Table II—Acute Toxicity in Female Mice

Compound	LD_{50} and 95% Confidence Limits, mg./kg. as Base	
	Intravenous	Subcutaneous
Compound I	6.7 (5.8–7.5)	99 (85–147)
Lidocaine	26 (23–33)	211 (183–256)
Bupivacaine	6.4 (5.5–7.3)	45 (38–54)
Tetracaine	4.1 (2.9–5.3)	32 (25–42)

pared with lidocaine, indicating that the agent should have a long duration of action in peripheral nerve blocks. The long duration of anesthesia is confirmed by the results obtained in guinea pig wheals, and testing in the surgically prepared cat shows that the compound should have a rapid onset and long duration of blocks in peridural anesthesia. In addition, its tissue irritation propensities and acute toxicity are within acceptable limits as judged by comparison with bupivacaine and tetracaine. Further studies of the pharmacology and toxicology of this compound are in progress.

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GLC Determination of Sorbitol and Mannitol in Aqueous Solutions

G. MANIUS, F. P. MAHN▲, V. S. VENTURELLA*, and B. Z. SENKOWSKI

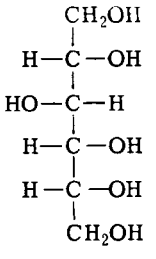
Abstract □ A rapid and specific method is described for the determination of the concentration of sorbitol and mannitol in aqueous solutions. The procedure was also applied to sorbitol USP powder. The developed GLC procedure requires the preparation of the acetate derivative, the addition of an internal standard (dioctyl sebacate), and the use of a hydrogen flame-ionization detector. The liquid phase, ECNSS-M, 5% on Gas Chrom Q, operated at 175°, is well suited for the separation of sorbitol acetate and mannitol acetate, although it is subject to gradual depletion with time. A study was made showing how the loss of this liquid phase affects peak efficiency and resolution. The GLC method is easier, more specific, and less time consuming than the official USP procedure.

Keyphrases □ Sorbitol—GLC determination from aqueous solutions □ Mannitol—GLC determination from aqueous solutions □ GLC—determination of sorbitol and mannitol in aqueous solutions

A quantitative GLC method was developed for the routine control of aqueous solutions containing sorbitol and mannitol. The present USP procedure is a laborious and highly exacting two-step process. Step 1 involves column chromatography to remove interfering excipients. This process includes the sectioning-off of column material assumed to contain all of the sorbitol and then the quantitative transfer back to the column. A final water wash is expected to desorb all of the sorbitol. Step 2 involves the determination of the amount of sorbitol by titration.

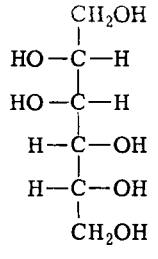
As will be shown, the GLC procedure entirely eliminates Step 1 because the sorbitol is successfully partitioned directly on the GLC column from mannitol, lower reduced sugars, and other excipients. Furthermore, GLC is well suited as a quantitative tool, particularly when an internal standard is used.

The GLC method outlined in this paper is demonstrative of a direct approach requiring neither extractions, column chromatographic purifications, nor recrystallizations. Sample preparation is restricted solely to a simple acetylation reaction, necessitating approximately 30 min. for completion. The described conditions offer an analytical alternative that greatly re-



