free fraction values of salicylic acid and sulfisoxazole, which are much less extensively protein bound, exhibited a statistically significant negative correlation with the concentration of albumin in rat serum.

Phenobarbital treatment, which is an effective means of lowering the serum bilirubin concentration in certain types of unconjugated hyperbilirubinemia (11, 12), has no apparent effect on the serum protein binding of bilirubin in rats. This information will facilitate the interpretation of results of phenobarbital–bilirubin interaction studies in normal rats. It remains to be determined if phenobarbital treatment affects bilirubin binding in the Gunn rat, the most important animal model of physiological unconjugated hyperbilirubinemia.

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[x] To whom inquiries should be directed.

Rapid GLC Determination of Therapeutic Plasma Glycerin Levels

THOMAS J. GOEHL [x], WILLIAM H. PITLICK, GERALD K. SHIU, and PICHAI PIRAKITIKULR

Abstract □ A new rapid and inexpensive method for the determination of therapeutic plasma glycerin concentrations is described. In this method, acetic anhydride and pyridine are added to 15 μl of plasma. After brief incubation and centrifugation, an aliquot of the supernate is injected directly onto the 3% OV-1 column. A linear calibration curve was found in the 0.05–3-mg/ml range, with the precision of the assay estimated to be ±5.5% (*RSD*). The method was used in determining preliminary pharmacokinetic data in the rabbit.

Keyphrases □ Glycerin—GLC analysis, plasma □ GLC—analysis, glycerin in plasma □ Hyperosmolar dehydrating agents—glycerin, GLC analysis in plasma

Cerebral edema, or swelling of brain tissue, increases intracranial pressure, which, if not medically corrected, leads to herniation of brain tissue with irreversible brain damage and subsequent death. Deterioration of brain function and progressive deepening of coma in acute neurologic emergencies are often results of increased intracranial pressure due to cerebral edema. Medical management is a triad composed of hypothermia, hypocapnea, and hyperosmolar dehydration of cerebral tissues (1). Glycerin has been particularly useful for the treatment of cerebral edema by hyperosmolar dehydration because of its physical properties, but a lack of clinical knowledge has hindered its maximum use (2, 3).

At present, empirical relationships govern the use of glycerin in life-threatening situations. The efficacy of treatment with glycerin probably could be improved with quantitative assessment of glycerin therapy, including monitoring of plasma levels. It is now possible, and preferable, to individualize the dosage regimen for each patient based on a desired therapeutic plasma concentration range known to produce a desired response. In life-threatening situations, such as increased intracranial pressure, it is imperative to have rapid assessment of plasma glycerin levels for effective therapy.

Currently, the most commonly used methods for the determination of plasma glycerin levels are enzymatic (4–11). The first such method (4) was developed for determining endogenous glycerin levels, normally in the 5–17-μg/ml range. For assaying therapeutic plasma glycerin levels (milligram per milliliter range) with enzymatic methods, it is necessary to find the proper dilution factor for each sample, which is both time consuming and expensive. Thus, another method was needed that would be

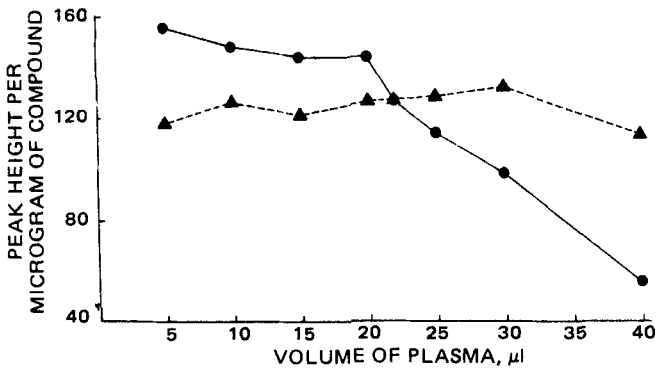

Figure 1—*Effect of plasma volume on acetylation of glycerin* (●) *and 1,6-hexanediol* (▲).

applicable over a wide range of plasma glycerin concentrations. In addition, it was highly desirable to reduce the cost of each assay by eliminating the use of relatively expensive enzymes. Consequently, a rapid, specific, and inexpensive method for the determination of glycerin in microliter volumes of plasma was developed.

