Renal or biliary clearance can be calculated by plotting the rate of urinary excretion or biliary excretion against the blood concentration at the midpoint of each urinary or biliary collection, respectively. The resulting slopes represent each corresponding clearance value (Fig. 2 illustrates the calculation of renal clearance). However, Cl_H, Cl_M, and Cl_0 cannot be calculated by conventional methods.

Percent net tubular secretion can be calculated using the following relationship:

$$\text{percent net tubular secretion} = \frac{Cl_R - Cl_{cr} \times \text{fraction of unbound compound}}{Cl_R} \times 100 \quad \text{(Eq. A19)}$$

REFERENCES

(1) R. K. Ferguson, H. R. Brunner, G. A. Turini, H. Gavras, and D. N. McKinstry, *Lancet*, **1**, 775 (1977).

(2) D. B. Case, S. A. Atlas, J. H. Laragh, J. E. Sealey, P. A. Sullivan, and D. N. McKinstry, *Progr. Cardiovasc. Dis.*, **21**, 195 (1978).

(3) H. Gavras, H. R. Brunner, G. A. Turini, G. R. Kershaw, C. P. Tifft, S. Cuttelod, I. Gavras, R. A. Vukovich, and D. N. McKinstry, *N. Engl. J. Med.*, **298**, 991 (1978).

(4) H. R. Brunner, H. Gavras, B. Waeber, G. R. Kershaw, G. A. Turini, R. A. Vukovich, and D. N. McKinstry, *Ann. Intern. Med.*, **90**, 19 (1979).

(5) K. J. Kripalani, D. N. McKinstry, S. M. Singhvi, D. A. Willard, R. A. Vukovich, and B. H. Migdalof, *Clin. Pharmacol. Ther.*, **27**, 636 (1980).

(6) B. H. Migdalof, S. M. Singhvi, and K. J. Kripalani, *J. Liq. Chromatogr.*, **3**, 857 (1980).

(7) L. E. Anderson and W. A. McClure, *Anal. Biochem.*, **51**, 173 (1973).

(8) K. Wong, S. J. Lan, and B. H. Migdalof, *Pharmacologist*, **21**, 173 (1979).

(9) A. J. Rommel, A. M. Pierides, and A. F. Heald, *Clin. Pharmacol. Ther.*, **27**, 282 (1980).

(10) S. M. Singhvi, A. E. Peterson, J. M. Shaw, and B. H. Migdalof, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **39**, 538 (1980).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mr. C. J. Mondi and Mr. R. J. Ratoff and the editorial assistance of Dr. J. Dreyfuss and Dr. P. L. Sibley. The synthesis of radiolabeled captopril was carried out by Mr. P. Egli.

Quantitation of Daunorubicin, Doxorubicin, and Their Aglycones by Ion-Pair Reversed-Phase Chromatography

A. CAROL HANEKE ˣ, J. CRAWFORD, and A. ASZALOS

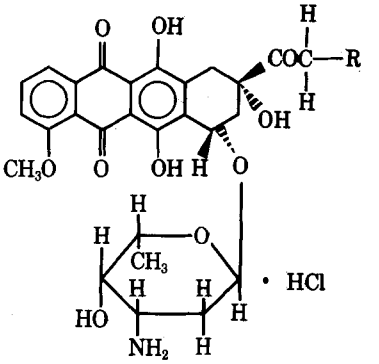
Received January 23, 1981, from the *Food and Drug Administration, National Center for Antibiotics Analysis, Washington, DC 20204.* Accepted for publication March 9, 1981.

Abstract □ A fast and sensitive high-pressure liquid chromatographic method was developed for the quantitation of doxorubicin, daunorubicin, and their aglycones in pharmaceutical preparations. Because its higher pH extends column life while permitting determination of impurities, this system represents an improvement over previously published methods. It utilizes a C_{18} bonded silica gel column and a solvent system consisting of methanol–0.01 *M* monobasic ammonium phosphate aqueous solution (65:35) at pH 4.0 and 1600 psi of pressure. The accuracy of the doxorubicin and daunorubicin determinations, expressed as the coefficient of variation, is 1.65 and 1.27%, respectively. The aglycones can be determined with a precision of <1.3%.