D-sorbitol,
$C_6H_{14}O_6$

mol. wt. 182.17
anhydrous m. p. 110–112°



D-mannitol,
$C_6H_{14}O_6$

mol. wt. 182.17
m. p. 166–168°

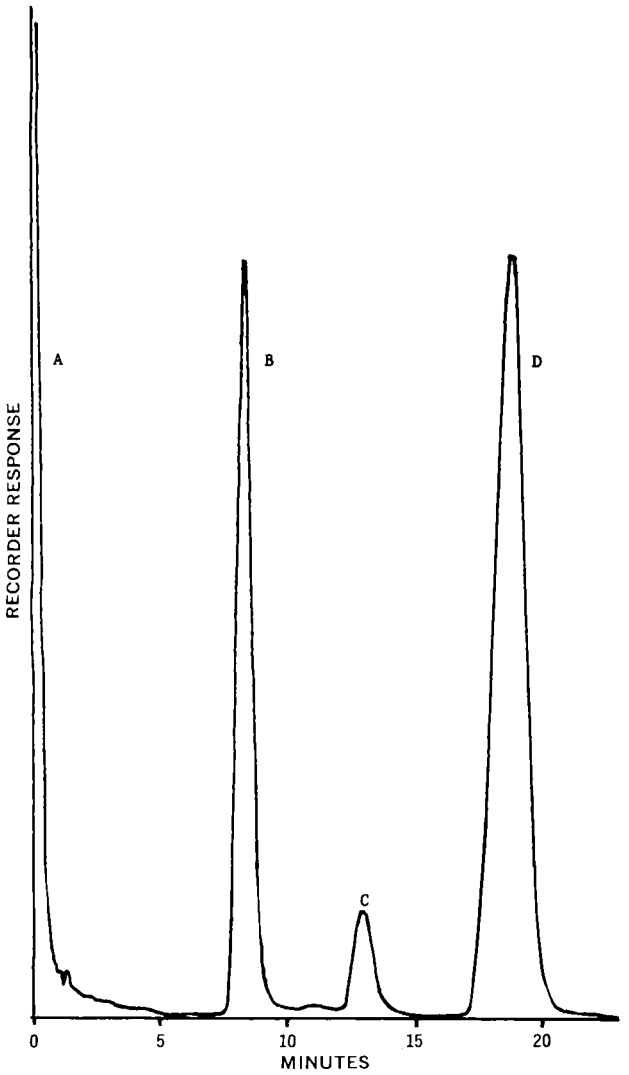


Figure 1—*Standard chromatogram showing the relative retention times of the sorbitol acetate derivative, mannitol acetate derivative, and internal standard. Key: A, solvent; B, dioctyl sebacate; C, mannitol acetate; and D, sorbitol acetate.*

duces the time factor as well as the consumption of reagents, chemicals, and glassware.

Formation of the hexaacetate derivative is shown to be a necessary step prior to GLC analysis. Experimental trials to achieve baseline separation of mannitol and sorbitol are described. Included also are the results of a study on column aging and optimum values that can be expected for efficiency and resolution. Detector linearity, data on different levels of samples, and a statistical evaluation of the method are reported.

EXPERIMENTAL

Chromatographic Conditions—The gas chromatograph[1] was equipped with a hydrogen flame-ionization detector. The recorder[2], 0–1 mv., was equipped with a disk integrator. The chart speed used was 30.48 cm. (12 in.)/hr. A stainless steel column, 0.61 m. × 0.32 cm. (2 ft. × 0.125 in.) o.d., was packed with 5% ECNSS-M on Gas Chrom Q, 80–100 mesh, fitted for "on-column" injection.

The gas flows used were: carrier gas, nitrogen, 30 ml./min.; detector gas, hydrogen, 30 ml./min.; and air, 300 ml./min. The column temperature used was isothermal, 175°, or adjusted accordingly to obtain a retention time of approximately 8 min. for the internal standard, dioctyl sebacate. The detector temperature was 260°, and the injector temperature was 175° or adjusted to the column temperature. Attenuation was 10^{-11} amp./mv., 64×, or adjusted accordingly to obtain the maximum scale recorder response. The injection volume was 1–2 μl., and the retention times[3] were: dioctyl sebacate, approximately 8 min.; mannitol acetate, approximately 13 min.; and sorbitol acetate, approximately 18 min.

A typical chromatogram is shown in Fig. 1.

Reagents—Mannitol NF was used. Sorbitol[4] and dioctyl sebacate[5] were purchased from commercial sources. All other compounds were reagent grade.

Standard Preparation—*I. Internal Standard*—Weigh accurately approximately 100 mg. of dioctyl sebacate into a 50-ml. volumetric flask. Dissolve and dilute to volume with acetone. Mix well (internal standard stock solution).

II. Reference Standard—Weigh accurately approximately 4 g. of sorbitol reference material and 300 mg. of mannitol reference material into a 25-ml. volumetric flask, tapping gently to compact the material to the bottom of the flask. Dissolve and dilute to volume with water. Caution should be exercised so that the water is added gradually to dissolve the mixture (reference standard solution).

