problem because the citrate assay and subsequent calculation directly reflect the citrate content, regardless of the original free citric acid concentration.

Approximate calculation of the pH at the equivalence point of the compendial citrate titration showed that the value differed markedly from the stated pH of 1.55. The calculated value of 1.81 was obtained when the preparation was assumed to contain the labeled quantities of citrate and citric acid, using a K_{a_1} value of 7.45×10^{-4} and a K_{a_2} value of 1.73×10^{-5} at 25° for citric acid (5) and assuming that the third dissociation was negligible. Applying the same approximate calculation to compute the pH at the equivalence point of a similar electrometric titration of citrate in anticoagulant citrate–dextrose solution gave a value of 2.07. This value was relatively close to the stated pH of 1.98 ± 0.02 (6).

Since commercial systemic alkalizer solutions contain additives such as preservatives, flavoring, and coloring agents, the assay of a solution placebo is recommended. The placebo should be subjected to the same analytical steps as the sample, and necessary corrections should be made for titrant consumption, if any, by the placebo. In contrast, the compendium directs the use of water as a blank in the electrometric titration of citrate and no blank runs for the titration of citric acid.

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Effect of Elevated Blood Glucose Levels on Hepatic Microsomal Enzyme System in Rats

MARTIN B. SIDEMAN

Received August 21, 1980, from the *Division of Pharmacotherapeutics, Arnold & Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, Brooklyn, NY 11201.* Accepted for publication November 11, 1980.

Abstract □ Elevated blood glucose levels attained by *ad libitum* drinking of 20% glucose solution inhibited *in vivo* *O*-dealkylation associated with the hepatic microsomal enzyme system. Significant inhibition of biotransformation was demonstrated after 1 day, with the maximum occurring at 3 days. Inhibition was followed by a tendency to return to normal activity. Serum levels of substrate and product were determined by liquid chromatographic techniques using 55% acetonitrile and 45% 0.1 *M* acetic acid as the mobile phase.

Keyphrases □ Glucose levels, blood—effect of elevation on hepatic microsomal enzyme system, rats □ Microsomal enzyme system—effect of elevated blood glucose levels, rats □ Enzyme systems—effect of elevated blood glucose levels on hepatic microsomal enzymes, rats □ Biotransformation—effect of elevated blood glucose levels on hepatic microsomal enzyme system, rats

The following reactions (1) are catalyzed by the mixed-function oxidase enzymes of the hepatic microsomal system: *N*- and *O*-dealkylation, aromatic ring side-chain hydroxylation, sulfoxide formation, *N*-oxidation, *N*-hydroxylation, deamination of primary and secondary amines, and replacement of a sulfur by an oxygen atom. Previous investigators showed that elevated blood glucose levels have inhibitory effects on several of the biotransformations mentioned in addition to depressing the cytochrome P-450 levels of the system. Hartshorn *et al.* (2) demonstrated, by *in vitro* microsomal studies in rats, the inhibition of *N*-demethylation of ethylmorphine and aniline hydroxylase as well as depression of the cytochrome P-450 content. *In vitro* microsomal studies in mice (3) showed the inhibition of hexobarbital biotransformation, primarily oxidation of the side chain at C-5, and the *N*-dealkylation of benzphetamine in addition to cytochrome P-450 depression. Strother *et al.* (4), using the *in vivo* technique of increased sleep time, also showed that elevated blood glucose levels delayed the biotransformation of various barbiturates in mice.

This investigation established, by direct measurements in blood using liquid chromatography, that elevated blood glucose levels inhibit *O*-dealkylation associated with the hepatic microsomal system.

EXPERIMENTAL

Animals—Adult male Sprague–Dawley rats, 180–200 g, were individually housed in temperature-, light-, humidity-, and air-controlled quarters. Commercial laboratory chow and drinking liquid, either water or 20% glucose solution, were given *ad libitum*.

Liquid Chromatography—A liquid chromatographic system containing a C_{18} column and an absorbance detector fixed at 254 nm was used isocratically[1]. The mobile phase consisted of 55% acetonitrile (HPLC grade) and 45% of 0.1 *M* acetic acid. Prior to use, the mobile phase was passed through a solvent-clarification apparatus to remove gases and particulates which might affect the column. Solvent delivery was at a rate of 1 ml/min. Recordings were made at a chart speed of 0.5 cm/min.