Keyphrases □ Doxorubicin—high-pressure liquid chromatographic assay, quantitation of impurities □ Daunorubicin—high-pressure liquid chromatographic assay, quantitation of impurities □ High-pressure liquid chromatography—quantitation of doxorubicin, daunorubicin, and their aglycones □ Antibiotics—quantitation of doxorubicin, daunorubicin, and their aglycones by high-pressure liquid chromatography

Doxorubicin (adriamycin) is an anthracycline antibiotic (I) consisting of the tetracyclic quinoid aglycone doxorubicinone (adriamycinone) in a glycosidic linkage to the amino sugar daunosamine (1, 2). Doxorubicin has been shown to be effective against various human neoplasms (3, 4). Pharmaceutical preparations of doxorubicin contain several impurities such as daunorubicin, daunorubicinone, and doxorubicinone, as well as other minor contaminants. Some of these impurities, particularly daunorubicin and daunorubicinone, are present because doxorubicin is prepared from daunorubicin by chemical synthesis (5), and the parent compound and its impurities are carried through to the product during the manufacturing process.

Daunorubicin (II) is a recently approved drug used in the treatment of acute leukemia and certain solid tumors in humans (6, 7). Daunorubicin preparations contain impurities such as daunorubicinone, which may be carried through the isolation process of the drug from the fermentation broth (8). It is important that the potency of both doxorubicin and daunorubicin be determined because clinical treatment relies on an accurate dosage schedule (9). It is equally important to determine the impurities in these pharmaceutical preparations, particularly the aglycone content, because these impurities are mutagenic and have no antitumor activity (10).



I: R = OH
II: R = H

0022-3549/81/1000-1112$01.00/0

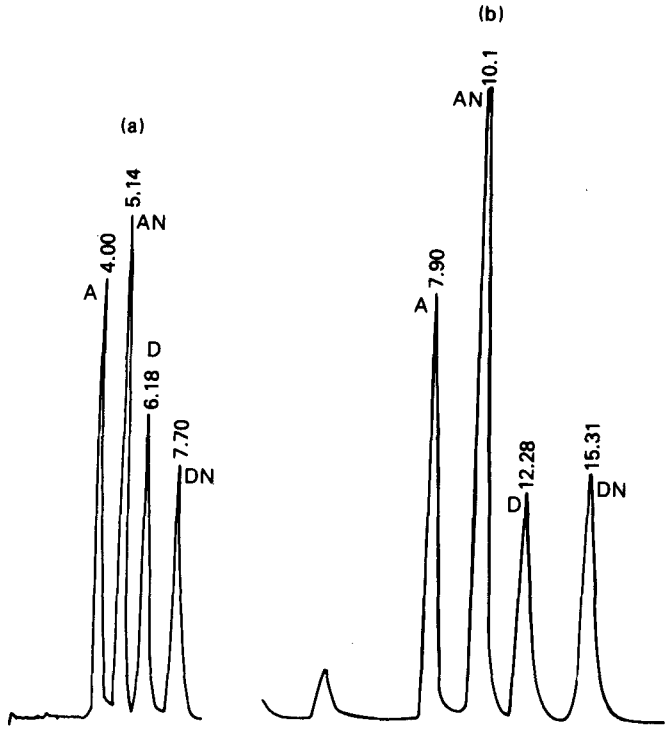


Figure 1—*HPLC separation profile of daunorubicin (D), doxorubicin (A), daunorubicinone (DN), and doxorubicinone (AN) with C_{18} bonded silica gel, methanol–0.01 M monobasic ammonium phosphate aqueous solution (65:35), pH 4.0, and flow rate of 2 ml/min (a) and 1 ml/min (b). Numbers given are retention times in minutes.*

BACKGROUND

The *Code of Federal Regulations* (CFR) (11) specifies that high-pressure liquid chromatography (HPLC) be used to establish the composition of doxorubicin and daunorubicin preparations but does not require determination of the aglycones. The official method utilizes a pH 2.0 mobile phase, which can adversely affect the useful life of the chromatographic column and may also hydrolyze daunorubicin to its aglycone and daunosamine during the long analytical process.

