed for the quantitative analysis of the neutral sugars L-fucose (6-deoxygalactose), mannose,

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galactose, and glucose in ethanol precipitates of human serum proteins. The chromatographic conditions and sample preparation resulted in short analysis times (20 min per run) and made routine analyses practicable (twelve samples per day). The alditol acetate derivatization yielded single derivatives for each sugar. Complete separation was achieved on a 2.0 m × 2 mm I.D. column with 2.0% Silar-7CP on Chromosorb W AW 80—100 mesh. The results of hydrolysis showed that the release of fucose and galactose preceded the release of mannose. Hydrolysis with AG 50W-X8 (H^+) ion-exchange resin in 0.5 *N* HCl at 100°for 7 h optimized glycosidic bond cleavage with only minimal destruction of fucose, mannose and galactose. A combination of strong cation- and anion-exchange resin columns was used to remove chromatographic background of peptides, amino acids, amino sugars, and inorganic ions. An average R.S.D. of less than 4% with recovery of >86% for the three sugars was achieved. The homogeneity of the chromatographic peaks for the neutral sugars of normal human serum glycoproteins was confirmed by GLC—mass spectrometry. Significantly elevated ratios of fucose, galactose, and mannose to serum protein were observed for patients with small cell lung and ovarian carcinomas.

INTRODUCTION

Glycoproteins are macromolecules with one or more polypeptide chains to which sugars are covalently bonded. These sugars form branching chains where the terminal sugar appears to be limited to either sialic acid or L-fucose [1]. Glycoproteins comprise a large number of compounds. They occur biologically as enzymes, hormones, and immunoglobulins. The role of carbohydrates in glycoproteins of the hemagglutination system, as carriers, immunoglobulins, and in other biologically active glycoproteins, is not well understood [2]. However, studies show that carbohydrates in glycoproteins from cell surfaces may function in growth behavior of tissue cells [2] and thus be of importance in cancer cell division.

The levels in serum for protein-bound carbohydrates have been shown to be frequently elevated in patients with metastatic cancer [3—9]. Increases are not common, however, early in the course of malignant disease when the tumor is small, localized, and prior to any tissue invasion or metastases [6, 10—13]. Similar elevations of protein-bound carbohydrates have also been found in patients with active metabolic or acute inflammatory diseases [4, 6, 7, 12, 14]. In view of the latter findings, the study of these compounds for diagnostic purposes was largely abandoned. These shortcomings, nevertheless, do not eliminate the potential value of serum carbohydrates in the clinical management of patients with metastatic disease who are free of such disorders. The majority of previous investigations have involved serum fucose. Recently, studies by Rosato and co-workers [15—17], and Barlow and Dillard [18] showed that serum fucose levels could be used as an additional means of assessing the disease status in patients with breast and cervical carcinoma, respectively. Evans et al. [19] in 1974, measuring serum fucose in combination with neutral hexoses, reported that these materials had further application for determining the metastatic status of patients with cancer. This investigation included a variety of neoplastic diseases.

The methods for analysis of protein-bound carbohydrates [13—19, 20—23] in human sera generally have been non-specific colorimetric measurements. Consequently, these procedures have distinct disadvantages due to mutual

interferences by the individual carbohydrates although corrective factors can be applied to reduce this problem [19] and/or to spurious chromogens developed from protein degradation products. Utilizing the original method of Rosato [15—17] modified by Evans et al. [19], we have recently confirmed that serum fucose analysis can be useful as an aid in the clinical management of patients with advanced breast cancer [24]. In order to circumvent the methodological difficulties of the colorimetric procedures, Mrochek et al. [25] developed a high-resolution anion-exchange separation method. A sensitive cerate oxidimetric detector was coupled to the chromatographic column, and the eluted oxidizable constituents monitored by means of the fluorescence of Ce^{3+}. Serum protein-bound fucose and the neutral hexoses, mannose, and galactose were separated and measured by this means. Modification of the hydrolysis procedure prior to chromatography permitted the further analysis of sialic acid (N-acetylneuraminic acid). These methods and their preliminary application to patients with breast cancer have been reported [26]. Although providing specificity and sensitivity, this analytical technique tended to be too time-consuming for the routine analysis of a large number of samples obtained sequentially at frequent intervals. In order to develop a method that could be carried out rapidly and applied to large numbers of samples, yet retaining sensitivity and specificity, we have turned to gas—liquid chromatography (GLC).

GLC is desirable, due to its simplicity, speed, and sensitivity compared to other analytical methods. Cited in the literature are several methods for the derivatization and GLC analysis of neutral sugars [27—32], amino sugars [30, 31, 33—35], and sialic acids [36, 37]. Amino sugars and neuraminic acid (sialic acid) are most conveniently analyzed as the trimethylsilyl (TMS) ether derivative of the corresponding methyl glycosides or ketosides [34]. A disadvantage of methyl glycosides is that multiple derivatives for each component sugar are produced as a result of anomerization, thus making quantitation difficult. Serum amino sugars and neutral sugars may be determined as the alditol acetate derivatives [28—32, 35] while serum sialic acids have been determined separately as TMS derivatives [36, 37]. The advantage of the alditol acetate derivatives is that one derivative is formed for each sugar (neutral and amino), whereas the disadvantage is that the sialic acids cannot be determined as the alditol acetate derivatives [30].

Several methods have been developed for the identification and quantitation of sugars from glycoproteins [27—38], and all these methods were applied in the analysis of partially purified homogeneous glycoproteins. Our investigations showed that these alditol acetate derivatization GLC methods were not reproducible and low yields of neutral sugars were obtained when analyzing sugars from human serum protein.

Our method is a further development of the procedures used by Lehnhardt and Winzler [28]. We employed the same alditol acetate derivatization, but different hydrolysis conditions, more rigorous cleanup procedures, and improved GLC techniques in the analysis of neutral sugars from serum glycoproteins. Our GLC sample turnover rate for analysis is 20 min, and twelve samples can be processed and analyzed daily by one analyst.

The purpose of this paper is to present in detail the GLC method we have

developed, including alditol acetate derivatization, and mixed-bed ion-exchange cleanup, and to demonstrate its application to normal control subjects. Included as examples are preliminary results for patients with either small cell carcinoma of the lung or ovarian carcinoma in order to show changes from normal which may be found in patients with malignant diseases.

EXPERIMENTAL

Apparatus

A Bendix Series 2500 gas chromatograph equipped with hydrogen flame detectors was used (Bendix, Ronceverte, W.Va., U.S.A.).

This instrument was interfaced with a Hewlett-Packard 3352B Laboratory Data System with 24 K memory (Hewlett-Packard, Avondale, Pa., U.S.A.) for identification and quantitation of the neutral sugars.

Samples were analyzed on a 2.0 m × 2.0 mm I.D. borosilicate glass column packed with 2.0% (w/w) Silar-7CP on 80—100 mesh Chromosorb W AW. The injection port temperature was maintained at 220°, the detector at 250°. The column was temperature programmed from 160° to 220° at 12°/min. A carrier gas (nitrogen) flow-rate of 20 ml/min was used.