Using a suitable microliter syringe[6], transfer exactly 25 μl. of the reference standard solution into a small vial, fitted with a screw-type, foil-lined cap. Add 0.2 ml. of anhydrous pyridine and 1 ml. of acetic anhydride. Tighten the cap, swirl to mix the contents, and place in a constant-temperature bath at 100° for 30 min. Remove, cool, and evaporate the contents to dryness on a steam bath under nitrogen. The residue is dissolved in 1 ml. of internal standard stock solution and chromatographed (working standard solution).

Sample Preparation—(Perform in Duplicate.) The aqueous samples to be assayed must be kept under refrigeration and analyzed before mold formation occurs in the solution.

A. Experimental: 40% (w/v) Aqueous Sorbitol Solutions—Accurately pipet 10 ml. of the 40% solution into a 25-ml. volumetric flask, dilute to volume with water, and mix well. Using a suitable microliter syringe[6], transfer exactly 25 μl. of this solution into a small vial fitted with a screw-type, foil-lined cap. Add 0.2 ml. of anhydrous pyridine and 1 ml. of acetic anhydride. Tighten the cap, swirl to mix the contents, and place in a constant-temperature bath at 100° for 30 min. Remove, cool, and evaporate the contents to dryness on a steam bath under nitrogen. The residue is dissolved in 1 ml. of internal standard stock solution and chromatographed (working sample solution).

B. USP: 70% (w/w) Aqueous Sorbitol Solutions—Weigh accurately approximately 6 g. of the viscous aqueous sorbitol sample solution, equivalent to about 4 g. of sorbitol, into a 25-ml. volumetric flask. Dilute to volume with water and mix well. Using a suitable microliter syringe[6], transfer exactly 25 μl. of this solution into a small vial fitted with a screw-type, foil-lined cap. Then proceed as described in *Sample Preparation A*, beginning with: "Add 0.2 ml. of anhydrous pyridine. . . ."

Procedure—The working standard solution is chromatographed and the response factor for mannitol and sorbitol is determined under the prescribed instrument parameters. After the elution of the sorbitol acetate, the column is ready for the next injection. Once a constant response is obtained, the working sample solution is chromatographed and the percent composition of the mannitol and sorbitol is determined.

Calculation—Determination of response factor for sorbitol and mannitol:

$$RF_S = \frac{A_S\,(\text{std}) \times C_{IS}}{A_{IS}\,(\text{std}) \times C_S} \qquad \text{(Eq. 1}a\text{)}$$

$$RF_M = \frac{A_M\,(\text{std}) \times C_{IS}}{A_{IS}\,(\text{std}) \times C_M} \qquad \text{(Eq. 1}b\text{)}$$

[1] Varian Aerograph, model 1200.
[2] Texas Instrument.
[3] Retention times diminish noticeably with column age.
[4] Matheson, Coleman and Bell.
[5] Varian Aerograph.
[6] Hamilton.

where:

A_S (std) = area of sorbitol acetate in the working standard solution
A_M (std) = area of mannitol acetate in the working standard solution
A_{IS} (std) = area of internal standard in the working standard solution
C_S = concentration of sorbitol in milligrams per milliliter of the working standard solution
C_M = concentration of mannitol in milligrams per milliliter of the working standard solution
C_{IS} = concentration of internal standard in milligrams per milliliter of the internal standard stock solution

Analysis for sorbitol and mannitol in aqueous preparations:

1. Experimental 40% (w/v) solutions:

$$\text{percent sorbitol (w/v)} = \frac{A_S\,(\text{spl}) \times C_{IS} \times 10{,}000}{A_{IS}\,(\text{spl}) \times RF_S \times 1000} \quad \text{(Eq. 2}a\text{)}$$

$$\text{percent mannitol (w/v)} = \frac{A_M\,(\text{spl}) \times C_{IS} \times 10{,}000}{A_{IS}\,(\text{spl}) \times RF_M \times 1000} \quad \text{(Eq. 2}b\text{)}$$

where A is area, C is concentration, and RF is the response factor as already described; 10,000 = dilution factor and conversion to percent; 1000 = conversion to grams; and (spl) = sample.

