**Table I—Products from Photolytic and Thermal Decomposition of Indapamide**

| Photochemical Decomposition in $N_2$-Flushed Methanol | Photochemical Decomposition in $O_2$-Flushed Methanol | Thermal Decomposition | Product $R_f$ Value[a] | Method of Structure Assignment[b] |
|---|---|---|---|---|
| II | II | II | 0.19[c] | NMR, IR, UV, and mass spectra |
| III | III | III[d] | 0.68 | Comparison of IR, UV, and mass spectra with those of a reference sample |
| | | IV | 0.75 | Comparison of IR, UV, and mass spectra with those of a reference sample |
| | | V[d] | 0.26 | UV and mass spectra |
| VI | VI | VI | 0.19[c] | NMR, IR, UV, and mass spectra |
| VII[e] | VII[e] | | 0.35 | Mass spectrum[f] |
| | VIII[e] | | 0.45, 0.44* | Mass spectrum[f] |
| | IX | | 0.12* | Comparison of IR, UV, and mass spectra with those of a reference sample |
| | X | | 0.65 | NMR, IR, UV, and mass spectra |
| | XI[e] | | 0.27* | Mass spectrum[f] |

[a] The $R_f$ values refer to qualitative TLC plates developed in Solvent System 1, except for those marked with an asterisk, which refer to Solvent System 2. [b] Unless otherwise stated, spectra were obtained for products isolated from each of the three decomposition systems. [c] The product at $R_f$ 0.19 was a mixture of II and VI. Compound VI was sublimed out of the mixture and obtained as a white crystalline compound. [d] Product only observed in those thermal decomposition samples maintained at 453°K for 12 hr. [e] Minor product as judged by quantity of material isolated from TLC plates as compared to other products. [f] Only sufficient material could be isolated for a mass spectrum to be recorded.

Type A cleavage leads to 3-sulfamoyl-4-chlorobenzamide (II and III), type B cleavage leads to 3-sulfamoyl-4-chlorobenzoic acid, and type C cleavage yields 1-(*N*-formamido)-2-methylindoline (V) and 1-aminocarboxymethyl-2-methylindoline (VI). Methyl-3-sulfamoyl-4-chlorobenzoate (VIII) presumably arises as a consequence of esterification of VII by the solvent, while IX arises as a consequence of indoline ring oxidative cleavage in species such as III or I. At present, the origin of IV is not clear.

Thermal decomposition of I yields only II–IV besides 2-methylindole (X) and *N*-(3-sulfamoyl-4-chlorobenzamido)-2-methylindole (XI). Thus, type A cleavage can occur from vibrationally excited levels of the ground ($S_0$) state, and II and III can arise by this mechanism in photolytic reactions. Such species could be generated by internal conversion from the photolytically generated $S_1$ state. Presumably, the appropriate $S_0$ levels for dehydrogenation are not accessible *via* internal conversion. Type B and C cleavages only occur under photolysis and must, therefore, arise from the first excited states. Since such reactions are not inhibited by oxygen, which is known to quench triplet-state molecules (4), the $S_1$ state is implicated.

Thus, the study has demonstrated the complexity of photolysis of a simple pharmaceutical and has shown the wide range of compounds to be screened in photosensitization tests of such molecules.

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# Improved Spectrophotometric Determination of Glycerol and Its Comparison with an Enzymatic Method


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Received November 9, 1978, from *McGaw Laboratories, Irvine, CA 92714.* Accepted for publication February 5, 1979.



**Abstract** □ A chemical method for the determination of glycerol was developed and compared to an enzymatic assay for sensitivity and reproducibility. The chemical assay is based on glycerol oxidation to formaldehyde and subsequent reaction with chromotropic acid to yield a colored product. With this method, as little as 5 μg of glycerol/ml can be detected. The enzymatic assay is based on enzymatic glycerol phosphorylation followed by glycerol phosphate dehydrogenation by nadide (nicotinamide adenine dinucleotide). The reduced nadide is used to reduce iodonitrotetrazolium violet to its colored formazan product. The enzymatic method can be used to determine 50 μg of glycerol/ml in aqueous samples.



**Keyphrases** □ Glycerol—analysis, spectrophotometry, comparison with enzymatic method □ Spectrophotometry—analysis, glycerol, comparison with enzymatic method


Glycerol is widely used in pharmaceuticals as a vehicle, sweetening agent, emollient, and humectant. Various analysis methods for glycerol have been reported (1–5), but they are usually nonspecific and are limited to a narrow concentration range that gives linear results. Enzymatic methods (2, 4, 5) for glycerol and glycerol-releasing compounds (*i.e.*, triglycerides) have been used widely but are usually expensive and require rigid temperature control and timing of reagent addition to obtain reproducible results.