Chemicals

Alditol acetate standards were obtained from Regis (Morton Grove, Ill., U.S.A.). L-Fucose, 2-deoxyribose, 2-deoxyglucose, mannose, galactose, glucose, xylose, and arabinose were purchased from Sigma (St. Louis, Mo., U.S.A.).

Other chemicals were purchased from the following sources: chloroform, sodium hydroxide, methanol, and ethanol A.C.S. certified grade, from Fisher Scientific (St. Louis, Mo., U.S.A.); pyridine from J.T. Baker (Phillipsburg, N.J., U.S.A.); glacial acetic acid from Mallinckrodt (St. Louis, Mo., U.S.A.); acetic anhydride A.C.S. certified grade from Aldrich (Milwaukee, Wisc., U.S.A.); sodium borohydride from Sigma; anion-exchange resin AG 1-X8, 100—200 mesh (CH_3COO^-) and cation exchange resin AG 50W-X8, 100—200 mesh (H^+) from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). All-glass double-distilled deionized water (d.d. H_2O) was used for preparing aqueous solutions.

Calibration and internal standard solutions

A stock solution of neutral sugars was prepared to give concentrations of 0.400 mg/ml of L-fucose, 2.00 mg/ml of mannose, 2.00 mg/ml of galactose and 0.800 mg/ml of glucose in d.d. H_2O.

A standard solution was prepared from this stock solution by diluting 20.0 ml of stock solution to a final volume of 100 ml. Therefore, the working standard solution was composed of 80.0 μg/ml of L-fucose, 400 μg/ml of mannose, 400 μg/ml of galactose, and 160 μg/ml of glucose. Thus, 1.0 ml of the working solution contains similar levels of the protein-bound neutral sugars in 0.5 ml of normal serum. Three 1-ml aliquots of the standard working solution were derivatized and used for the calibration of the GLC system.

Single internal-standard stock solutions of 2-deoxyglucose and 2-deoxyribose were prepared to yield concentrations of 2.00 mg/ml and 4.00 mg/ml, respectively, in d.d. H_2O. The working internal standard solution was prepared by

diluting 10.0 ml of each stock solution to a final volume of 100 ml.

The stock solutions were stored at −20°, and the working solutions were held at 4° and prepared freshly every two weeks.

Representative serum samples for analytical determinations

Blood was obtained from 22 normal control male and female subjects free of any known disorders in order to establish the normal mean and range [22] (serum carbohydrate/protein), and from patients with histologically documented small cell carcinoma of the lung, and from patients with carcinoma of the ovary. The patients with cancer were included on the basis of their known disease status or response category as determined by clinical and laboratory parameters. The selection of disease status or response categories was made initially in order to show both maximal differences from normal if these occurred, and to demonstrate the subsequent changes associated with response to therapy. All patients were free of other diseases and had normal renal function. Patients with abnormal liver function were excluded unless secondary to proven liver involvement with tumor.

Samples were obtained from three categories of patients with small cell carcinoma of the lung: (1) prior to any therapy; (2) patients with measurable disease who were in partial or complete remission after treatment; and (3) patients who after treatment had recurrent disease or progressive disease. Partial remission was defined as a reduction by at least 50% of the product of the longest perpendicular diameters of the most clearly measurable mass lesion with no increase (of 25% or more) in any other indicator lesion, no new clinically apparent areas of malignant disease, and no significant deterioration in weight (<10%), symptoms or performance status. For classification as a complete response, all clinically detectable tumors had disappeared. Progressive disease included any of the following criteria: increase in any measurable lesion by more than 50%, or appearance of new areas of malignant disease, along with significant deterioration in symptoms, decrease in weight (>10%), or decrease of one level in performance status. Those patients whose measurable tumor mass disappeared following treatment and who had no evidence of disease elsewhere were considered in complete remission. Patients with carcinoma of the ovary who were in complete remission, as defined for small cell carcinoma of the lung, and those who had recurrent disease after therapy or progressive disease before or after treatment were included. All patients who fulfilled the criteria for admission to therapeutic protocol studies were considered as candidates for determination of serum protein-bound carbohydrates if qualified on the basis of the disease categories under investigation. All patients with ovarian carcinoma, prior to any treatment were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Of those with progressive disease, six had been originally classified as Stage IV, fourteen as Stage III, and three as Stage II; of those in complete remission, nine had been originally Stage III, one Stage II, and Three Stage I. Anti-tumor chemotherapy was used to treat both the patients with small cell carcinoma of the lung and those with ovarian carcinoma. In general, this consisted of multiple drugs given in courses at specific intervals. All blood samples were drawn prior to therapy, and at least 14 to 28 days following the previous course of treat-

ment. After clotting occurred, serum was separated by centrifugation in a refrigerated centrifuge and aliquot samples frozen at —50° to —70° until analysis.

Preparation of ion-exchange resins

Cation-exchange resin

(1) Place the resin, about 500 g of AG 50W-X8, 100—200 mesh (H^+), in a 2000-ml Erlenmeyer flask. Wash the resin by addition of 750 ml of methanol and swirling. Discard the methanol solution and repeat the methanol wash. After discarding the methanol solution, wash the resin with 1 l of d.d. H_2O followed by discarding the aqueous solution and repeating the H_2O wash. (This step is for new resin only.)

(2) Exhaust the resin with 1 l of 2 *N* NaOH for 1 h in a water-bath, at 70°.

(3) Discard the NaOH solution, add 1 l of 2 *N* NaOH and hold for 2 h at 70°. Then discard the supernatant NaOH solution.

(4) Add 1 l of 2 *N* NaOH containing 2% EDTA in acid form and maintain for 3 h at 70°. (Note: steps 2, 3, and 4 should be carried out at 70° with gentle swirling every 15—30 min.)

(5) Discard the NaOH solution and rinse resin three times with excess d.d. H_2O. This can be done in the 2 l Erlenmeyer flask or in a 2—4 l graduated cylinder.

(6) Add to the resin 1 l of 6 *N* HCl, place in a 100° water-bath, swirl every 15 min for 1 h, discard HCl solution, and add 1 l of 6 *N* HCl for 3 h at 100°. Discard HCl solution. Add 1 l of 6 *N* HCl and hold for 12 h (overnight) at 100°.

(7) Rinse resin with d.d. H_2O until neutral.

Anion-exchange resin

(1) Same as step 1 for cation-exchange resin, except anion-exchange resin, AG 1-X8, 100—200 mesh (Cl^-), is used.

(2) Exhaust resin with 2 *N* NaOH at room temperature for 1, 2 and 3 h, as for cation-exchange resin. Then rinse four times with d.d. H_2O.

(3) Add 1 l of 2 *N* acetic acid for 1, 3, and 6 h, respectively, with gentle swirling occasionally during the regeneration. (Note: all anion-exchange steps were performed at room temperature.)

Storage of regenerated resins

Keep regenerated resins at 4°, cation-exchange resin in 2 *N* HCl, anion-exchange resin in 2 *N* acetic acid. Before use place resin in regeneration column and rinse with d.d. H_2O until neutral.