EXPERIMENTAL

Apparatus—A gas chromatograph, equipped with a flame-ionization detector[1] and a 2-mm i.d. × 180-cm coiled glass column packed with 3% OV-1 on 80–100-mesh Supelcoport[2], was used. Prior to packing, the column was thoroughly rinsed with methanol and acetone, dried, and conditioned for 1 hr with 5% dimethyldichlorosilane[3] in toluene. Then the column was rinsed with toluene and acetone and dried. A silane-treated glass wool[3] plug was placed in the exit end of the column, but none was used in the injection port side. Prior to use, the column was conditioned by injecting 30 μl of a trimethylsilylating mixture[4].

Chromatographic Conditions—The column oven, injection port, and detector were maintained at 120, 250, and 250°, respectively. Nitrogen, the carrier gas, was maintained at 60 ml/min, with hydrogen and air at 20 and 240 ml/min, respectively.

Reagents—Glycerin[5], the internal standard 1,6-hexanediol[6], pyridine[7], and acetic anhydride[5] were of reagent grade and were used without further purification. Stock solutions containing 1 and 2 mg of 1,6-hexanediol/ml of pyridine were prepared. Small aliquots of stock aqueous solutions of glycerin were added to control citrated plasma to prepare plasma standards.

GLC Assay Procedure—To 15 μl of plasma in a 12-ml Pyrex glass-stoppered centrifuge tube were added 100 μl of acetic anhydride and 10 μl of the stock pyridine solution of 1,6-hexanediol. The glass stoppers were then inserted, and the test tubes were mixed well and heated for 15 min at 80°. After centrifugation at about 3000 rpm for 5 min, 4 μl of the supernate was injected onto the GLC column.

Enzymatic Assay Procedure—This newly developed GLC method was compared to an established enzymatic method. A commercial glycerin assay kit[8], which had been developed based on the Bucolo and David method (10), was used.

Calculations—Peak height ratios were calculated by dividing the peak height corresponding to the acetylated glycerin by the peak height corresponding to the acetylated 1,6-hexanediol. Calibration curves were constructed daily from results of spiked control plasma samples by plotting peak height ratios *versus* concentrations of glycerin.

RESULTS AND DISCUSSION

Quantification of glycerin in lipids by GLC was reported previously (12–14); glycerin was methylated (12), silylated (13), or acetylated (14) prior to chromatography. However, these methods do not lend themselves to an easy direct adaptation to the assay of glycerin in plasma.

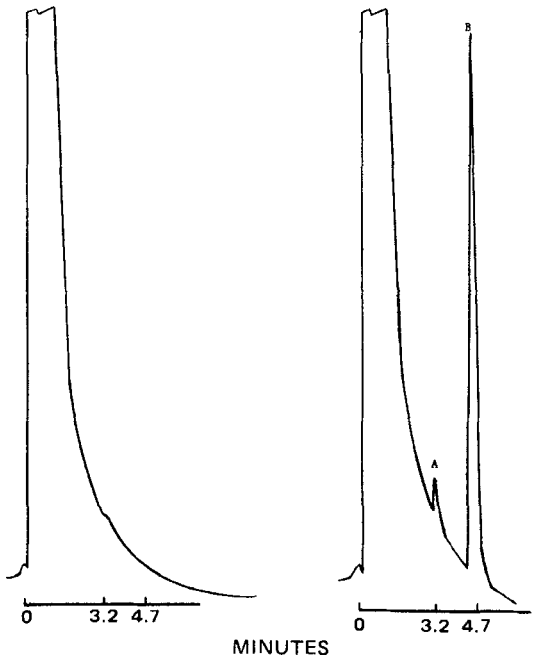

Figure 2—*Chromatograms showing peaks from glycerin analysis. Key: left, control plasma; and right, plasma containing 0.09 mg of glycerin/ml and 2.0 mg of 1,6-hexanediol/ml.*

Since removal of the glycerin from its aqueous environment in plasma proved to be difficult without a lengthy procedure, an attempt was made to derivatize the glycerin in plasma directly. A major factor that would seem to make this approach feasible was the high plasma glycerin concentrations found when glycerin was used to produce hyperosmolar dehydration of cerebral tissue. With such high concentrations, only small volumes of plasma are needed.

