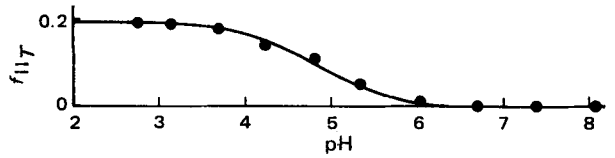


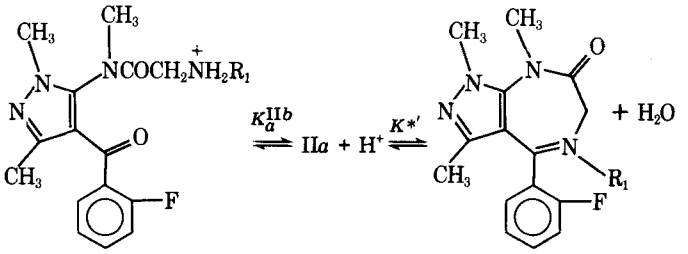
Figure 2—*Plot of* f_{II_T} versus *pH*.

As indicated previously, at pH values below the pKa values of open and closed forms, Eq. 1 reaches a limit:

$$\lim f_{II_T}^{a\,H^+\to\infty} = \frac{1}{1 + K^{*\prime}K_a^{IIb}} \qquad \text{(Eq. 5)}$$

As seen from Eq. 5, the maximum concentration of the open form (II) that can exist in these systems depends not only on the pKa of the open form (K_a^{IIb}) but also on the value of the ring closure constant ($K^{*\prime}$). If, as is the case here, the value of $K^{*\prime}$ is very large ($1.38 \times 10^8\ M^{-1}$), then the maximum concentration of the open form at equilibrium is small irrespective of the solution's pH. The ring form then dominates. This situation is best understood by considering the reaction shown in Scheme I. An increase in hydrogen-ion activity serves not only to convert II to its unreactive protonated form but also to promote dehydration.

The marked increase in the value of the ring closure constant observed here may be attributed to a methyl group in position 4 of the diazepinone ring (1). Also important is the electronic distribution around the imino carbon atom (position 8). Zolazepam is a stronger base than ripazepam by a factor of 10 (1). This finding suggests that electron withdrawal from this carbon is less here than in the previous system. Attack by water would be hindered, and the ring form would be stabilized.



IIa: R_1 absent, no charge
IIb: R_1 = H, charge +1
IIc: IIa · H_2SO_4

Ia: R_1 absent, no charge
Ib: R_1 = H, charge +1

Scheme I

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Improved Assay for Mixtures of Citrate and Citric Acid in Systemic Alkalizer Solutions

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Abstract □ A modification of the USP method for the assay of systemic alkalizer solutions containing mixtures of citrate and citric acid is presented; it involves two titrations and ion-exchange chromatography. A diluted sample is titrated with 0.02 *N* NaOH to find the free citric acid content. The eluate from cation-exchange chromatography of an equal volume of diluted sample is titrated with 0.02 *N* NaOH to assay for total citric acid. Subtracting the results of the first titration from the second provides the citrate content. Synthetic mixtures of potassium citrate–citric acid, potassium citrate–sodium citrate–citric acid, and sodium citrate–citric acid were prepared and assayed. The method was applied to commercially available preparations. The proposed method eliminates some significant errors of the compendial method, and the accuracy and reproducibility are equal or better than those obtained with the compendial method. Theoretically, the result obtained by the compendial method does not necessarily give the citrate content.

Keyphrases □ Citrate–citric acid mixtures—improved assay of systemic alkalizer solutions □ Citric acid–citrate mixtures—improved assay of systemic alkalizer solutions □ Alkalizer—improved assay of citrate–citric acid mixtures

The compendial method for the assay of mixtures of citrate and citric acid solutions is a combination of an electrometric and visual titration (1). Direct application of this compendial method for the assay of citrate and citric acid to recently available commercial systemic alkalizer solutions such as potassium citrate–citric acid oral solution (I), potassium citrate–sodium citrate–citric acid oral solution (II), and sodium citrate–citric acid oral solution (III) is not practical since the preparations are generally colored and are assayed in undiluted form. Moreover, the titrants used are 1 *N* in strength. Since these difficulties allow for many sources of error, a more appropriate assay is desired for the quality control of these commercial preparations.