Storage of exhausted resin

Store both types of resin at 4°, the cation-exchange resin under 3 *N* HCl and the anion-exchange resin under 1 *N* HCl. Never allow resins to remain in the laboratory at room temperature for long periods of time.

Rotary evaporation system

All rotary evaporations were performed on a system assembled by our laboratory for this method. The system consists of a Model KLRC-3 Compound Liquid Ring Vacuum Pump (Kinney, Boston, Mass., U.S.A.), six CaLab rotary evaporators (CaLab, Oakland, Calif., U.S.A.), six 100-ml glass evaporator traps with vacuum-tight PTFE plugs (Kontes Glass Co., Vineland, N.J., U.S.A.), and two Model 1225 RS water heating baths (William Boekel, Philadelphia, Pa., U.S.A.).

Included in the system are six pyrex Kjeldahl connecting bulbs, 100 × 45 mm O.D. (Corning 2020), which prevent cross-contamination between samples, a vacuum gauge, and an automatic shut-down control, which allows the pump to run for 3 min after switching the pump to the off position, thus flushing out corrosive material.

Analytical procedure

Sample preparation for serum protein-bound neutral sugars

(1) Remove the serum sample and thaw in running warm water and allow to come to ambient temperature.

(2) Mix sample on a vortex mixer for 15 sec. Pipet 500 μl of the sample with a 500 μl Eppendorf pipet into a 16 × 100 mm pyrex screw-cap tube.

(3) Add 5.0 ml of 95% ethanol and mix on vortex mixer. Allow sample to stand at ambient temperature for 15 min.

(4) Centrifuge the sample for 15 min at 1500 *g* with a clinical centrifuge. Carefully decant the supernatant avoiding loss of precipitate.

(5) Invert the pyrex tube on tissue paper and allow precipitate to drain dry for 15 min at ambient temperature.

(6) Add 2.0 ml of 0.1 *N* NaOH to the precipitate. Let stand at ambient temperature with periodic vortex mixing until the precipitate is completely dissolved (usually 1—2 h).

Hydrolysis

(7) Add 2.0 ml of dry AG 50W-X8 (H^+) 100—200 mesh resin to the sample solution. Pipet 400 μl of 3.53 *N* HCl with a 200 μl Eppendorf pipet into the sample solution. Solution is 0.50 *N*.

(8) Tightly cap the tube with a PTFE-lined screw-cap and mix sample on a vortex mixer for 15 sec.

(9) Hydrolyze the sample for 7 h in a heating block at 100±1° (Scientific Instruments Shop, University of Missouri, Columbia, Mo., U.S.A.). Vortex sample for 15 sec every 30 min.

Cleanup of hydrolysate

(10) Allow hydrolysate to come to ambient temperature. Quantitatively transfer the hydrolysate onto a column (30.0 × 1.0 cm I.D. pyrex glass fitted with PTFE stopcock) packed with 15.0 ml of AG 50W-X8 (H^+) 100—200 mesh resin, which is in series with another glass column packed with 10.0 ml of AG 1-X8 (CH_3OO^-) 100—200 mesh resin.

(11) Wash column series with 35 ml of 50% (v/v) methanol—H_2O and collect

effluent in a 125 ml $ 24/40 pyrex-glass flat-bottomed rotary evaporator flask (Corning 1100). Allow solutions to drain by gravity flow. The flow-rate was about 1 ml/min.

Derivatization

(12) Pipet 1.00 ml of 2-deoxyribose solution, 200 μg/ml, (used as an internal standard to monitor the recovery) into the rotary evaporator flask containing the effluent. Take contents of flask to dryness on rotary evaporator (water-bath at 60°).

(13) Pipet 1.00 ml of 2-deoxyglucose, 200 μg/ml (internal standard) into the flask. Add 1.0 ml of d.d. H_2O and mix flask contents well by swirling to contact walls.

(14) Reduce the sugars by adding 2.0 ml of 0.22 *M* $NaBH_4$ and mix by swirling. Allow to stand for 30 min at ambient temperature [39].

(15) Add 1.0 ml of glacial acetic acid and evaporate to dryness on rotary evaporator.

(16) Remove borate by adding 2.0 ml of HCl—methanol (1:1000, v/v) to the dry flask, swirling, and taking to dryness by rotary evaporation. Repeat this step two times.

(17) Acetylate the sample by adding 1.0 ml of acetic anhydride and 1.0 ml of pyridine. With a glass stopper on the flask, mix by swirling. Heat flask in a 100° oil-bath for 30 min.

(18) Remove flask from oil-bath and transfer contents with a disposable pipet into a disposable pipet fitted with a glass-wool plug (1/4") in the constricted tip end and collect the sample in a 16 × 75 mm pyrex tube.

(19) Evaporate the sample just to dryness under a gentle stream of dry nitrogen while in a heating block at 60°.

(20) Redissolve the sample by pipetting 1.00 ml of $CHCl_3$ with a 1000 μl Eppendorf pipet into the tube. Cap the tube with a PTFE-lined screw-cap and mix sample on a vortex mixer for 15 sec.

(21) Inject 5.0 μl into the gas—liquid chromatograph for analysis.

Protein analyses

The procedure used for protein determination was a semi-automated Technicon System for nitrogen determination described by Wall and Gehrke [40].

RESULTS AND DISCUSSION

GLC of alditol acetates

Fig. 1 shows the separation of six alditol acetates achieved in 12 min on a glass column packed with 2.0% (w/w) Silar-7CP on Chromosorb W AW, 80—100 mesh. An internal standard, 2-deoxyglucose, was included for accurate quantitation of the neutral sugars.

Precision of GLC analysis with standards

Repeated injections of 5.0 μl of a standard solution (0.4—2.0 μg each) of six neutral sugars gave relative standard deviations of 0.3—1.9% (Table I). These data show excellent precision of the GLC analysis for standards over the range 80—400 μg of sugar per ml.

TABLE I

PRECISION OF GLC ANALYSIS OF NEUTRAL SUGAR STANDARDS USING AN INTERNAL STANDARD METHOD

	Sugar (μg/ml)*					
	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
$\bar{x}$	80	82	81	396	405	186
σ	1.5	0.6	0.6	1.2	2.1	0.6
R.S.D. (%)	1.9	0.7	0.7	0.3	0.5	0.3

*Reference standard analyzed independently three times.

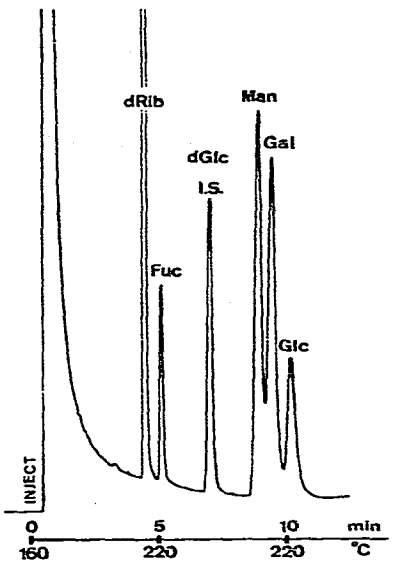


Fig. 1. GLC separation of alditol acetates of neutral sugars. Sample: 5 μl (≈1 μg) each. Program: from 160° at 12°/min to 220°. Attenuation: 1 × 10^{-10} A.f.s. Column: 2.0% (w/w) Silar-7CP on Chromosorb W AW, 80—100 mesh, 2 m × 2 mm glass.