Chemical methods (1, 3) usually utilize glycerol oxidation to formaldehyde and subsequent reaction with a chromogenic reagent. These methods generally have been shown to produce linear results over a very limited concentration range. The present investigations were undertaken to develop a chemical analysis for glycerol with improved reaction conditions and to compare this method with a standard enzymatic assay.

## EXPERIMENTAL

**Instrumentation**—All absorbance measurements were taken on a




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Table I—Effect of Sulfuric Acid Volume on Absorbance (300 μg of Glycerol/ml, 0.5 ml of 10% Chromotropic Acid)

| Sulfuric Acid, ml | Absorbance (570 nm) |
|---|---|
| 0 | 0.00 |
| 1 | 0.37 |
| 2 | 0.50 |
| 4 | 0.52 |
| 5 | 0.54 |
| 7 | 0.52 |

double-beam spectrophotometer[1] using 1-cm quartz cells.

**Reagents**—All inorganic reagents were analytical reagent grade, were used without further purification, and were prepared in distilled deionized water. These reagents included sodium metaperiodate, sulfuric acid, sodium sulfite, and hydrochloric acid[2]. Glycerol[2] was obtained as the analytical reagent and was used without further purification.

Sodium 1,8-dihydroxynaphthalene-3,6-disulfonate[3] (chromotropic acid) was recrystallized several times from water–isopropyl alcohol by dissolution in hot water and then slow addition of an equivalent volume of alcohol until precipitation. The crystals were collected and dried overnight in a vacuum oven.

ATP[4] ($8.9 \times 10^{-3}$ *M*) and nadide (nicotinamide adenine dinucleotide)[4] ($1.56 \times 10^{-2}$ *M*) were obtained as a mixture and reconstituted with distilled water just prior to use. Iodonitrotetrazolium violet[4] and *N*-methylphenazonium methosulfate[4] were supplied in solution as analytical reagents. Glycerol kinase[4] (1780 units) and glycerol phosphate dehydrogenase[4] (150,000 units) were supplied in solution and mixed with the ATP–nadide solution immediately prior to use.

**Procedures**—*Chemical Assay*—The standard, 2.0 ml, or appropriately prepared sample containing 0–1000 μg of glycerol/ml was pipetted into a 16 × 100-mm test tube. One milliliter of 1% $NaIO_4$ was added and mixed thoroughly, and the mixture was allowed to stand at room temperature for ~15 min. Then 1 ml of 5% $Na_2SO_3$ was added and mixed, and the mixture was allowed to stand for 5 min.

One milliliter of this solution was pipetted into a 100-ml volumetric flask, and 0.50 ml of a 10% chromotropic acid solution was added. Then 5.0 ml of concentrated $H_2SO_4$ was added slowly, and the solution was heated at 100° in a water bath for 30 min. After heating for 30 min, air was bubbled through the solution for ~20 min. Distilled water was added to raise the volume to 90%. The solution was allowed to cool to room temperature and raised to volume with distilled water. The solution absorbance was measured at 570 nm.

*Enzymatic Assay*—Fifty microliters of the standard or appropriately prepared sample was pipetted into a 16 × 100-mm test tube. Glycerol kinase–glycerol phosphate dehydrogenase, 0.5 ml, was added, and the solution was placed in a 37° water bath. Exactly 10 min after the addition of the enzyme mixture, the test tube was removed from the water bath, 0.5 ml of 0.03% iodonitrotetrazolium violet and 0.005% *N*-methylphenazonium methosulfate was added, the test tube was returned immediately to the water bath.

Exactly 10 min after the last addition, the test tube was removed from the water bath, and 2.0 ml of 0.1 *N* HCl was added and mixed. Then the test tube was allowed to stand for 5 min. Within 30 min, the solution absorbance was measured at 505 nm.

Special care must be taken to ensure exact time intervals and proper temperature controls.

## RESULTS AND DISCUSSION

After glycerol oxidation to formaldehyde by sodium periodate, the formaldehyde reacts with chromotropic acid in the presence of excess acid to yield a colored product (6). This reaction produces a product that follows Beer's law in the range of 5–1000 μg of glycerol/ml. Several investigators (7–9) have dealt with the optimization of the formaldehyde reaction with chromotropic acid. The critical factors found in the chemical assay procedure reported in this paper are: (*a*) the sulfuric acid concentration in the reaction mixture, (*b*) the chromotropic acid to glycerol concentration ratio, and (*c*) the time of heating at 100°.