Alkali salts of organic acids have been analyzed by passing an aqueous solution of the salt through an ion-exchange column followed by titration of the eluate with a base (2, 3). The ion-exchange principle is included in the analysis of commercial systemic alkalizer solutions containing mixtures of citrate and citric acid. The method is simple, and the reproducibility and accuracy are equal or better than those obtained with the USP method.

EXPERIMENTAL

Reagents and Chemicals—Citric acid monohydrate[1], potassium citrate monohydrate[1], sodium citrate dihydrate[1], a sulfonated polystyrene copolymer strong cation-exchange resin in the hydrogen form[2] (medium porosity), a 0.02 *N* NaOH volumetric solution obtained by quantitative dilution of 1 *N* NaOH (4), and phenolphthalein indicator solution were used. All other chemicals were reagent grade and were used without purification.

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Rexyn 101 (H), Fisher Scientific Co., Fair Lawn, N.J.

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Table I—Recovery Data from Synthetic Mixtures of I–III

Sample	Amount Analyzed[a], mg/ml		Amount Found[b], mg/ml				Recovery ± *SD*, %			
			Proposed Method		USP XX Method		Proposed Method		USP XX Method	
	A	B	A	B	A	B	A	B	A	B
I	220.2	67.3	220.4	67.5	220.6	67.2	100.1 ±0.9	100.3 ±0.3	100.2 ±0.6	99.9 ±0.2
II	128.9[c]	66.0	125.6[c]	66.5	127.5[c]	67.7	97.4 ±0.7	100.7 ±0.2	98.9 ±0.6	102.6 ±0.2
III	100.2	62.4	99.7	63.2	97.9	62.2	99.5 ±0.7	101.2 ±0.4	97.7 ±0.8	99.7 ±0.5

[a] A = potassium citrate monohydrate or sodium citrate dihydrate; B = citric acid monohydrate. [b] Average of three determinations. [c] Milliequivalents of total citrate per milliliter.

Table II—Recovery Data from Commercial Preparations of I–III

Sample	Label Claim[a], mg/ml		Amount Found[b], mg/ml				Label Claim ± *SD*, %			
			Proposed Method		USP XX Method		Proposed Method		USP XX Method	
	A	B	A	B	A	B	A	B	A	B
I[c]	220.0	66.8	207.2	92.9	210.9	93.1	94.2 ±0.4	139.0 ±0.2	95.8 ±0.4	139.4 ±1.0
II[c]	128.4[d]	66.8	124.8[d]	68.3	123.7[d]	67.2	97.2 ±0.4	102.2 ±0.1	96.3 ±0.6	100.7 ±0.4
III[c]	100.0	60.1	98.6	64.2	102.1	64.7	98.6 ±0.9	106.8 ±0.0	102.1 ±0.3	107.6 ±0.8

[a] A = potassium citrate monohydrate or sodium citrate dihydrate; B = citric acid monohydrate. [b] Average of three determinations. [c] Sample colors: I, wine red; II, green; and III, bright yellow. [d] Milliequivalents of total citrate per milliliter.

Cation-Exchange Column—Ten grams of cation-exchange resin was hydrated by mixing with 50 ml of glass-distilled water in a 100-ml beaker. The resin was allowed to settle, and the excess water was decanted until a slurry of resin remained in the beaker. The slurry was poured into a 15-mm i.d. × 300-mm long glass column, provided with a fritted disk and polytef stopcock, and the resin beads were allowed to settle into a homogeneous bed. Approximately 100 ml of glass-distilled water was used to wash the resin bed. The stopcock was closed when the water level was 1–2 mm above the resin bed.

Assay for I and II—A 15.0-ml aliquot of sample was transferred to a 250-ml volumetric flask and diluted to volume with distilled water.

Free Citric Acid—Five milliliters of the diluted sample was pipetted into a 250-ml erlenmeyer flask, and 25 ml of distilled water and 5 drops of phenolphthalein were added. The contents of the flask were swirled and titrated with 0.02 *N* NaOH. The quantity, in milligrams per milliliter, of citric acid monohydrate in the original sample was found using the formula $233.50 \times A$, where A is the number of milliequivalents of sodium hydroxide[3] consumed in the titration.