Most previously published HPLC procedures for the analyses of these drugs are suitable only for the estimation of doxorubicin and its metabolites in plasma (12–15). Some also use a detrimental pH 2 mobile phase (12, 13). Others use a quaternary (14) or a gradient (15) solvent system and do not provide rapid quantitation of pharmaceutical preparations of doxorubicin or daunorubicin. A low pH system was used by Eksborg (16), who achieved good separations of daunorubicin, doxorubicin, and their C-13 hydroxylated metabolites by HPLC (16). Other methods were developed to determine daunorubicin and its C-13 hydroxylated metabolite in plasma (17, 18). However, none of these published methods was designed to quantitate doxorubicinone and daunorubicinone.

This report describes a new HPLC method for the quantitative determination of daunorubicin, doxorubicin, and their aglycones in pharmaceutical preparations.

EXPERIMENTAL

Reagents—Spectrograde methanol and deionized water were used throughout the experiments. Monobasic ammonium phosphate[1], acetic acid[2], and heptanesulfonic acid[3] were used without further purification. Daunorubicin[4] and doxorubicin[5] were obtained commercially. Daunorubicinone and doxorubicinone were prepared as described previously (19 and 2, respectively) and checked for purity by melting-point, IR, and TLC [single spot at R_f 0.92 and 0.78, respectively; methanol–chloroform–formic acid (15:85:2); silica gel] analyses.

[1] Fisher Scientific Co., Pittsburgh, Pa.
[2] Mallinckrodt Chemical Co., St. Louis, Mo.
[3] Eastman Chemicals, Rochester, N.Y.
[4] Rhodia Inc., New York, N.Y.
[5] Farmitalia, Milan, Italy.

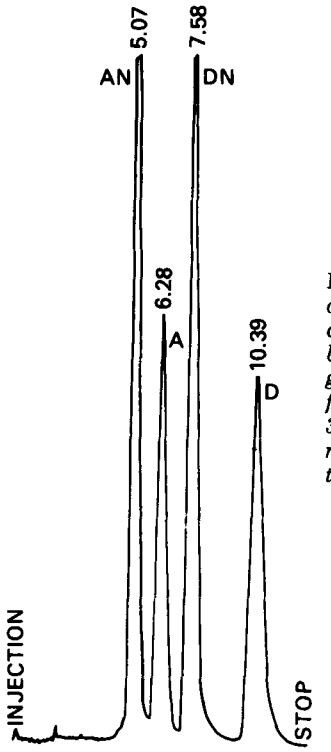


Figure 2—*HPLC separation profile of daunorubicin (D), doxorubicin (A), daunorubicinone (DN), and doxorubicinone (AN) with C_{18} bonded silica gel, methanol–0.005 M heptanesulfonic acid aqueous solution (62.5:37.5), pH 3.5, and flow rate of 2 ml/min. Numbers indicate retention times in minutes.*

Apparatus—A modular high-pressure liquid chromatograph[6] was equipped with a constant-flow pump, a valve-type injector[7], and a 254-nm fixed-wavelength detector[8]. Data were recorded and reduced by a microprocessor[9] set for peak area calculations at a chart speed of 0.5 cm/min and attenuation of 128.

Chromatographic Conditions—A 25-cm × 4.6-mm i.d. stainless steel column, packed with 10-μm particle-size C_{18} reversed-phase packing material[10], was used for all assays. The isocratic mobile phase consisted of 650 ml of methanol and 350 ml of water to which was added 1.15 g of monobasic ammonium phosphate dissolved in 5 ml of acetic acid (final pH 4.0). The mobile phase was filtered through a 0.2-μm filter and degassed by vacuum for 20 min. The flow rate was 2 ml/min, which produced a pressure of ~1600 psi. All separations were performed at ambient temperature. A 5-μl sample was injected into the chromatograph.