Acetylation of glycerin and 1,6-hexanediol, the internal standard, was accomplished with acetic anhydride in the presence of pyridine. Within 15 min at 80°, the reaction was complete. The effect of plasma volume on the acetylation was then studied. Various aliquots (5–40 μl) of plasma spiked with 1.15 mg of glycerin/ml were transferred to glass-stoppered test tubes. To each test tube were added 100 μl of acetic anhydride and 10 μl of a pyridine solution of the internal standard. Then these test tubes were incubated at 80° for 15 min.

After GLC analysis, the peak heights were measured and divided by the absolute amount of compound injected onto the column. A plot of peak height (millimeters) per microgram of compound *versus* plasma volume (microliters) was constructed (Fig. 1). The reaction with glycerin was minimally affected with up to 20 μl of plasma; with 1,6-hexanediol, up to 30 μl of plasma was tolerated. As a precaution, it is recommended that 15 μl of plasma be used. Acetic anhydride serves as an acetylating agent for the glycerin and 1,6-hexanediol and also as a precipitating agent of the plasma protein.

The recovery of glycerin and 1,6-hexanediol in this direct derivatization method was virtually 100%. In a stability study, glycerin was added to control plasma; aliquots were frozen and assayed periodically. Glycerin was stable in frozen plasma for at least 1 month.

Chromatograms of control human plasma and human plasma containing 0.09 mg of glycerin/ml are shown in Fig. 2. The chromatogram of control plasma shows no interfering peaks. In the other chromatogram, two peaks with retention times of 3.2 and 4.7 min correspond to the derivatives of glycerin and 1,6-hexanediol, respectively.

Since glycerin is often given in a glucose solution, the effect of high concentration of glucose in plasma was studied. Blood glucose levels as high as 500 mg % did not interfere with the glycerin determination.

The standard curve for the assay of glycerin in human plasma was linear and showed good reproducibility.

Table I shows a comparison of the precision and accuracy of the GLC and enzymatic assays. The enzymatic assay includes a series of reactions. First, glycerin in the sample solution is phosphorylated by adenosine triphosphate in a reaction catalyzed by glycerin kinase forming adenosine diphosphate and glycerin phosphate. Second, adenosine diphosphate reacts with phosphoenol pyruvate in a reaction mediated by pyruvate

[1] Model 5720A, Hewlett-Packard, Avondale, Pa.
[2] Supelco, Bellefonte, PA 16823.
[3] Applied Science Laboratories, State College, Pa.
[4] Pierce Chemical Co., Rockford, IL 61105.
[5] Mallinckrodt, St. Louis, Mo.
[6] Eastman-Kodak, Rochester, NY 14650.
[7] Fisher Scientific, Fair Lawn, NJ 07410.
[8] Calbiochem, La Jolla, CA 92037.

Table I—Comparison of the Precision and Accuracy of GLC and Enzymatic Assays for Glycerin

Actual Concentration, mg/ml	GLC Assay		Enzymatic Assay	
	Concentration, mg/ml ± % *RSD*	Error, %	Concentration, mg/ml ± % *RSD*	Error, %
Control	—[a]	—	—[b]	—
0.052	0.052 ± 5.8	0	0.052 ± 0	0
0.073	0.075 ± 10.7	+2.7	0.086 ± 0	+18
0.100	0.10 ± 8.0	0	0.12 ± 0	+18
0.17	0.17 ± 4.7	0	0.20 ± 2.0	+18
0.37	0.38 ± 6.6	+2.7	0.36 ± 0	−2.8
0.73	0.74 ± 5.0	+1.4	0.73 ± 1.1	0
1.3	1.2 ± 2.7	−7.7	1.2 ± 0.7	7.7
1.7	1.6 ± 0.9	−5.9	1.5 ± 0.8	−12
2.0	2.0 ± 5.5	0	2.0 ± 0.4	0
2.5	2.7 ± 3.2	+8.0	2.2 ± 1.1	−12
3.2	3.1 ± 2.4	−3.1	3.2 ± 0.9	0
Mean *RSD*, %	5.5		0.6	
Mean error, %	2.9		7.1	

