High-Pressure Liquid Chromatographic Assay for Chloramphenicol, Chloramphenicol-3-monosuccinate, and Chloramphenicol-1-monosuccinate

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Abstract □ A high-pressure liquid chromatographic method for the sensitive and rapid determination of chloramphenicol, chloramphenicol-3-monosuccinate, and chloramphenicol-1-monosuccinate is presented. The procedure utilizes a reversed-phase chromatographic column with UV absorption detection. The assay is useful for monitoring patients receiving chloramphenicol, determining the pharmacokinetics of parenteral chloramphenicol sodium succinate, and certifying sterile chloramphenicol sodium succinate.

Keyphrases □ Chloramphenicol—simultaneous high-pressure liquid chromatographic assay with chloramphenicol-3-monosuccinate and chloramphenicol-1-monosuccinate □ High-pressure liquid chromatography—analysis, chloramphenicol, chloramphenicol-3-monosuccinate, and chloramphenicol-1-monosuccinate □ Antibiotics—chloramphenicol, simultaneous high-pressure liquid chromatographic assay with chloramphenicol-3-monosuccinate and chloramphenicol-1-monosuccinate

Chloramphenicol, a broad spectrum antibiotic, is available for parenteral administration as the sodium salt of the 3-monosuccinate ester[1]. This prodrug is biologically inactive and must be converted to chloramphenicol base by hydrolysis of the ester in the liver (1). Glazko *et al.* (2) reported that ~30% of a dose administered to normal adults is excreted unhydrolyzed in the urine. Consequently, impaired renal and/or hepatic function may affect the bioavailability of active chloramphenicol when administered parenterally as the sodium succinate salt.

Chloramphenicol-3-monosuccinate (III) exists in equilibrium with chloramphenicol-1-monosuccinate (I) at pH values near neutrality (3) (Scheme I). A colorimetric assay (4) for the simultaneous measurement of chloramphenicol and chloramphenicol succinate is tedious and lacks specificity. Numerous chromatographic methods have been developed for determining chloramphenicol in biological fluids (5–14), but none included the quantification of chloramphenicol succinate. The current Food and Drug Administration spectrophotometric assay for certifying chloramphenicol sodium succinate for injection (15) does not differentiate among chloramphenicol, III, and I. This paper presents a high-pressure liquid chromatographic (HPLC) method that allows the simultaneous quantification of these compounds.

EXPERIMENTAL

Materials—Reagent grade acetic acid[2], sodium acetate[3], deionized water, and HPLC grade acetonitrile[3] were used to prepare the mobile phase. Pure chloramphenicol[4] and chloramphenicol-3-monosuccinate[5] (III) were dissolved in ethyl acetate[3] to prepare the standard solutions. The internal standard solution was benzocaine[6] in methanol[3] (0.06 mg/ml). Trichloroacetic acid[3] (30%) was used for protein precipitation.

Instrument Conditions—The liquid chromatograph[7] was equipped with a variable-wavelength UV detector. The effluent was monitored at 275 nm with 0.05 aufs. An integrator[8] and a recorder[9] were used to monitor the detector output. A reversed-phase column[10] (25 cm long × 4 mm i.d.) was maintained at 50°. The mobile phase consisted of a 22% solution (v/v) of acetonitrile in 0.05 *M* sodium acetate. The pH of the final solution was adjusted to 5.7 with acetic acid. The flow rate was 1.25 ml/min.

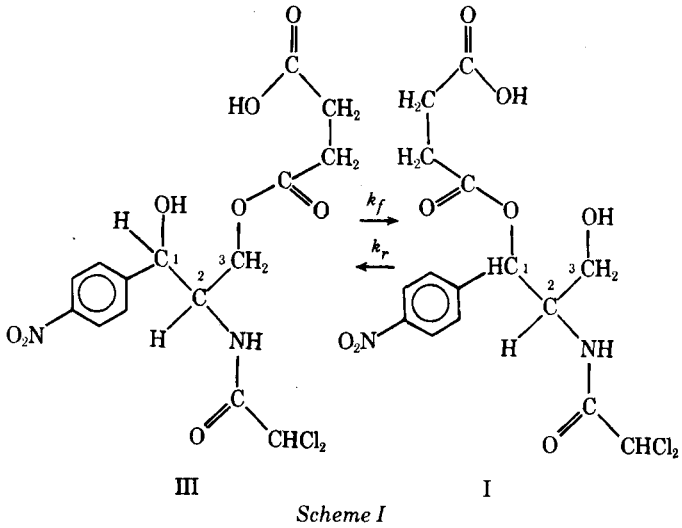
Determination of Equivalence of Molar Absorbance of I and III—A 0.05 *M* phosphate buffer adjusted to pH 7.4 was used to prepare 13 1.0-ml samples of 9.08×10^{-5} *M* III. Immediately after preparation, the samples were incubated at 37° for various periods. The internal standard (50 μl) was added to the sample at the end of the incubation, and a 90-μl portion was injected immediately onto the high-pressure liquid chromatograph.