Before the assay was begun, a resolution factor for the system was determined at ambient temperature with a flow rate of 1.0 ml/min. To facilitate measurements of peak width, the detector was set to give at least 50% response full scale and a chart speed of 2 cm/min. The column was purged with mobile phase until a steady baseline was established. Then 5 mg of doxorubicin and 5 mg of doxorubicinone were accurately weighed into a 10-ml volumetric flask and diluted to volume with methanol. Then 5 μl of this solution was injected into the chromatograph. The retention time for the doxorubicin and the doxorubicinone peaks should be ~470 and 600 sec, respectively.

The resolution factor (R_s) is calculated as follows:

$$R_s = 2(t_2 - t_1)/(W_1 + W_2) \qquad \text{(Eq. 1)}$$

where $(t_2 - t_1)$ is the difference in retention times measured in centimeters at peak maxima between the doxorubicin and the doxorubicinone peaks and W_2 and W_1 are the peak widths at the base of the peaks in centimeters.

A resolution factor of 2.2 is recommended. Experiments evaluated in statistical terms were performed eight to 10 times each.

Sample Preparation—*Vials*—To obtain a concentration of 0.5 mg/ml, the finished products were diluted as follows: 4, 8, or 10 ml of methanol was added to a 10-, 20-, or 50-mg vial, respectively. Then 2, 2, or 1 ml of the resulting solution was transferred to a 10-ml volumetric flask and diluted to volume with methanol. Dilution factors were 20, 40, or 100 ml, respectively. A 5-μl sample size was used. Peak areas of the

[6] Model 6001A, Waters Associates, Milford, Mass.
[7] Model U6K, Waters Associates, Milford, Mass.
[8] Model 240, Waters Associates, Milford, Mass.
[9] Integrator 3380A, Hewlett-Packard Co., Palo Alto, Calif.
[10] μBondapak, Waters Associates, Milford, Mass.

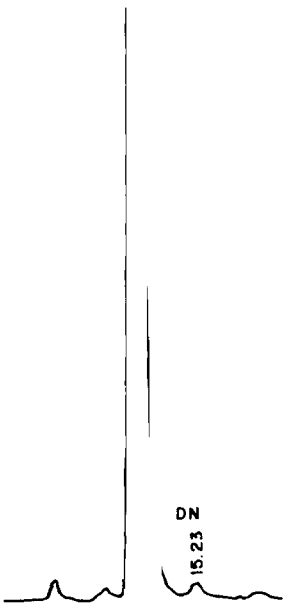


Figure 3—*Chromatogram of daunorubicinone estimated at 0.5% of the parent compound with the HPLC conditions given under* Experimental *for estimation of the aglycones. Numbers indicate retention times in minutes.*

samples were compared to those of the standard, and the concentrations were calculated as follows:

$$\text{mg/vial} = \frac{A_u \times W_s \times DF \times P}{A_s \times 1000\ \mu\text{g/mg}} \quad \text{(Eq. 2)}$$

where A_u is area of the sample peak, W_s is milligrams per milliliter of the standard, DF is the dilution factor in milliliters, P is purity of the standard in micrograms per milligram, and A_s is area of the standard peak.

Bulk Samples—The standard and bulk samples were diluted with methanol to a concentration of 0.5 mg/ml, and a 5-μl sample size was used. Peak area data of the bulk samples were compared to those of the standard and calculated as follows:

$$\mu\text{g/mg} = \frac{A_u \times W_s \times P}{A_s \times W_u} \quad \text{(Eq. 3)}$$

where W_u is milligrams per milliliter of the sample.

Estimation of Aglycones—The aglycone determination for each drug was developed for the range in which they usually occur in the preparations analyzed (0.5–3.0% of the parent compound). Because of the relatively low aglycone concentrations in the preparations and the limitations of the microprocessor in calculating relatively small areas, the attenuation was changed to 32 and the flow rate was changed to 1.0 ml/min to ensure good precision.