Calculation of sugar concentration

Quantitation of the neutral sugars was based upon an internal standard method using 2-deoxyglucose as internal standard (I.S.). The relative weight response (RWR) values compared to 2-deoxyglucose were as follows: $RWR_{Fuc/I.S.}$ = 1.01; $RWR_{Gal/I.S.}$ = 0.91; $RWR_{Man/I.S.}$ = 0.96; $RWR_{Glc/I.S.}$ = 1.11. The RWR values for the four neutral sugars were determined by at least three independent analyses of calibration standards, and remained constant over a three-month period.

Hydrolysis of serum proteins

Hydrolysis with 1.0 *N* HCl at 100° for 10 h was compared to hydrolysis with 1.0 *N* HCl plus 2.0 g AG 50W-X8 (H^+) resin at 100° for 10 h. Both studies were performed in triplicate.

2-Deoxyribose was added as an internal standard after ion-exchange cleanup to each sample and its percentage recovery was monitored. In general, higher values for the neutral sugars mannose and galactose were obtained when hydrolyzing with AG 50W-X8 (H^+) resin than without. Better precision, lower R.S.D. (%) values, and improved recoveries for 2-deoxyribose (mean 94%) and higher recovery values for the neutral sugars were also obtained when hydrolyzing with the aid of AG 50W-X8 (H^+) resin (Table II).

The use of AG 50W-X8 (H^+) in hydrolysis resulted in higher yields with less neutral-sugar destruction, and removed chromatographic interferences which resulted in better precision. Thus, hydrolysis results with this resin gave better precision and neutral sugar recoveries than hydrolysis without it.

The hydrolysis of ethanol precipitates of serum proteins was investigated to determine the best conditions for maximum glycosidic bond cleavage and minimal destruction of the neutral sugars. Aliquots of a pooled control serum were used for this study. Different acid concentrations and times of hydrolysis at 100° were investigated. Samples were hydrolyzed with 1.0 *N* HCl for 2—14 h with 0.5 *N* HCl for 4—15 h, and with 0.05 *N* HCl for 15—60 h. Except for these different hydrolysis conditions, the procedure followed was identical to the analytical procedure previously described.

The results of hydrolysis (Fig. 2) show that the release of fucose and galactose preceded the release of mannose at each hydrolysis acid concentration. A slight loss of fucose can be observed with each acid concentration at longer hydrolysis times. The 0.5 *N* HCl condition gives as high or higher values for all three sugars than does the 0.05 or 1.0 *N* HCl. Therefore, for convenience samples were hydrolyzed for 7 h and routine analyses used hydrolysis with 0.5 *N* HCl and 2.0 g AG 50W-X8 (H^+) 100—200 mesh at 100° for 7 h.

Method linearity

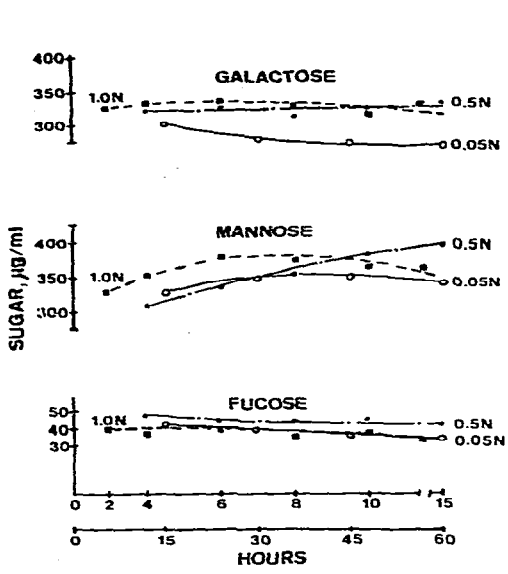
Duplicate analyses of 0.10—58.0 mg ovomucoid (egg white; Sigma) hydrolysates were performed to establish the working range of the method. A linear relationship for mannose and galactose was obtained (Fig. 3). Precision between duplicates was quite good, as shown by the range given on the graph.

TABLE II

COMPARISON OF HYDROLYSIS WITH CATION-EXCHANGE RESIN AND 1.0 *N* HCl

A and B were different pooled control sera.

10 h at 100° with 2.0 g AG 50W-X8 (H^+) 100—200 mesh and 1.0 *N* HCl					10 h at 100° with 1.0 *N* HCl				
Serum A	Neutral sugars (μg/ml)				Serum B	Neutral sugars (μg/ml)			
	dRib	Fuc	Man	Gal		dRib	Fuc	Man	Gal
$\bar{x}$	748	38.3	365	317		615	43.5	352	310
σ	23	2.1	2.9	3.5		86	2.9	18	19
R.S.D. (%)	3.2	5.5	0.8	1.1		14	6.7	5.2	6.2
Recovery of spike (Mean %)	94	80	89	90		77	82	76	79



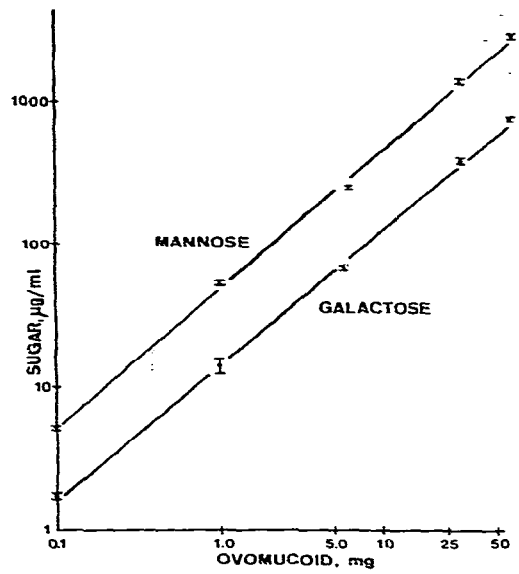


Fig. 2. Hydrolysis of serum protein with 2.0 g of AG 50W-X8 (H^+) at 100°.

Fig. 3. Linearity of GLC method.

Precision of analysis for neutral sugars in serum — Matrix-independent and matrix-dependent

Neutral sugars were determined using 0.5 ml serum of individual samples (matrix-dependent) and pooled control serum (matrix-independent). A chromatogram for a matrix-dependent analysis is shown in Fig. 4.