[a] Not detectable. [b] Values of 0.0009 and 0.0005 mg/ml were obtained in the low and high range validation runs, respectively.

kinase, forming pyruvate and regenerating adenosine triphosphate. Third, pyruvate is reduced to lactate by lactate dehydrogenase. The concomitant oxidation of NADH to NAD^+ results in an absorbance change at 340 nm proportional to the glycerin content of the sample.

Two validation runs were made; one covered the 0.05–0.20-mg/ml range, and the other covered the 0.2–3.5-mg/ml range. The amount of internal standard used for the higher concentration range was twice that of the lower range. With the GLC assay, a mean percent *RSD* of 5.5 was found with a mean percent error of 2.9. These results compared favorably with the mean percent *RSD* of 0.6 and mean percent error of 7.1 for the enzyme assay.

A problem of sample carryover was encountered in the chromatography of these samples. This carryover could not be eliminated by on-column silylation or by increasing the injection port temperature, but it could be eliminated by removing the glass wool from the injection end of the column. In practice, a very small plug of silane-treated glass wool was used, and 5 μl of solvent was injected between samples to flush the system.

The method was used to monitor glycerin in clinical situations and to study glycerin pharmacokinetics in the rabbit. Glycerin, 10% (w/v), in normal saline solution was infused intravenously into the marginal ear vein of three rabbits (1 g/kg). Blood samples were withdrawn from the opposite marginal ear vein into small heparinized capillary tubes. After centrifugation, 15 μl of plasma was transferred into a glass-stoppered test tube and derivatized by the direct acetylation method.

A standard curve was prepared by assaying control plasma to which had been added known amounts of glycerin. A linear plot of peak height ratio *versus* concentration was prepared and used to determine the concentration of glycerin in the rabbit plasma. A semilogarithmic plot of the plasma concentration–time profile for three rabbits is shown in Fig. 3. From the mean concentration values, the half-life of glycerin in the rabbit was about 41 min with a volume of distribution of about 0.6 liter/kg, corresponding to a clearance ($K_E V_d$) of about 9 ml/kg/min.

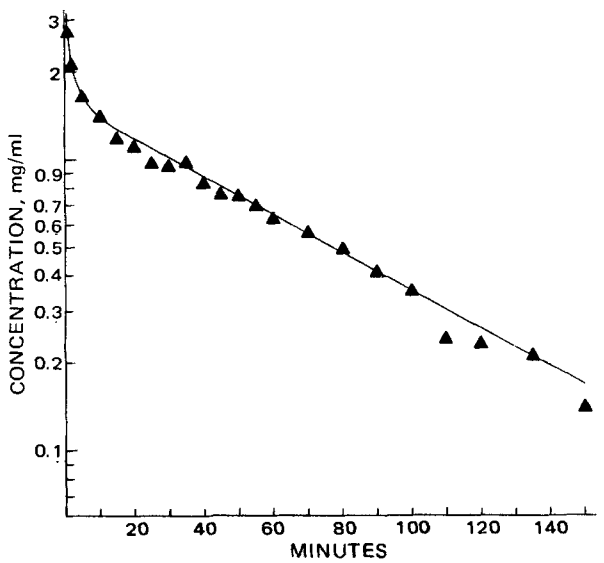

Figure 3—*Plot of plasma glycerin levels* versus *time in rabbits after intravenous medication (1 g/kg).*

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[x] To whom inquiries should be directed.