Table I contains the results of varying the sulfuric acid concentration during the reaction with a sample originally containing 300 μg of glycerol/ml and after addition of the 0.50 ml of 10% chromotropic acid. The results indicate that 5.0 ml of sulfuric acid is an appropriate volume. Table II indicates that a chromotropic acid–glycerol ratio by weight of at least 50:1 should be used. These data were generated for 500-μg of glycerol/ml samples and 5.0 ml of sulfuric acid. From Table III, it can be seen that the reaction mixture using 300-μg of glycerol/ml samples, 0.5 ml of chromotropic acid, and 5.0 ml of sulfuric acid should be heated approximately 30 min. The color developed in this manner is stable for at least 48 hr. Other references for the reaction of formaldehyde with chromotropic acid (7, 10) have reported very limited concentration ranges over which the reaction will yield linear results. The sodium chromotropate recrystallization is essential to obtain reproducible results and greatly extends the linearity range.

Table II—Effect of Chromotropic Acid Concentration (5 ml of Sulfuric Acid, 500 μg of Glycerol/ml)

| 10% Chromotropic Acid, ml | Absorbance (570 nm) |
|---|---|
| 0 | 0.00 |
| 0.1 | 0.67 |
| 0.2 | 0.81 |
| 0.5 | 0.84 |
| 1.0 | 0.83 |
| 2.0 | 0.86 |

Table III—Effect of Heating Time at 100° (5 ml of Sulfuric Acid, 300 μg of Glycerol/ml)

| Minutes | Absorbance |
|---|---|
| 0 | 0.288 |
| 5 | 0.478 |
| 10 | 0.522 |
| 15 | 0.520 |
| 30 | 0.543 |
| 45 | 0.532 |

Table IV—Day-to-Day Reproducibility of the Chemical and Enzymatic Assays Using 300-μg of Glycerol/ml Samples

| Day | Absorbance | |
|---|---|---|
| | Chemical Assay | Enzymatic Assay |
| 1 | 0.525, 0.526 | 0.271, 0.23 |
| 2 | 0.527, 0.526 | 0.230, 0.250 |
| 3 | 0.523, 0.523 | 0.249, 0.210 |
| 4 | 0.532, 0.525 | 0.230, 0.235 |
| 5 | 0.526, 0.526 | 0.260, 0.230 |
| Mean | 0.526 | 0.235 |
| *SD* | 0.0025 | 0.025 |
| *CV*, % | 0.48 | 7.46 |

Thus, glycerol in concentrations as low as 5 μg and as high as 1000 μg/ml was determined. The chemical method yields a linear relationship between the glycerol concentration and absorbance, with a correlation coefficient of 0.997. The results from two determinations per day for 5 days of 300-μg of glycerol/ml samples are listed in Table IV and give a standard deviation of 1.4 μg/ml and a coefficient of variance of 0.48%.

In the enzyme assay, following glycerol phosphorylation and glycerol phosphate dehydrogenation by nadide, the reduced nadide reduces iodonitrotetrazolium violet to a colored product (11). This procedure yields a product that follows Beer's law from 50 to 600 μg of glycerol/ml. Of critical importance in enzyme assays is the maintenance of temperature at 37° during the reaction and the time intervals at which reagents are added. The time intervals and temperature were already optimized for triglyceride testing (12) and were not investigated further.

In this method, as low as 50 μg and as high as 600 μg of glycerol/ml were determined. The enzymatic methods yields a linear relationship between the concentration of glycerol and absorbance, with a correlation coefficient of 0.975. As can be seen in Table IV, 10 determinations (two per day for 5 days) of 300-μg of glycerol/ml samples yielded a standard deviation of 22.4 μg/ml and a coefficient of variance of 7.5%.

Both the chemical and enzymatic assays are linear over the regions analyzed, with the working curve slopes being statistically different. The average response factor over the region analyzed in the chemical assay was $1.73 \pm 0.095 \times 10^{-3}$ absorbance unit/μg of glycerol/ml, with a coefficient of variance of 5.5%. The average response factor for the enzymatic assay was $8.3 \pm 1.5 \times 10^{-4}$ absorbance unit/μg of glycerol/ml, with a coefficient of variance of 18.2%. The chemical assay method is more sensitive due to a greater response per unit change in concentration and shows a wider linear concentration range than the enzymatic assay.