2. USP 70% (w/w) solutions:

$$\text{percent sorbitol (w/w)} = \frac{A_S\,(\text{spl}) \times C_{IS} \times 100{,}000}{A_{IS}\,(\text{spl}) \times RF_S \times \text{spl. wt. (mg.)}} \quad \text{(Eq. 3}a\text{)}$$

$$\text{percent mannitol (w/w)} = \frac{A_M\,(\text{spl}) \times C_{IS} \times 100{,}000}{A_{IS}\,(\text{spl}) \times RF_M \times \text{spl. wt. (mg.)}} \quad \text{(Eq. 3}b\text{)}$$

where A is area, C is concentration, and RF is the response factor as already described; 100,000 = dilution factor and conversion to percent.

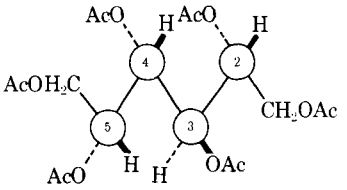
RESULTS AND DISCUSSION

Numerous GLC methods have been reported for the monosaccharides and their reduced sugars (sugar alcohols). These reports indicated favorably the use of acetate derivatives over trimethylsilyl derivatives. There are advantages and disadvantages to each.

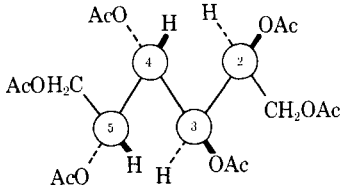
Crowell and Burnett (1) cited some disadvantages of trimethylsilyl derivatization, especially the extensive drying procedures necessary to remove all of the water, which would compete in the silylation reaction to form hexamethyldisiloxane. In addition, the results may not always be quantitative and reaction times can be quite long. Ellis (2) listed the advantages of trimethylsilyl derivatives as: (*a*) the simplicity of the reaction (as with hexamethyldisilazane and trimethylchlorosilane reagents), (*b*) higher volatility of the final products, and (*c*) wider choice of liquid phases with less column bleed.

Our trials indicated satisfactory silylation but little or no separation of the trimethylsilyl derivatives of mannitol and sorbitol on several columns of varying polarity, including OV-17, QF-1, XE-60, DOW-710, and PEG-4000 MS. Sweeley *et al.* (3) obtained no separation with SE-52 and virtually none with ethylene glycol succinate. Clearly, silylation, while it does make a suitable derivative for the sugar alcohols, will not permit effective separation of the two compounds in question. It can be reasoned that the trimethylsilyl derivatives, because of the equivalence of the methyl groups, would have little affinity for the liquid phase. In addition, reagent tailing and poor reproducibility were evident with the trimethylsilyl derivative.

Separation of mannitol and sorbitol is much more likely when the hexaacetate derivative is made. This is explained stereochemically by Gunner *et al.* (4) in their paper using the zig-zag conformational approach. They showed that the retention times of sugar alcohol molecules (in this case, mannitol and sorbitol acetates) depend on the arrangement of acetoxy groups attached to the nonterminal carbon atoms. The more acetoxy groups in the same plane, the greater is the affinity for the liquid phase of the column and, hence, the longer is the retention time. The retention time may also be affected by the closeness of the acetoxy groups to each other. In our experimentation, as expected, sorbitol with three acetoxy groups in the same plane did indeed result in a longer retention time than mannitol, which contains only two acetoxy groups in the same plane. The difference in the structure between sorbitol and mannitol is at the number two carbon atom.



sorbitol hexaacetate



mannitol hexaacetate

During this investigation, it was found that most liquid phases result in poor separation of the reduced sugars, even as their acetates. In addition, tailing occurs on some phases, including XE-60, which prevents baseline separation and quantitation of mannitol and sorbitol[7]. Etched glass beads (GLC 110) coated with 0.1% XE-60 were tried, as well as 0.1% OV-17 and 0.1% OV-225. Only the latter separated the two acetates completely and with symmetry, but the column rapidly deteriorated at the necessary operating temperature of approximately 200° (possibly due to the lean loading). The separation of mannitol and sorbitol acetates was also reported (5) using QF-1 as the liquid phase packed into a 1.8-m. (6-ft.) × 6-mm. i.d. column.

Complete separation of the alditol acetates (including mannitol, galactitol, and sorbitol) on organosilicone polymer liquid phases has been reported. Sawardeker *et al.* (6) found that the two most promising phases of this type were EGSS-X and ECNSS-M. Shaw and Moss (7) used EGSS-X to resolve their alditol acetates after having first reduced the parent monosaccharides with sodium borohydride. They found the relative response factors of mannitol to sorbitol to be 1.08 to 1.00, corresponding to results achieved in this study and confirming maximum elution of the sorbitol acetate.