Reagents—Five liters of 20% glucose solution was prepared at one time and stored in the refrigerator until needed. Methanol (HPLC grade) was used as a solvent for the internal standard and as a protein precipitant.

Indomethacin[2] (I) [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] was used as a substrate for biotransformation and as a reference standard.

O-Desmethylindomethacin[3] (II) was used as a reference standard for the biotransformation product. Compound II is a major product of the

[1] The liquid chromatographic system (Waters Associates, Milford, Mass.) was composed of a solvent delivery system (M 6000 A), a stainless steel μBondapak C_{18} column, an absorbance detector (M 440), and an omniscribe recorder (Houston Instruments).

[2] Lot L-590,226-00125A, generously supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

[3] Lot L-594-957-00R10, generously supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

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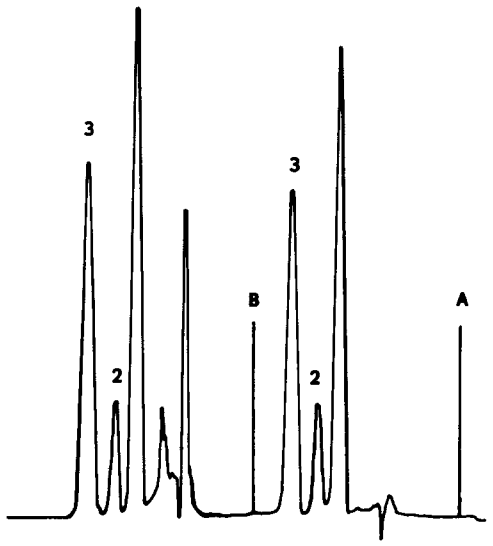

Figure 1—*Chromatogram of indomethacin, desmethylindomethacin, and the internal standard. Key: A, 0.5 ml of water used as the aqueous medium; B, 0.5 ml of serum used as the aqueous medium; 1, desmethylindomethacin; 2, internal standard; and 3, indomethacin.*

biotransformation of I in humans (5) and in various laboratory animals (6).

1-(*p*-Fluorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid[4] (III) was used as the internal standard for chromatography.

***In Vivo* Biotransformation**—The animals drank the 20% glucose solution for varying times (1, 3, 5, 7, and 10 days). Each experimental group consisted of six rats chosen at random from the pool. The control group contained eight rats. To reduce the workload on the day of sacrifice, each group was divided in half, with the second half starting 1 day later. All groups in each half were sacrificed the same day. Controls were started at the same time as the 10-day experimental group. The glucose solutions were replaced daily to avoid bacterial growth and contamination. On the morning of the day of sacrifice, all animals were tested for glucose in their urine.

Following the urine test, the animals were weighed and injected with a saline solution of I (10 mg/kg ip) solubilized by titrating to pH 7.6–8.0. The animals then were returned to their cages and sacrificed by decapitation 30 min later. The blood was collected, allowed to clot, and centrifuged; the serum was stored at from −10 to −20° until analyzed. During collection, an aliquot of whole blood was removed and tested for glucose with glucose reagent strips[5] and a reflectance colorimeter[6].

A preliminary study of blood I and II levels *versus* time showed an increase in concentration of both to a maximum at 15 min. The succeeding fall in concentration was measurable to at least 2 hr. The 0.5-hr point was chosen because of relatively high I and II concentrations. It was also assumed that little if any I was removed from the injection site and that the blood levels were the result of cellular uptake, biotransformation, and excretion on the maximum levels, in short, normal metabolic pathways.

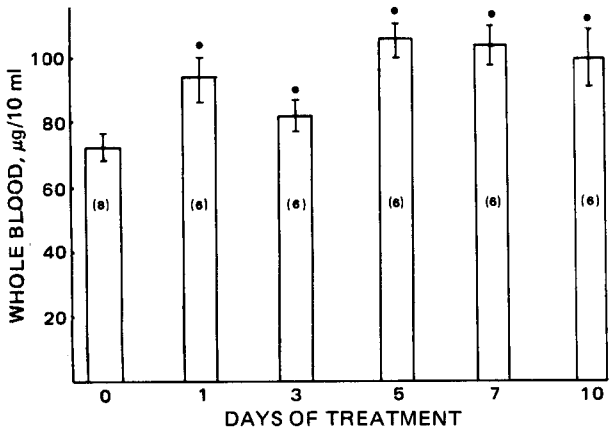

Figure 2—*Serum glucose levels. Key: ●, significant differences from control, p < 0.05, as determined by the Student t test.*

[4] Lot 620-370-00203, generously supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J.
[5] Dextrostix, Ames Division of Miles Laboratories, Elkhart, Ind.
[6] Eyetone, Ames Division of Miles Laboratories, Elkhart, Ind.