[1] Beckman model 25.
[2] Mallinckrodt, St. Louis, Mo.
[3] Eastman Kodak Co., Rochester, N.Y.
[4] Hycel Inc., Houston, Tex.

The reproducibility of the chemical assay (on a day to day basis ) is superior to the enzyme assay, as seen by comparing the standard deviations and coefficients of variance from the data obtained from five consecutive test periods. The need for rigid controls of temperature and timing and the low reproducibility of the enzyme assay dictate that a standard working curve be generated each time numerous samples are run. The chemical assay offers a significant improvement in reproducibility and requires only an initial standard curve. The chemical assay described in this paper offers an easy, inexpensive, and reproducible method of determining glycerol in aqueous samples.

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# Clearance Constants in Physiologically Based Pharmacokinetic Models


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Received December 18, 1978, from the *Departments of Chemical Engineering and Internal Medicine, University of Arizona, Tucson, AZ 85721.* Accepted for publication February 14, 1979.



**Abstract** □ The intrinsic clearance of an organ is usually approximated by the apparent clearance from that organ in the development of a physiologically based pharmacokinetic model. In this study, the exact relationship between the two clearances was derived and analyzed. When the extraction ratio of the drug was small (<0.05), the approximation was reasonable. However, when the extraction ratio was high (>0.2), serious errors could be made by using the approximation. These errors could be as much as 50% reduction in the estimated extraction ratio and as much as an order-of-magnitude difference in the intrinsic clearance.



**Keyphrases** □ Pharmacokinetics—drug clearance through an organ, estimation □ Drug clearance—estimation of clearance through an organ, pharmacokinetics □ Antineoplastic agents—estimation of clearance through an organ, pharmacokinetics


In a typical linear physiologically based pharmacokinetic model (1), the drug concentration in an organ such as the kidney is governed by the differential equation:

$$V\frac{dC}{dt} = Q\left(C_p - \frac{C}{R}\right) - K\frac{C}{R} \qquad \text{(Eq. 1)}$$

where $C$ and $C_p$ are the drug concentrations in the organ and plasma (or blood), respectively; $V$ is the physiological volume of the organ; $Q$ is the plasma (or blood) flow rate through the organ; $R$ is the equilibrium partition coefficient for drug distribution between the organ tissue and its venous plasma, and the constant $K$ is a clearance term for drug elimination from the organ.

The development of a physiological model for predicting drug concentration–time histories requires the estimation of $V$, $Q$, $R$, and $K$. The parameters $V$ and $Q$ are the physiological volumes and blood flow rates through the organs for the subject to be simulated; $R$ can be estimated from animal experiments and calculated according to a recently developed method (2). The clearance $K$ is usually assumed to be equal to $K_{app}$, the apparent drug clearance from the organ. For example, if the organ is the kidney, $K$ is usually calculated from:

$$K = K_{app} \equiv \text{total urinary excretion} \Big/ \int_0^\infty C_p\,dt \qquad \text{(Eq. 2)}$$

Several questions can be posed regarding $K$ and its significance. What is its physical meaning? Is it equal to the apparent drug clearance from the organ? Can it be greater than $Q$, the plasma flow rate through the organ? How can $K$ be estimated from experimental data? These questions will be discussed in the present paper.

## THEORETICAL

Without loss of generality, the typical organ to be studied will be the kidney. The total cumulative urinary excretion, $U$, is then given as:

$$\frac{dU}{dt} = K\frac{C}{R} \qquad \text{(Eq. 3)}$$

or:

$$U = \frac{K}{R}\int_0^T C\,dt \qquad \text{(Eq. 4)}$$

where $T$ is the urine collection time interval. Chen and Gross (2) showed recently that the drug concentration in the organ is related to the plasma drug level by:

$$C = \frac{QRC_p}{Q + K - \beta VR} \qquad \text{(Eq. 5)}$$

during the terminal elimination phase after intravenous bolus injection and that:

$$C = \frac{QRC_p}{Q + K} \qquad \text{(Eq. 6)}$$

at steady state after a constant-rate infusion. The parameter $\beta$ is the apparent elimination rate constant for the terminal phase after intravenous injection. Therefore, Eq. 4 may be approximated as:

$$U = \frac{QK}{Q + K}\int_0^T C_p\,dt \qquad \text{(Eq. 7)}$$

for all modes of drug administration if $K + Q \gg \beta VR$ and if the tissue




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