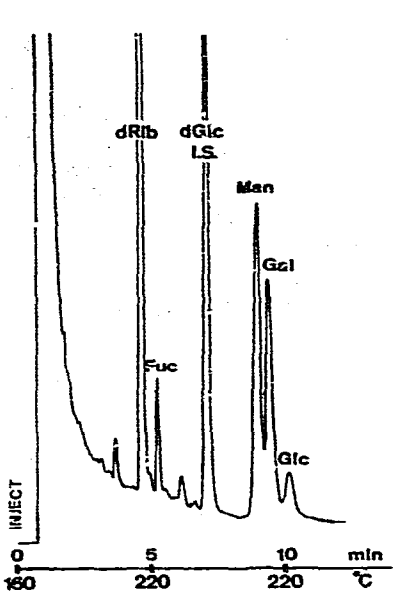
The matrix-independent and matrix-dependent precisions for the GLC analysis of three neutral sugars in serum protein are given in Table III. The data for the matrix-independent samples were obtained by analyzing the same sample of pooled control serum independently 16 times, whereas the data for the matrix-dependent samples were obtained from 30 different serum samples each analyzed independently twice over three months. Excellent precision of analysis was achieved.

Recovery of neutral sugars from serum

Recoveries of neutral sugars were determined by spiking serum samples with standards. Chromatograms for non-spiked and spiked serum samples are shown in Figs. 5 and 6, respectively. The recoveries obtained from 30 different serum samples are given in Table IV.

Precision and recovery of protein analyses from serum of cancer patients

The matrix-dependent precision and recovery data for the protein analyses are given in Tables V and VI. The precision values for the matrix-dependent



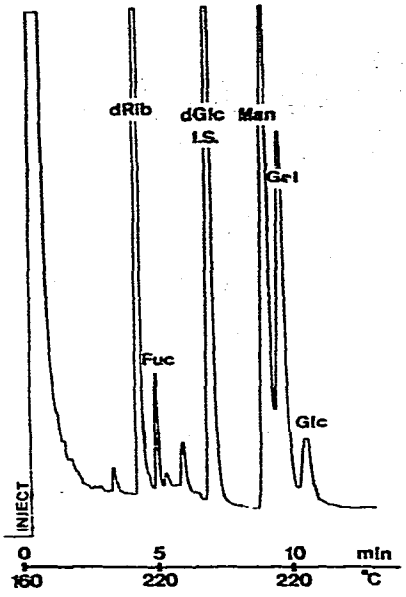


Fig. 4. GLC of neutral sugars in serum protein from breast cancer patient. Conditions as in Fig. 1.

Fig. 5. GLC of neutral sugars in serum protein from breast cancer patient. Conditions as in Fig. 1.

TABLE III

PRECISION OF GLC ANALYSIS OF SERUM PROTEIN-BOUND NEUTRAL SUGARS

	Sugar (μg/ml)		
	Fucose	Mannose	Galactose
Matrix-independent (n = 16)			
$\bar{x}$	42.9	374	340
σ	2.73	13.0	11.7
R.S.D. (%)	6.4	3.5	3.4
Matrix-dependent (n = 30)*			
$\bar{x}$	68	577	551
σ**	2.5	16.5	11.6
R.S.D. (%)	3.6	2.9	2.1

*Precision for different serum samples over three months.

**$\sigma = \sqrt{\frac{\Sigma(x_2 - x_1)^2}{2P}}$ where P is the number of pairs (P = 30); each pair was analyzed on the same day.

TABLE IV

RECOVERY OF ADDED NEUTRAL SUGARS FROM CANCER SERUM

Recoveries were from different serum samples over three months (n = 30). Added at the following levels of μg per 0.5 ml serum were fucose 80, mannose 400, and galactose 400.

	Recovery (%)		
	Fucose	Mannose	Galactose
$\bar{x}$	86	85	91
σ	6.0	3.9	3.5
R.S.D. (%)	6.9	4.6	3.9

TABLE V

PRECISION OF PROTEIN ANALYSES FROM CANCER SERUM

Precision is for different serum samples over three months.

Matrix-dependent	Protein (mg/ml serum)
n = 38	
$\bar{x}$	73
σ^*	1.4
R.S.D. (%)	1.9

*$\sigma = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2P}}$ where P = 38.

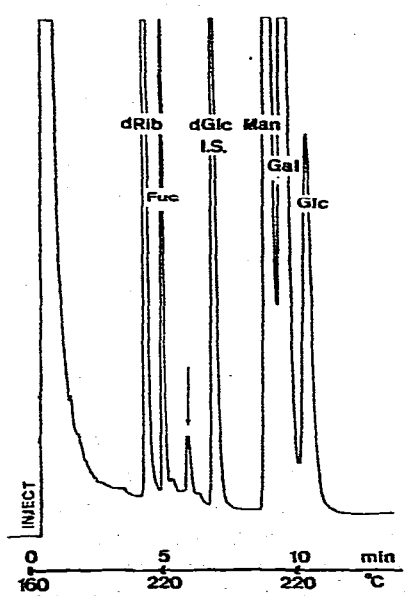


Fig. 6. GLC of neutral sugars in serum protein from breast cancer patient spiked with neutral sugars. Conditions as in Fig. 1. Spiked sugars (μg added per ml serum): Fuc, 160; Man, 800; Gal, 800; Glc, 320.

TABLE VI

RECOVERY OF BOVINE SERUM ALBUMIN ADDED TO CANCER SERUM PROTEIN

Recovery is calculated from different serum samples over a period of three months. Ten mg of bovine serum albumin were added to each serum sample.

	Recovery (%)
$n = 34$	
$\bar{x}$	101
σ	3.54
R.S.D. (%)	3.5

samples were obtained from 38 different serum samples analyzed independently twice over three months, whereas the data for the recoveries for matrix-dependent samples were obtained by analyzing 34 spiked and non-spiked individual serum samples. Excellent precision and recoveries were obtained.

Effect of storage temperature on the serum protein-bound sugar levels

The influence of serum storage temperature on the serum protein-bound sugar levels was investigated. The serum was from a freshly prepared pooled normal sample from our laboratory personnel. Fresh whole blood was taken from two of the Experiment Station Chemical Laboratories' staff. The blood was transferred to 30 ml Corex centrifuge tubes and allowed to clot for 15 min. The blood was centrifuged at 5000 *g* for 15 min, then the serum was decanted into a 125 ml Erlenmeyer flask. With continuous stirring, 0.5-ml aliquots of serum were pipetted into 13 × 75 mm culture tubes. The serum was divided and aliquots were stored at room temperature, 4° (refrigerator temperature), -20°, and -70°.

Three aliquots of the freshly prepared serum were analyzed to establish the original values for the sugars. The stored serum samples were analyzed in triplicate at the following specified storage times and temperatures: room temperature, 2, 4, 8, and 24h; +4°, 1, 3, 7, and 28 days; -20°, 1, 3, 7, and 28 days; —70°, 1, 3, 7, and 28 days.

The serum-bound sugar levels in serum stored at room temperature for up to 24 h and at +4°, —20°, and —70° for up to 28 days were found to have essentially the same levels as freshly prepared serum.

Effects of successive freezing and thawing on the total protein and protein-bound sugar levels in serum

A pooled control serum sample was used. The frozen sample was thawed at room temperature, and aliquots were taken for the determination of sugars and protein. The serum sample was then frozen again for 24 h and the same process was repeated. For each of the four freezing and thawing cycles independent duplicate analyses were made for total protein and sugars. The average values are presented in Table VII. The data indicate no change in the levels of fucose, mannose, galactose and total serum protein after repeated freezing and thawing. This information is helpful in knowing the integrity of the sample during storage.