Total Citric Acid—Five milliliters of the diluted sample was added carefully to the top of the resin bed in the column. A 250-ml erlenmeyer flask was placed below the column, and the stopcock was opened until the sample entered the resin bed. The sample was eluted with 60 ml of distilled water at a flow rate of ≃5 ml/min, and ~65 ml of eluate was collected. Then 5 drops of phenolphthalein was added to the eluate, and the contents were swirled and titrated with 0.02 *N* NaOH.

Calculation of Citrate Content—The quantity in the original sample, in milligrams per milliliter, of potassium citrate monohydrate in I was calculated using the formula $360.46 \times (B - A)$, or the quantity of total citrate ($C_6H_5O_7$) in II was calculated using the formula $210.10 \times (B - A)$, where A is as defined previously and B is the number of milliequivalents of sodium hydroxide[3] consumed in the titration of the cation-exchange column eluate.

Assay for III—Ten milliliters of the sample was transferred to a 100-ml volumetric flask and diluted to volume with distilled water. The directions given for the assay for I and II, beginning with *Free Citric Acid*, were followed with the exception of the calculation formulas. The quantity in the original sample, in milligrams per milliliter, of citric acid monohydrate was calculated using the formula $140.10 \times A$, and the quantity of sodium citrate dihydrate was calculated using the formula $196.07 \times (B - A)$, where A and B are as defined previously[4].

[3] Milliequivalents of sodium hydroxide equal milliliters of sodium hydroxide consumed in the titration × normality of sodium hydroxide. Constant equals equivalent weight × dilution factor. Dilution factor = (250 ml/5 ml) (1 ml/15 ml). Equivalent weight for citric acid monohydrate is 70.05; for potassium citrate monohydrate, it is 108.14; and for $C_6H_5O_7$, it is 63.03.

[4] Dilution factor = (100 ml/5 ml) (1 ml/10 ml). Constant equals equivalent weight times dilution factor. Equivalent weight of sodium citrate dihydrate is 98.04.

RESULTS AND DISCUSSION

In the assay of citrate, the sample solution was passed through a strong cation-exchange resin. In the process, the cations such as potassium and sodium ions of the citrate were replaced by protons from the resin, resulting in an eluate containing citric acid. Since the sample also contained free citric acid, the titration of the eluate with base gave the total citric acid concentration. The free citric acid of the sample was titrated directly with base. When equal volumes of samples were used for the determination of free citric acid and of total citric acid, the difference in titrant consumption was equivalent to the quantity of citric acid derived from citrate. Studies indicated that citrate did not interfere in the direct titration of free citric acid with 0.02 *N* NaOH. Quantitative results also were obtained when pure potassium or sodium citrate solutions were passed through the column and the eluate was titrated with base.

Recovery data for synthetic mixtures containing mixtures of potassium and/or sodium citrate and citric acid are shown in Table I. The accuracy and precision were identical to those obtained by the compendial method. The proposed method was applied to the assay of commercial systemic alkalizer solutions (Table II). The compendial assay of these commercial preparations is usually subject to greater variations between individual samples, particularly in determining the end-point of the citric acid titration with base. The commercial preparations are generally colored and are analyzed in undiluted form. For example, one commercial product is a wine-red solution. As the wine-red solution is titrated with base to the phenolphthalein end-point, the color change to pink (or red) at the equivalence point is difficult to observe and a sharp end-point cannot be obtained. Since a 1 *N* NaOH titrant is used, each drop (~0.05 ml) of titrant from the equivalence point is equivalent to 3.5 mg of citric acid monohydrate. The coloring agent present in other commercial products also masks the color change at the equivalence point. This problem accounts for the relatively larger standard deviations for the compendial results as compared to those obtained by the proposed method (Table II). No problems were encountered in the titrations following the proposed method since a sharp end-point was obtained in each case.

The compendial assay for the citrate content in the mixture involves an electrometric titration to a predetermined pH of 1.55. Since the product of this titration is citric acid, the pH at the equivalence point is simply the pH of a solution of the total citric acid, which includes citric acid formed from citrate plus free citric acid already in the sample. Theoretically, the compendial method does not necessarily give the actual citrate content. A preparation containing the correct quantity of citrate but an incorrect amount of citric acid (as stated on a label claim) would give a wrong citrate content when the titration is conducted to a predetermined pH. Titration gives a true indication of the citrate content only when the quantity of free citric acid is correct. An electrometric titration to a predetermined pH is useful if the species analyzed is the only one that determines the end-point pH. The proposed method eliminates this

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

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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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