The selected liquid phase, ECNSS-M, is an organosilicone polyester of ethylene glycol succinate chemically combined with a silicone of the cyanoethyl type. It is reported to have a normal operating temperature of 190° and a maximum temperature of 210°. Because of the slow column bleed rate observed at the operating temperature of 175°, there could be baseline drift, thereby necessitating periodic monitoring of the baseline. It is theorized that there is a steady "bleeding-off" of the ethylene glycol succinate component of the liquid phase.

Since a continual decrease in retention times had also been observed, a study of column depletion was undertaken. Two columns of identical dimensions [stainless steel, 0.61 m. × 0.32 cm. (2 ft. × 0.125 in.) o.d.] were prepared. One was packed with 2% ECNSS-M on Gas Chrom Q; the other, as the procedure specifies, was packed with 5% ECNSS-M on Gas Chrom Q. After conditioning both overnight at 140°, the leaner loaded column was operated isothermally at 160° over 10 days, while the other column was operated at 175° over the same period of time. These temperatures initially yielded comparable retention times.

Calculations of column efficiency for all three components were made using the following formulas:

$$n = 16\left(\frac{L}{M}\right)^2 \quad \text{(Eq. 4}a\text{)}$$

[7] P. Weinert, W. Szkrybald, and G. Jaffe, Hoffmann–La Roche Inc., Nutley, N. J., reported data using XE-60, which included a separation of mannitol acetate and sorbitol acetate.

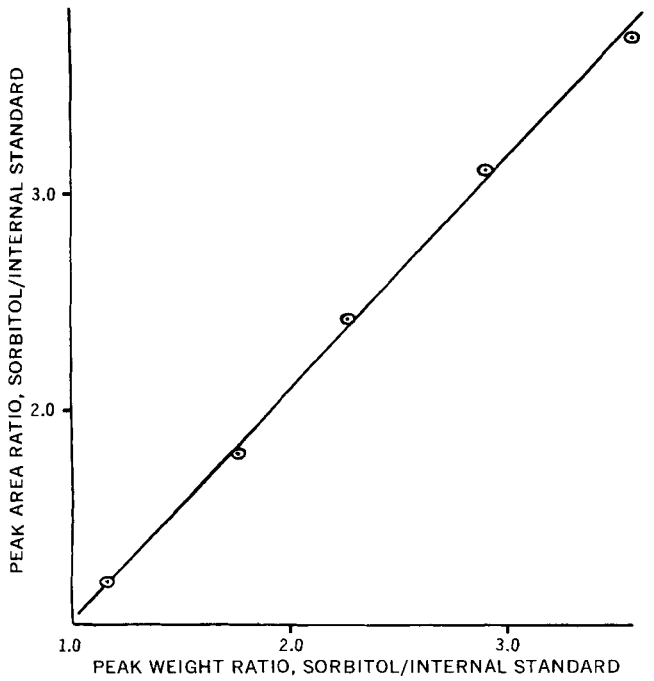


Figure 2—*Linearity of response of sorbitol with concentration—column, 5% ECNSS-M on Gas Chrom Q; length, 61 cm. (2 ft.); temperature, 175°. Sorbitol was injected as the acetate derivative with the internal standard as a constant.*

and:

$$\frac{n}{l} = \text{number of theoretical plates per column foot} \quad \text{(Eq. 4}b\text{)}$$