TABLE VII

EFFECT OF FREEZING AND THAWING ON STABILITY OF PROTEIN-BOUND NEUTRAL SUGARS AND SERUM PROTEIN

Freezing and thawing cycle	Sugar (μg/ml)			Protein (mg/ml)
	Fucose	Mannose	Galactose	
1	41.9	361	339	60
2	40.5	340	323	59
3	42.4	361	337	61
4	40.0	356	325	61
$\bar{x}$	41.2	355	331	60
σ	1.1	10	8.2	0.96
R.S.D. (%)	2.8	2.8	2.5	1.6

Analysis for free and bound neutral sugars

Investigations were then made on the levels and nature of the covalently bound neutral sugars in serum glycoproteins, to verify and differentiate the free and covalently bound sugars. Also, experiments were made on lipid bound neutral sugars in the ethanol precipitates of the proteins.

Precipitation of glycoprotein with ethanol

The protein in duplicate aliquots of a control pooled serum sample was precipitated with ethanol, analyzed, and additional aliquots were precipitated with ethanol and the precipitates washed once with ethanol. Other precipitates were washed with ethanol two and four times, then analyzed. The results are given in Table VIII.

The data show that fucose, mannose, and galactose are protein-bound. The level of these neutral sugars in the protein does not significantly change after four washings with ethanol; a decrease of about 10% was noted for mannose and galactose. However, the level of glucose does change significantly after two washings (>50%). Most of the glucose can be removed by washing with ethanol and most of it is present as the free neutral sugar. Thus, during precipitation of glycoprotein with ethanol, glucose coprecipitates or is occluded.

Dialysis of neutral sugars from serum

A pooled control serum sample was used (different from that used for the ethanol-washing experiment). The serum was dialyzed using seamless cellulose tubing with an average pore size of 24Å. The cellulose tubing was permeable to water and compounds of low molecular weight, but not to proteins. Serum (15.0 ml) was pipetted into a dialysis tube (1 in. flat width × 8 in.), tied at one end and dialyzed at 4° against 1.0 l of d.d. H_2O for over 48 h. The dialysis water was replaced with fresh d.d. H_2O after 2, 4, 6, 8, 24 and 48 h. Then, aliquots of serum were taken from the dialysis tube after each designated time period, precipitated with ethanol, and analyzed for protein-bound neutral sugars by GLC (Table IX).

TABLE VIII

PRECIPITATION OF SERUM PROTEINS WITH ETHANOL: EFFECT OF MULTIPLE ETHANOL WASHINGS

Five ml of ethanol were added to 0.5 ml of serum, shaken vigorously, allowed to stand 15 min, then centrifuged at 1500 *g* for 30 min. Each value is the average of two independent analyses on a pooled control serum.

No. of ethanol washings	Neutral sugars (μg/ml)			
	Fucose	Mannose	Galactose	Glucose
1	44.0	265	294	111
2	47.9	266	272	56.3
4	48.5	237	264	44.7

Dialysis of isolated serum glycoprotein for neutral sugars

The glycoprotein was dialyzed in seamless cellulose tubing with an average pore size of 24 Å. A pooled serum aliquot (0.5 ml) was precipitated with ethanol, then suspended in 2.0 ml of d.d. H_2O with sonication, and dialyzed (dialysis tube: 1 in. flat width × 8 in., tied at one end) at 4° against 1.0 l of d.d. H_2O over 24 h with occasional agitation. The dialysis water was replaced with fresh d.d. H_2O after 2, 4, 6 and 8 h. Then the dialysate containing the protein was transferred to a Virtis 150-ml flask and lyophilized to near dryness. The lyophilized sample solution was transferred into a 16 × 100 mm culture tube, 2.0 ml of 0.1 *N* NaOH were added, and the dialyzed protein was analyzed for neutral sugars by GLC.

The results of this experiment are given in Table IX together with data from experiments for neutral sugars in the ethanol-washed precipitates, and dialyzed serum. The data from multiple ethanol washings of the glycoprotein, dialysis of pooled serum, and dialysis of isolated glycoprotein are all comparable; that

TABLE IX

NEUTRAL SUGARS IN SERUM PROTEIN

Each control a different pooled serum; 0 = control.

Sugar	Ethanol-precipitated serum proteins washed with ethanol		Dialysis of neutral sugars from serum		Dialysis of isolated serum glycoproteins	
	0 Washings	4 Washings	0 h	24 h	0 h	24 h
Fucose (μg/ml)	44.0	48.5	42.9	43.3	45.8	42.8
Mannose (μg/ml)	265	237	374	378	345	335
Galactose (μg/ml)	294	264	340	340	330	332
Glucose (μg/ml)	111	44.7	257	55.0	263	76.0

is, all of the fucose, mannose, galactose, and some of the glucose, in ethanol precipitates of serum are covalently bound to serum proteins.

The levels of fucose, mannose, and galactose were not changed on dialysis of serum or serum proteins, but the level of glucose changed significantly, and so confirmed that about 25% of the glucose in serum is covalently bound in serum glycoprotein.

Lipid-bound neutral sugars

Ethanol precipitates of pooled control serum were dissolved in 0.1 *N* NaOH and extracted for lipids according to the Folch method [41] with chloroform—methanol (2:1, v/v). The lipid extract was analyzed for covalently bound neutral sugars, but none were found above the background level. Thus, the neutral sugars are not present in serum in the form of glycolipids.

Analysis of alditol acetates by mass spectrometry

The mass spectra of high purity alditol acetates of 2-deoxyribose, L-fucose, arabinose, xylose, 2-deoxyglucose, mannose, galactose, and glucose were obtained by GLC—electron-impact mass spectrometry (MS). Alditol acetates of sugar standards and sugars from glycoproteins of serum from cancer patients were prepared according to the previously described analytical method. The mass spectral fragmentation pattern of the derivatized standards and samples from cancer patients were compared with those of commercially available (Regis) high purity neutral sugar alditol acetates. Comparison of these sets of spectra based on spectra published by Lonngren and Svensson [42] showed very good agreement, thus confirming the identity of the peaks of interest and their homogeneity. A small unknown peak in glycoprotein from cancer samples (indicated by an arrow in Fig. 6) was analyzed by GLC—MS and the elucidation of its structure was attempted. The unknown compound showed deviations from the characteristic fragmentation pattern of alditol acetates [42] with the largest detectable fragment at *m/e* 331. High-resolution GLC—MS of the *m/e* 331 ion indicates a nitrogen-containing compound with the formula $C_{17}H_{17}NO_6$.

Normal levels of protein-bound neutral sugars in sera

The protein and neutral sugar levels for normal non-fasting males, normal non-fasting females, and normal fasting females were determined (Table X). The $\bar{x}$, σ, and R.S.D. (%) values for a population of n are also presented in the table. On comparing a group of normals analyzed by Mrochek's high-performance liquid chromatographic (HPLC) method [25] with data for our group of normals by GLC (Table XI), no significant differences were observed. The GLC method gave slightly higher values for all the neutral sugars. This may be attributed to the difference in hydrolysis conditions.