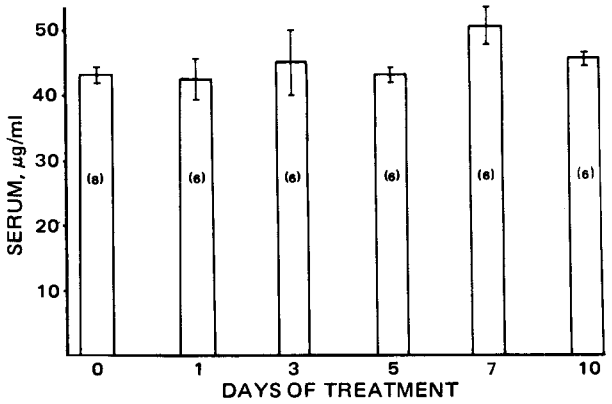

Figure 3—*Serum indomethacin levels.*

Analysis—To analyze the serum for I and II properly, substances that would interfere with the proper function of the column, principally protein, had to be removed. Methanol was added to the serum to precipitate the protein. Furthermore, methanol increased the solubility of the compounds under investigation and reduced the number of transfers needed to put them on the column. Four volumes of methanol served adequately.

The procedure used 0.5 ml of serum and 2 ml of methanol containing a fixed concentration of III. The mixture was agitated on a vortex mixer to ensure complete mixing and was centrifuged for 10 min, and the supernate was filtered to remove any particulate material. A 10-μl aliquot was placed on the column, and the peak areas were determined from standard curves.

Standard curves were prepared by plotting the ratio of the peak area produced by I or II divided by the peak produced by III *versus* the standard concentration. Recovery experiments showed that there was no loss of the three compounds if the 0.5-ml portion was methanol, water, or serum. Thus, no corrections for recovery had to be made. Figure 1 shows chromatograms of substrate, product, and internal standard added to either water or serum from a rat receiving no injections, the differences being the substances present in serum that do not interfere with the compounds under investigation. A complete chromatogram required 7 min.

All data are reported as the group mean ± *SE*. Significance to $p < 0.05$ was determined by the Student *t* test.

RESULTS AND DISCUSSION

All animals showed good growth patterns with no significant difference in body weight. The average weight at sacrifice ranged from 263 ± 6 to 278 ± 5 g; the control group was not one of the extremes. Whole blood

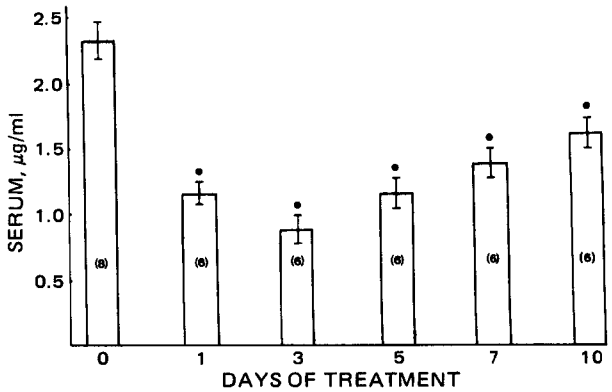

Figure 4—*Serum desmethylindomethacin levels. Key: ●, significant differences from control, p < 0.05, as determined by the Student t test.*

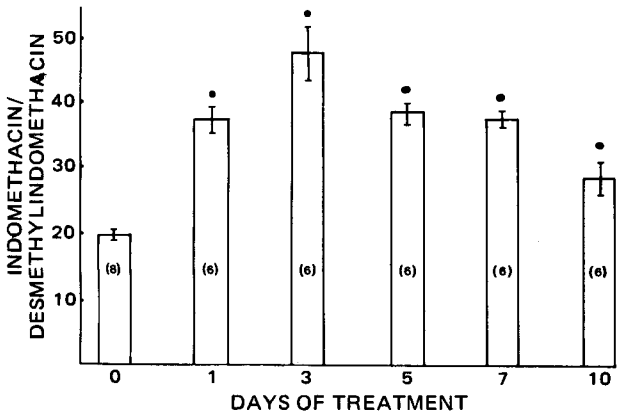

Figure 5—*Ratio of indomethacin divided by desmethylindomethacin. Key: ●, significant differences from control, p < 0.05, as determined by the Student t test.*

glucose levels (Fig. 2) were significantly elevated after 1 day and remained so. Although their blood levels were higher than those of the controls, none of the experimental animals had glucose in their urine on the day of sacrifice.