RESULTS AND DISCUSSION

Optimal HPLC conditions established for this assay consisted of a mobile phase acidity of 3.5–7.0 to ensure a reasonably long column lifetime, a turnaround time of <15 min to enable high productivity, and a resolution factor of at least 2.0 for accurate quantitation.

Figure 1 shows the optimum HPLC profile achieved with the best ammonium sulfate solvent system. The pH was 4.0 and retention times for the last appearing peaks were 8 and 15 min at 2- and 1-ml/min flow rates, respectively. The resolutions for the first two peaks were 2.4 (Fig. 1*a*) and 2.7 (Fig. 1*b*) at 2- and 1-ml/min flow rates, respectively. Figure 2 shows the optimum HPLC profile achieved with the heptanesulfonic acid system. The separation time was slightly over 10 min, the pH was 3.5, and the resolution was 2.1 for the first two peaks with a 2-ml/min flow rate. No correlation between resolution and pH, flow rate (column pressure), or percent methanol could be found for either solvent system. However, the resolution was generally poorer at higher pH values.

Since the ammonium sulfate solvent system at pH 4.0 and 65% methanol gave slightly superior resolution and acidity than the heptanesulfonic acid solvent system, it was used for the rest of the study. This system was evaluated for quantitative estimation of doxorubicin, daunorubicin, and their aglycones. The aglycones were assayed at low levels relative to the parent compound since pharmaceutical preparations contain small amounts of these substances.

Table I—Comparative Results by the Ammonium Sulfate and the CFR Methods

Sample	Ammonium Sulfate Method		CFR Method	
	%	μg/mg	%	μg/mg
Standard	100.0	912	100.0	912
Sample A: 1	97.5	889	97.6	890
2	97.8	892	97.5	890
3	98.2	896	98.7	901
Sample B: 1	98.7	900	98.0	894
2	97.9	893	97.7	891
3	98.1	896	99.2	904
Sample C: 1	98.0	894	99.6	908
2	96.2	879	97.0	885
3	97.9	892	99.1	904

Doxorubicin was assayed in the 1–5-μg/injection range. There is a linear relation between the peak area and the quantity of doxorubicin injected within this range. A standard curve must be determined for every combination of parameters. The standard deviation of these results was 0.71%, and the coefficient of variation was 1.65%. Similar results, a standard deviation of 0.77% and a coefficient of variation of 1.27%, were obtained for daunorubicin.

Doxorubicin preparations are dispensed in 10- and 50-mg vials. Daunorubicin is obtained as a bulk preparation or in 20-mg vials. Both drugs are dissolved in methanol to obtain a 0.5-mg/ml solution, and a 5-μl sample is injected. The content of the vials can be calculated as shown in the *Experimental* section.

The precision for the estimation of doxorubicinone in the presence of a relatively large amount of doxorubicin and of daunorubicinone in the presence of a relatively large quantity of daunorubicin, expressed as standard deviation, is 1.27%. A linear relationship is shown between peak area data and the quantity of the algycones injected. Assay of these aglycones in either preparation is possible under these experimental conditions provided a standard curve is determined for each new combination of parameters. Figure 3 shows a chromatogram of the assay of daunorubicinone at 0.5% of the parent compound.

Several pharmaceutical preparations were assayed by the new ammonium sulfate method, and the results (Table I) were compared to those obtained by the CFR method, which uses an internal standard. Comparison of these data shows that results fall within a desirable range and that the proposed new method produces assay values comparable to those found by the official method. The average coefficient of variation is 0.70% for these measurements. The aglycone content was also determined for two bulk preparations used in this study; Samples A and B contained 0.34 and 0.49% daunorubicinone, respectively.

REFERENCES

(1) F. Arcamone, A. DiMarco, and S. Penco, German offen. pat. 2,510,866 (1975).

(2) F. Arcamone, A. G. Franceschi, S. Penco, and A. Selva, *Tetrahedron Lett.*, **1969**, 1007.

(3) S. K. Carter, *J. Natl. Cancer Inst.*, **55**, 1265 (1975).

(4) "International Symposium on Adriamycin," S. K. Carter, A. DiMarco, M. Ghione, I. H. Krakoff, and G. Mathe, Eds., Springer Verlag, New York, N.Y., 1972.