Sugar:protein ratios in serum from ovarian and small cell lung carcinoma

In Fig. 7 are given the serum fucose:protein ratios for patients with small cell carcinoma of the lung. Each patient is distributed in one of three disease or response categories: (1) pre-treatment, progressive disease; (2) complete or partial response following chemotherapy; and (3) post-treatment, recurrent, or

TABLE X

NORMAL LEVELS OF PROTEIN-BOUND NEUTRAL SUGARS IN SERA

The R.S.D. (%) values are for the respective population.

	Protein (mg/ml)	Sugar (μg/mg of protein)		
		Fucose	Mannose	Galactose
Non-fasting males (n = 11)				
$\bar{x}$	76	0.71	6.17	5.64
σ	6.8	0.07	0.56	0.63
R.S.D. (%)	8.9	10	9.1	11
Non-fasting females (n = 11)				
$\bar{x}$	72	0.73	7.1	6.6
σ	2.6	0.08	0.76	0.81
R.S.D. (%)	3.6	11	11	12
Fasting females (n = 9)				
$\bar{x}$	73	0.75	7.0	6.5
σ	3.4	0.18	0.73	0.67
R.S.D. (%)	4.6	24	10	10

TABLE XI

COMPARISON OF DATA BY HPLC AND GLC FOR PROTEIN-BOUND NEUTRAL SUGARS

The R.S.D. (%) values are for a population of n.

	Sugar (μg/ml of serum)*					
	Fucose		Mannose		Galactose	
	HPLC**	GLC***	HPLC	GLC	HPLC	GLC
Non-fasting females						
n	12	11	12	11	12	11
$\bar{x}$	48	52	444	511	436	477
R.S.D. (%)	25.7	9.9	17.6	9.4	19.5	11.2
Fasting females						
n	4	9	4	9	4	9
$\bar{x}$	40	55	392	514	414	473
R.S.D. (%)	20.2	25.0	16.2	10.9	15.7	10.2

*Each value is an average for n in a population.
**Determined by Mrochek's HPLC Method [25].
***Determined by our GLC method.

progressive disease. As shown, the majority of patients with progressive disease whether before or after treatment had elevated values. In contrast, the majority (13/16) of those patients in complete or partial remission had fucose:protein ratios within the normal range. Three patients had somewhat elevated values. Whether at the time blood was drawn these patients had occult disease not detected by the usual clinical means is not known. Similar results were found for patients with ovarian carcinoma. Both fucose:protein and galactose:protein

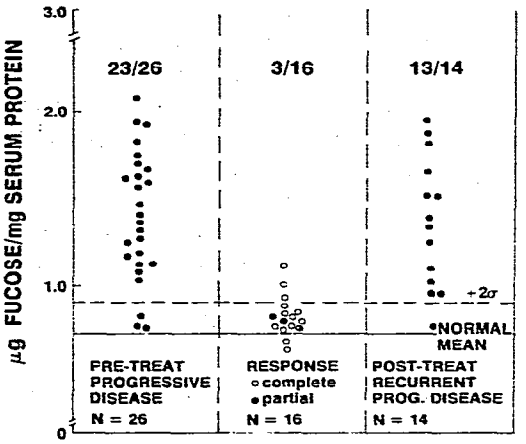


Fig. 7. Fucose levels in small cell carcinoma of lung.

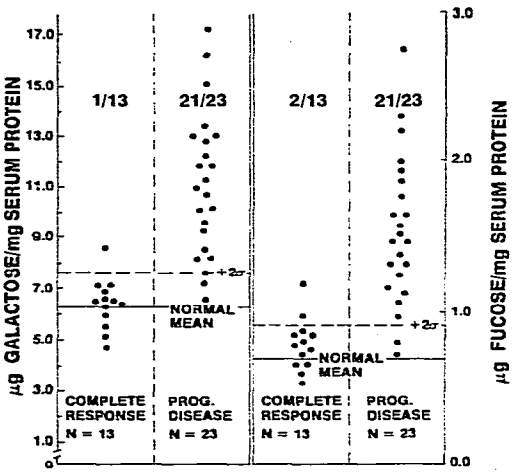


Fig. 8. Galactose and fucose levels in ovarian carcinoma.

values are plotted in Fig. 8 for 23 patients with progressive disease and 13 patients considered to be in complete clinical remission.

In Table XII the results for galactose:protein ratios are compared for serum and malignant effusions, ascites and/or pleural fluid, obtained from five patients with progressive ovarian cancer. As noted, the serum, ascites, and pleural fluid tend to parallel the value for the serum for each respective patient.

In our previous study of serum fucose:protein ratios involving patients with breast cancer [24] we confirmed that the ratio was elevated in acute metabolic and inflammatory diseases other than cancer, and demonstrated that (a) diet (i.e. fasting or non-fasting) had no effect on the ratio; (b) the ratio was elevated in close to 90% of the patients with metastatic breast disease; and (c) the ratio paralleled changes in tumor mass, or progression or regression after therapy.

TABLE XII

GALACTOSE:PROTEIN RATIO IN SERUM AND MALIGNANT EFFUSION FOR PATIENTS WITH OVARIAN CARCINOMA

Patient	μg Galactose per mg protein		
	Serum*	Ascites effusion	Pleural effusion
A	15.8	16.7	16.6
B	10.7	10.6	—
C	11.5	—	10.8
D	12.2	12.4	—
E	17.0	14.0	—

* $\bar{x}$ for normal females = 6.2 (range 4.8—7.6).

Studies to date indicate that in general both galactose:protein and mannose:protein ratios are lower and less frequently elevated for patients with either breast cancer or small cell carcinoma of the lung, in contrast to the fucose:protein ratios. However, for patients with ovarian cancer whose disease is progressing, in general we have found that both galactose:protein and mannose:protein ratios also were elevated if the fucose:protein ratios were raised.

Small cell carcinoma of the lung spreads to other organs or tissues early in the course of disease, with frequent involvement of liver, bone marrow and brain. However, this carcinoma responds rapidly to chemotherapy and remission rates of 50—70% have been reported. Nevertheless, the carcinoma is prone to recur and the possibility of occult disease even when the patient is considered in complete remission may be great. Carcinoma of the ovary tends to remain confined to the abdominal cavity without distant metastases. Patients, however, are most frequently present initially with advanced disease (stage III and IV), and it is very difficult to assess disease status early in its course or to determine if residual tumor remains after treatment.

It is evident that additional diagnostic information is needed in the clinical management of patients with small cell carcinoma of the lung and ovarian cancer. These preliminary results show the great potential of the fucose, mannose, and galactose:protein ratios in following the course of the disease.

Comments on the method

(1) The optimum times for complete reduction and acetylation of the neutral sugars were investigated and confirmed with literature conditions [13, 27, 35].

(2) 2-Deoxyglucose was added as internal standard for calculating the neutral sugar concentrations.

(3) 2-Deoxyribose was added to monitor the effectiveness of sample cleanup, and losses on evaporation of acetylating reagents.

(4) A strong cation exchange resin, AG 50W-X8 (H^+), was used to remove substances that interfered in derivatization.