Figures 3 and 4 represent the serum levels of I and II, respectively. There were no significant differences in the substrate during the experiment. However, there were significant differences in the product level from Day 1 on. The maximum inhibition occurred at Day 3, followed by a tendency toward control levels as time progressed.

Elevated blood glucose levels obtained by oral administration of the 20% glucose solution rapidly led to inhibition of the hepatic microsomal mixed-function oxidase, which catalyzed *O*-dealkylation within 1 day. Although serum insulin levels were not measured, it can be projected from the urine levels at sacrifice and from the data presented by Hartshorn *et al.* (2) that the results were not due to hypoinsulinemia. Inversion of the inhibition after the maximum level at Day 3 confirmed earlier findings (7) that inhibition is short lived and returns to normal even though glucose administration is prolonged.

Since there were no significant differences in the blood substrate levels, the biotransformation inhibition could only be accounted for by the glucose affecting enzymatic activity. Kinetic studies (3) indicated that glucose caused mixed inhibition (competitive and noncompetitive) and altered the enzyme at the active site and at an allosteric site. The inversion of inhibition may have been caused by induction of new enzyme as well as displacement of old enzyme, freeing more active sites. Figure 5 shows the ratio of substrate to product, representing the biotransformation of I to II under the influence of glucose and time.

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Simultaneous Determination of Hydroxyzine Hydrochloride and Benzyl Alcohol in Injection Solutions by High-Performance Liquid Chromatography

G. N. MENON and B. J. NORRIS ˣ

Received January 18, 1980, from the *Department of Analytical Research, Abbott Laboratories, North Chicago, IL 60064.* Accepted for publication November 19, 1980.

Abstract □ A stability-indicating, high-performance liquid chromatographic method was developed for the simultaneous determination of hydroxyzine hydrochloride and benzyl alcohol in injection solutions. Separation was achieved using a μBondapak C_{18} column and the eluent [60% water, 25% acetonitrile, and 15% methanol containing 0.06% (v/v) sulfuric acid, 0.5% (w/v) sodium sulfate, and 0.02% (w/v) heptanesulfonic acid sodium salt] at a flow rate of 2 ml/min. Isobutyrophenone and *p*-nitroacetophenone were used as internal standards. The UV detector response at 257 nm was linear for hydroxyzine hydrochloride in the 3–10-mg/ml range and for benzyl alcohol in the 0.54–1.8-mg/ml range under analysis conditions. The method is accurate, simple, and precise.

Keyphrases □ High-performance liquid chromatography—simultaneous determination of hydroxyzine hydrochloride and benzyl alcohol, injection solutions □ Analysis, simultaneous—high-performance liquid chromatographic method for hydroxyzine hydrochloride–benzyl alcohol injection solutions □ Injection solutions—high-performance liquid chromatographic method for simultaneous determination of hydroxyzine hydrochloride and benzyl alcohol

Hydroxyzine hydrochloride is usually formulated with benzyl alcohol as a preservative (bacteriostatic) in injection solutions. Depending on conditions such as pH, light exposure, and temperature, photolysis occurs in solution, resulting in the formation of *p*-chlorobenzophenone, *p*-chlorobenzaldehyde, *p*-chlorobenzoic acid, and 1-[2-(2-hydroxyethyl)ethyl]piperazine dihydrochloride (1, 2). The compendial assay for hydroxyzine hydrochloride injection, based on titration of the free base with perchloric acid, is not indicative of drug stability in solution (3). From an analytical standpoint, a simple, rapid, and precise method is desirable for the simultaneous determination of both the drug substance and the preservative in the formulation.

This paper describes the development of a high-performance liquid chromatographic (HPLC) method for hydroxyzine hydrochloride and benzyl alcohol in an injectable formulation. The method is stability indicating of both ingredients in solution.

EXPERIMENTAL

Instrumentation—The chromatographic system was equipped with a dual-piston reciprocating pump[1], a universal injector[2], and a vari-

[1] Model 6000A, Waters Associates, Milford, Mass.
[2] Model U6K, Waters Associates, Milford, Mass.

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