(5) F. Arcamone, A. G. Franceschi, and S. Penco, U.S. pat. 3,803,124 (1974).

(6) C. Tan, H. Tasaka, K. Yu, M. L. Murphy, and D. A. Karnofsky, *Cancer*, **20**, 333 (1967).

(7) H. S. Schwartz and P. M. Kanter, *Cancer Chemother. Rep. Part 3*, **8**, 107 (1975).

(8) A. DiMarco, M. Gaetani, P. Orezze, B. M. Scarpinato, R. Silvestrini, M. Soldati, T. Dasdia, and L. Valentini, *Nature*, **201**, 706 (1964).

(9) R. A. Minow, R. S. Benjamin, E. T. Lee, and J. A. Gottlieb, *Cancer*, **39**, 1397 (1977).

(10) C. Cordaro, T. Cebula, and J. Ramsey, FDA/PRT Semiannual Report, AIC-R-29, Apr. 14, 1980.

(11) Code of Federal Regulations, 21 Food and Drug 436.322.

(12) R. N. Pierce and P. I. Jatlow, *J. Chromatogr.*, **164**, 471 (1979).

(13) S. Eksborg, H. Ehrssom, and I. Anderson, *ibid.*, **164**, 479 (1979).

(14) J. H. Peters and J. F. Murray, Jr., *J. Liq. Chromatogr.*, **2**, 45 (1979).

(15) M. Israel, W. J. Pegg, P. M. Wilkinson, and M. B. Garnick, *ibid.*, **1**, 795 (1978).
(16) S. Eksborg, *J. Chromatogr.*, **149**, 225 (1978).
(17) R. Hulhoven and J. P. Desager, *ibid.*, **125**, 369 (1976).
(18) R. Baurain, A. Zenebergh, and A. Troulet, *ibid.*, **157**, 331 (1978).
(19) F. Arcamone, G. Franceschi, P. Orezze, G. Cassinelli, W. Barkieri, and R. Mondelli, *J. Am. Chem. Soc.*, **86**, 5334 (1964).

Extended Hildebrand Solubility Approach: Methylxanthines in Mixed Solvents

A. MARTIN *x, A. N. PARUTA ‡, and A. ADJEI *

Received July 24, 1980, from the **Drug Dynamics Institute, College of Pharmacy, University of Texas, Austin, TX 78712*, and the ‡*College of Pharmacy, University of Rhode Island, Kingston, RI 02881.* Accepted for publication March 16, 1981.

Abstract □ The solubility profiles of theobromine, theophylline, and caffeine at 25° were examined in binary solvent systems including dioxane–formamide, water–polyethylene glycol 400, and glycerin–propylene glycol. Theobromine solubility was studied in dioxane–water mixtures, a solvent system that was investigated earlier for the solubility of theophylline and caffeine. Solubilities were calculated in these polar systems by a regression method, based on an extension of the Hildebrand–Scatchard equation of regular solution theory. A linear relationship between the mixed solvent solubility parameter, δ_1, and dielectric constant, ϵ, was introduced earlier and was confirmed in the present study. In addition, it was observed that a regression of log(activity coefficient) on ϵ in a second or higher degree polynomial provides reasonable solubility values for the methylxanthines in mixed solvents. A direct regression of molal or mole fraction (but not molar) solubility against δ_1, ϵ, or against volume percent of one or the other solvent in a binary solvent mixture provided a suitable measure of solubility for these crystalline drugs in mixed polar solvents. The drug's solubility parameter as determined from peak solubility in mixed polar solvents varied somewhat, depending on the specific solvent system employed. It is suggested that a drug may exhibit one (or more) solubility parameters in nonpolar solutions and multiple solubility parameters in polar systems. The extended solubility approach serves for the back-calculation of solubilities in mixed solvent systems, even though the solubility parameter of the solute may vary from one solvent system to the next.