(5) A strong anion-exchange resin, AG 1-X8 (CH_3COO^-), removed chromatographic interferences.

(6) To assure accuracy and precision of this method, thoroughly mixed samples for homogeneity and addition of exact amounts of internal standard are mandatory.

(7) A pooled normal control sample with pre-determined neutral sugar levels was prepared and analyzed with each group of samples (about 20) to establish and monitor the performance of day-to-day analyses.

(8) The temperature for drying samples on the rotary evaporator and in heating blocks should not exceed 60°. After samples are taken to dryness in the heating block they should be removed immediately. Prolonged heating causes some loss of the neutral sugars.

(9) Each day the instrument settings, RWRs, and performance are checked and compared by injecting a standard solution.

(10) In our laboratory the Silar-7CP column has been used for more than 400 analyses without loss of separation efficiency.

CONCLUSIONS

The potential of the neutral sugar:protein ratios (fucose, mannose, and galactose) as markers for clinical cancer management has been demonstrated. Initial results for small cell lung and ovarian carcinoma showed that more than 88% of the patients with progressive disease had elevated fucose:protein values. In contrast, following chemotherapy more than 81% of those patients in complete or partial remission of disease had fucose:protein ratios within the normal range. In post-treatment small cell lung cancer patients with recurrent progressive disease more than 93% had elevated fucose:protein values. Thus, our studies to date indicate that the fucose:protein ratio is a good index for following changes in tumor burden in small cell lung and ovarian carcinoma. In general both the galactose:protein and mannose:protein ratios are lower and less-frequently elevated for patients with either breast cancer or small cell carcinoma of the lung, in contrast to the fucose:protein ratio. However, for following the progression and remission of ovarian carcinoma the sugar:protein ratios for fucose, mannose, and galactose serve as reliable markers. This is extremely helpful to the physician in following response to chemotherapy; as for patients with ovarian carcinoma it is very difficult to know the disease status early in its course or to determine if residual tumor remains after treatment.

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REFERENCES

1 A. White, P. Handler and E. Smith, Principles of Biochemistry, McGraw-Hill, New York, 5th ed., 1973, p. 56.
2 H. Faillard and R. Schauer, in A. Gottschalk (Editor), Glycoproteins: Their Composition, Structure, and Function, Part B, Elsevier, Amsterdam, 1972, p. 150.
3 C.A. Apffel and J.H. Peters, Progr. Exp. Tumor Res., 12 (1969) 1.
4 R.A.L. Macbeth and J.G. Bekesi, Cancer Res., 22 (1962) 1170.
5 R.A.L. Macbeth and J.G. Bekesi, Arch. Surg., 88 (1964) 633.
6 R.A.L. Macbeth and G. MacBride, Cancer Res., 25 (1965) 1779.
7 M.R. Shetlar, Ann. N.Y. Acad. Sci., 94 (1961) 44.
8 S. Snyder and G. Ashwell, Clin. Chim. Acta, 34 (1971) 449.
9 R.G. Spiro, New Eng. J. Med., 281 (1969) 991.
10 P.O. Almquist and E.A. Lansing, Scand. J. Clin. Invest., 9 (1957) 179.
11 A. Hadjivassilion, A. Costanaki, G. Hriston and B. Lissaios, Surg. Gynecol. Obstet., 140 (1975) 239.
12 M.R. Shetlar, J.U. Foster and K.H. Kelly, Cancer Res., 9 (1949) 515.

13 R.J. Winzler, in B. Glick (Editor), Methods of Biochemical Analysis, Vol. II. Interscience Publishers, New York, 1955, p. 294.
14 A. Saipr and S.K. Weintraub, Clin. Chim. Acta, 6 (1961) 174.
15 F.E. Rosato, Surg. Gynecol. Obstet., 124 (1967) 1291.
16 F.E. Rosato and M.H. Seltzer, Amer. J. Surg., 118 (1969) 61.
17 F.E. Rosato, M. Seltzer, J. Muller and E.F. Rosato, Cancer, 28 (1971) 1575.
18 J.J. Barlow and P.H. Dillard, Obstet. Gynecol., 39 (1972) 727.
19 A.S. Evans, M.F. Dolan, P.Z. Sobocinski and E.A. Quinn, Cancer Res., 34 (1974) 538.
20 P.Z. Sobocinski, K.M. Hartley, A.S. Evans and W.J. Canterbury, Armed Forces Radiobiology Research Institute, Scientific Report SR 72-13, Bethesda, Md., 1972.
21 P.Z. Sobocinski, W.J. Canterbury and K.M. Hartley, Armed Forces Radiobiology Research Institute, Scientific Report SR 72-6, Bethesda, Md., 1972.
22 G. Gyorky and J.C. Houck, Can. J. Biochem., 43 (1965) 1807.
23 Z. Dische and L.B. Shettles, J. Biol. Chem., 175 (1948) 595.
24 T.P. Waalkes, C.W. Gehrke, D.C. Tormey, K.B. Wood, K.C. Kuo, J. Snyder and H. Hansen, Cancer, 41 (1978) 1871.
25 J.E. Mrochek, S.R. Dinsmore and T.P. Waalkes, Clin. Chem., 21 (1975) 1314.
26 J.E. Mrochek, S.R. Dinsmore, D.C. Tormey and T.P. Waalkes, Clin. Chem., 22 (1976) 1516.
27 M.D.G. Oates and J. Schrager, Biochem. J., 97 (1965) 697.
28 W.R. Lehnhardt and R.J. Winzler, J. Chromatogr., 34 (1968) 471.
29 J.H. Kim, B. Shome, T. Liao and J.G. Pierce, Anal. Biochem., 20 (1967) 258.
30 J. Metz, W. Ebert and H. Weicker, Chromatographia, 4 (1971) 345.
31 W.H. Porter, Anal. Biochem., 63 (1975) 27.
32 R. Kannan, P.N. Serg and H. Debuch, J. Chromatogr., 92 (1974) 95.
33 K.G. Braund, P. Ghosh, T.K.F. Taylor and L.H. Larson, Anal. Biochem., 64 (1975) 10.
34 J.R. Etchison and J.J. Holland, Biochemistry, 66 (1975) 87.
35 R.S. Varma, R. Varma, W.S. Allen and A.H. Wardi, J. Chromatogr., 93 (1974) 221.
36 J. Casals-Stenzel, H. Buscher and R. Schauer, Anal. Biochem., 65 (1975) 507.
37 D.A. Craven and C.W. Gehrke, J. Chromatogr., 37 (1968) 414.
38 R. Varma, R.S. Varma, W.S. Allen and A.H. Wardi, J. Chromatogr., 86 (1973) 205.
39 J. Abdel-Akher, J.K. Hamilton and F. Smith, J. Amer. Chem. Soc., 73 (1951) 4601.
40 L.L. Wall and C.W. Gehrke, J. Ass. Offic. Agr. Chem., 58 (1975) 1221.
41 J.F. Folch, M. Lees and G.H. Sloane Stanley, J. Biol. Chem., 226 (1957) 497.
42 J. Lonngren and S. Svensson, Adv. Carbohyd. Chem. Biochem., 29 (1974) 41.