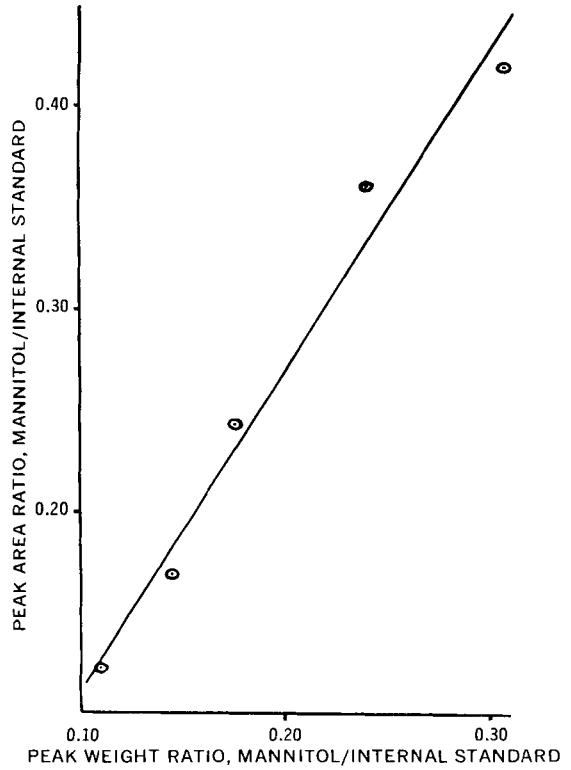


Figure 3—*Linearity of response of mannitol with concentration—column, 5% ECNSS-M on Gas Chrom Q; length, 61 cm. (2 ft.); temperature, 175°. Mannitol was injected as the acetate derivative with the internal standard as a constant.*

Table I—Determination of Sorbitol and Mannitol in Various Samples

Lot	Sorbitol, % (w/v)	Mannitol, % (w/v)
Experimental 40% Solutions		
N-3603	45.3	0.4
N-3606	44.0	0.6
N-3616	44.4	0.4
N-3618	41.1	0.4
Sample	Sorbitol, % (w/w)	Mannitol, % (w/w)
USP 70% Solutions		
1	70.4	2.0
2	68.5	2.1
3	70.8	2.2

where:

n = number of theoretical plates
L = distance from injection point to tangential half-peak width
M = tangential peak width
l = column length in feet

The drop in efficiencies in the columns, observed after 10 days, was as follows: (*a*) 2% column loading—dioctyl sebacate, initially 392 to 116 plates/ft.; mannitol, initially 620 to 225 plates/ft.; and sorbitol, initially 512 to 266 plates/ft.; and (*b*) 5% column loading—dioctyl sebacate, initially 261 to 155 plates/ft.; mannitol, initially 422 to 304 plates/ft.; and sorbitol, initially 353 to 288 plates/ft.

The resolution between mannitol and sorbitol also diminished and after a few days showed a general leveling-off of values above 1.5, which should be expected to hold fairly constant for some time. Column temperature might have to be lowered, however, to achieve proper retention times.

The 5% packing would seem to be the one of choice because its lifetime is longer (it would take a longer amount of time to lose its volatile liquid phase), it shows better efficiency over the long run, and, most important, it accommodates more sample without overload patterns which could impair quantitation.

It was reported (1) that sorbitol exhibits the slowest acetylation rate of the sugar alcohols. In our study, complete acetylation of sorbitol occurred in 30 min. at 100°. In addition, if the acetylation reagents are removed by evaporation following derivatization, no tailing of the solvent is observed in the chromatogram.

Detector linearity for both sorbitol acetate and mannitol acetate *versus* the internal standard as a constant is shown in Figs. 2 and 3, respectively.

Results obtained on experimental 40% aqueous sorbitol solutions and USP 70% solutions are listed in Table I. The GLC method, when applied to sorbitol powder USP, was quite satisfactory and no difficulty was encountered in handling. A typical sorbitol assay was 97.8%, well above the minimum USP level of 91%.

The internal standard, dioctyl sebacate, contained a slight impurity which did not interfere in the retention time of any of the peaks being measured. Dinonyl sebacate would be a better choice since its elution is closer to mannitol acetate; however, pure dinonyl sebacate could not be obtained.

Six replicates of an aqueous solution containing 40% sorbitol (w/v) showed a mean of 45.1 with a standard deviation of ±1.15. The limits of a single determination (*ts*) at the 95% confidence level for 5 degrees of freedom was ±2.97.

This method offers a more rapid and simple means of analysis of aqueous sorbitol solutions and dry sorbitol powder than existing techniques, including the USP method. Furthermore, in comparison to the USP procedure, the GLC assay is relatively inexpensive, requiring small amounts of reagents, column material, and packing. Column life itself can be extended by lower temperature operation without sacrificing efficiency or resolution. The GLC method is applicable to the analysis of both sorbitol and mannitol in the same chromatogram, while the USP procedure determines the sorbitol content.