Keyphrases □ Methylxanthines—solubility profiles using extended Hildebrand–Scatchard equation □ Solubility—methylxanthines in mixed solvents, extended Hildebrand–Scatchard equation □ Hildebrand–Scatchard equation—modified, solubility profiles of methylxanthines in mixed solvents

Previous reports (1–3) introduced an approach to estimate the solubility of drugs in mixed and pure solvent systems. The method employs the equation:

$$-\log X_2 = \frac{\Delta S^f_m}{R}\log\frac{T_m}{T} + \frac{V_2\phi_1^2}{2.303RT}(\delta_1-\delta_2)^2 + \frac{V_2\phi_1^2}{2.303RT}2(\delta_1\delta_2 - W) \quad \text{(Eq. 1a)}$$

or:

$$-\log X_2 = \frac{\Delta S^f_m}{R}\log\frac{T_m}{T} + \frac{V_2\phi_1^2}{2.303RT}(\delta_1^2+\delta_2^2-2W) \quad \text{(Eq. 1b)}$$

where X_2 is the mole fraction solubility of the drug, ΔS^f_m is the entropy of fusion, R is the molar gas constant, T_m is the melting point of the compound in Kelvin degrees, T is the absolute temperature at which the solubility is measured, V_2 is the molar volume of the drug as a hypothetical supercooled liquid solute at temperature T, ϕ_1 is the volume fraction of the solvent, δ_1 and δ_2 are the solubility parameters of the solvent and solute, and W is the solute–solvent interaction energy. Subscript 1 is used for solvent and subscript 2 for solute. The W value is computed for the drug in each solvent mixture, using Eq. 1*b*. It may, in turn, be back-calculated employing a power series regression in δ_1 to estimate mole fraction or molal solubilities. By knowing the density of the solution at a particular temperature, it is also possible to convert these calculations to molar solubilities. The term $V_2\phi_1^2/2.303RT$ is designated in this study by the symbol A. The present work tests the extended Hildebrand solubility approach (as this method is called) with various binary solvent mixtures.

EXPERIMENTAL

The sources and treatment of methylxanthines and some of the solvents used were given previously (2, 3)[1]. Additional solvents employed in the present study were propylene glycol[2], polyethylene glycol 400[2], and glycerin[2]. Pertinent physicochemical properties of the xanthine derivatives are recorded in Table I. The solubilities of the drugs were determined in a shaker bath employing 20-ml screw-capped vials containing an excess of the drug at 25 ± 0.2°. Equilibrium occurred well before 96 hr. Samples were withdrawn after 96 hr and filtered through a 0.22-μm filter, and aliquots were removed and diluted for spectrophotometric assay. Runs were carried out in quadruplicate, and the four results were averaged. Densities of the saturated solutions and of the solvent mixtures were determined in quadruplicate at 25 ± 0.2° in 10-ml pycnometers.

RESULTS AND DISCUSSION

Theobromine was dissolved in mixtures of dioxane and water at 25°. The solubility profile is shown in Fig. 1. Table II contains the data used to plot the back-calculation line of Fig. 1, including W_{calc} and A values. Solution densities and dielectric constants are also recorded in Table II.

Ideal Solubility in Relation to Maximum Solubility in Real Systems—The ideal mole fraction solubility of theobromine at 25° is 0.0029, a value well below the ideal solubilities of theophylline (0.0190) and caffeine (0.0685) at 25° (Table I and Fig. 1) because of the greater ΔH^f_m value and high melting point (348°) of theobromine. The peak solubility of theobromine in the best dioxane–water mixture (~70% dioxane) is well below ideal solubility (Fig. 1), as observed previously for caffeine and theophylline. This phenomenon was noted by Gordon and Scott (4) and by others (5, 6). Scatchard *et al.* (7) observed that the value of C_{12} (an-

[1] Reference 2 states that mean molar volumes of the binary solvent mixtures are calculated from Eq. 17, in which each molecular weight was multiplied by the mole fraction of that solvent in the mixture. This is an error; the quantity used in Refs. 2 and 3 and in the present study is volume fraction rather than mole fraction.
[2] Fisher Scientific.

0022-3549/81/1000-1115$01.00/0