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Phenylbutazone–Sodium Warfarin Binding Using a Fluorescent Probe Technique

H. W. JUN▲, L. A. LUZZI, and P. L. HSU

Abstract □ 1-Anilinonaphthalene-8-sulfonate was used to study the binding of phenylbutazone and sodium warfarin to bovine serum albumin. The protein molecules appeared to have an average of three binding sites for these compounds. The binding constant for phenylbutazone was found to be 2.5 times larger than that for sodium warfarin. Identical binding sites with a different binding affinity between the two drugs indicate a competition for the binding sites between phenylbutazone and sodium warfarin in favor of phenylbutazone. The mechanism of binding for these drugs may be of a hydrophobic nature.

Keyphrases □ Phenylbutazone–warfarin binding—studied using fluorescent probe technique, binding constants, proposed mechanism □ Warfarin–phenylbutazone binding—studied using fluorescent probe technique, binding constants, proposed mechanism □ Fluorescent probe technique—used to study phenylbutazone–warfarin binding, binding constants, proposed mechanisms □ Interactions—phenylbutazone–warfarin–protein binding constants, proposed mechanism

Phenylbutazone has been shown to displace sodium warfarin from plasma protein binding sites. Aggeler *et al.* (1) used dialysis techniques to demonstrate that sodium warfarin is displaced by phenylbutazone. O'Reilly and Levy (2), using *in vivo* studies, showed that the elimination half-life of warfarin is decreased, while its anticoagulant activity is increased, by concurrent administration of phenylbutazone. Other clinical studies (3, 4) also showed a marked increase in prothrombin time of sodium warfarin in the presence of phenylbutazone.

Fluorescence probe techniques were recently employed for the study of drug–protein binding (5) and for elucidation of the competitive binding of substrates and enzymes (6). Therefore, the binding of phenylbutazone and sodium warfarin to bovine serum albumin was studied by the use of 1-anilinonaphthalene-8-sulfonate, and the competition between the two drugs for the binding sites was examined.

EXPERIMENTAL

The general approach and techniques used were essentially the same as those employed by Brand *et al.* (6) in their study of the binding of rose bengal and anilinonaphthalene sulfonates to alcohol dehydrogenase. A few minor modifications were made.

Materials—1-Anilinonaphthalene-8-sulfonate[1] was employed as a fluorescent probe. This compound has minimal fluorescence in aqueous solution but fluoresces strongly when bound to bovine serum albumin. Phenylbutazone[2] and sodium warfarin[3] were used without further purification. Crystalline bovine serum albumin[4] was purchased. Methanol[5] was spectroscopic grade, and all other chemicals were reagent grade or of special purity. Water used in this study was double distilled from glass.

Apparatus—Fluorometric measurements were made with a spectrophotofluorometer[6]. The relative fluorescence intensities of bound probe were obtained directly from fluorometer readings of uncorrected excitation (375 nm.) and emission (475 nm.) wavelengths.

Methods—Fluorescence intensities of the protein–probe complex as a function of probe concentration ($1.0–15.0 \times 10^{-6}$ *M*) were measured at two protein concentrations (0.2 and 2.0 mg. protein/2 ml. solution) in pH 7.2 phosphate buffer. Two milliliters of each protein solution was titrated with successive additions of 2 μl. of 1×10^{-3} *M* probe in methanol. Titrations were performed manually with Hamilton microsyringes. Experiments were carried out at 28°.

Titrations of the protein solutions with probe were repeated in the presence of 1×10^{-4} *M* concentration of both phenylbutazone and sodium warfarin individually. The drugs were added to the solution prior to titration.

Treatment of Data—Enhancement of the fluorescence of the probe upon addition to bovine serum albumin at two concentrations and the subsequent decrease of fluorescence in the presence of the binding competitors, phenylbutazone and sodium warfarin, were used to calculate the binding constants for the probe and competitors. The fraction of probe bound, X, was calculated using the following

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Lot No. SN 46241, Ciba-Geigy Pharmaceuticals, Ardsley, New York, N. Y.
[3] Lot No. 833-1521, Abbott Laboratories, North Chicago, Ill.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Matheson, Coleman & Bell, Norwood, Ohio.
[6] Aminco-Bowman.