A standard curve for III and chloramphenicol was prepared in a 0.05 *M* phosphate buffer at pH 4.0. The concentrations of III, I, and chloramphenicol were determined by relating the peak area ratios of the unknowns to the internal standard and comparing them to the standard curves. The standard curve for III was used to quantitate III and I. The apparent first-order forward, k_f, and reverse, k_r, rate constants describing the equilibrium between III and I (Scheme I) were estimated using the NONLIN program (16) with the following equations and unweighted data:

$$A = \frac{A_0 k_r}{k_f + k_r} + \frac{A_0 k_f}{k_f + k_r} e^{-(k_f + k_r)t} \qquad \text{(Eq. 1)}$$

$$B = \frac{A_0 k_f}{k_f + k_r} - \frac{A_0 k_f}{k_f + k_r} e^{-(k_f + k_r)t} \qquad \text{(Eq. 2)}$$

$$K_{eq} = \frac{k_f}{k_r} \qquad \text{(Eq. 3)}$$



III ⇌ I

Scheme I

[1] The USAN name chloramphenicol sodium succinate refers to the sodium salt of the 3-monosuccinate ester. Chloramphenicol sodium succinate is marketed as Chloromycetin Sodium Succinate by Parke-Davis Co.
[2] J. T. Baker Chemical Co.
[3] Fisher Scientific Co.
[4] Lot 423256, courtesy of Parke-Davis Co.
[5] Lot 441339, courtesy of Parke-Davis Co.
[6] Eastman Kodak Co.
[7] Model 7000, Micromeritics Instrument Corp.
[8] CDS 111, Varian Associates.
[9] Fisher Recordall series 5000, Fisher Scientific Co.
[10] LiChrosorb RP-18, E. Merck.

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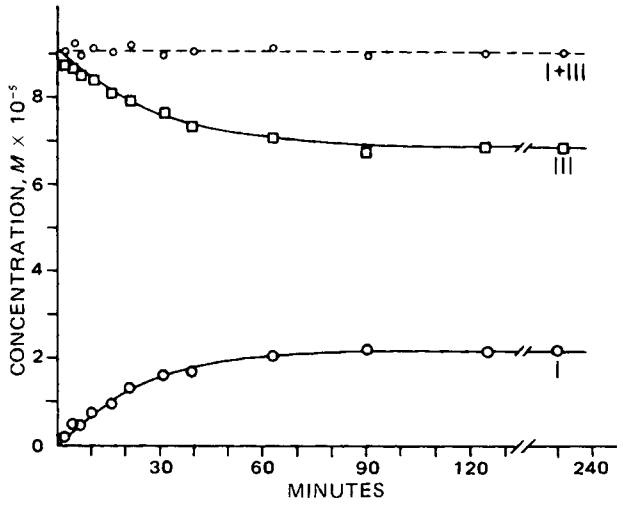

Figure 1—*Rearrangement of III to I in pH 7.4 phosphate buffer at 37°*

where A is the concentration of III, B is the concentration of I, and A_0 is the concentration of III at $t = 0$.

Quantitation of III, I, and Chloramphenicol in Biological Fluids—Blood samples treated with ethylenediaminetetraacetic acid were placed immediately in an ice bath. The plasma was separated and adjusted to pH ~4 by the addition of 20 μl of 4 *N* HCl/ml of plasma. A 250-μl sample was transferred to a 15-ml conical glass tube. The internal standard (25 μl) was added immediately before precipitation with 50 μl of a 30% solution of trichloroacetic acid. The sample was vortexed for 10 sec and then centrifuged for 10 min at 2500 rpm. A portion of the supernate (90 μl) was injected.

Urine samples were centrifuged to remove the sediment and diluted to concentrations of 1:100–1:500. The internal standard (25 μl) was added to 250 μl of the diluted urine sample, and 90 μl then was injected. Plasma standards containing III and chloramphenicol were prepared for each patient using a quantity of his own acidified plasma (pH 4.0). Phosphate buffer (pH 4.0) was used to prepare standards for urine assays.

Stability of III in Plasma—Two portions of fresh human plasma were adjusted to pH 4.0 and 7.4, respectively, and were used to prepare 250-μl samples containing 61.1 μg of III/ml[11]. Sets of plasma samples adjusted to pH 7.4 were incubated at 37, 25, and 0°. The pH 4.0 plasma samples were incubated at 25 and 0°. The concentrations of I, III, and chloramphenicol were determined at 0.5, 1.0, 2.0, 6.0, 12.0, and 24.0 hr.

Effect of Plasma Protein Concentration on Drug Recovery for Assay—Plasma samples from three normal volunteers were each diluted with normal saline to prepare 1:0, 3:1, 1:1, 1:3, and 0:1 dilutions (plasma to normal saline). The pH of each solution was adjusted to 4.0 with 4 *N* HCl and then spiked with 25 μg of chloramphenicol/ml and 19.1 μg of III/ml. Then 100 μl of trichloracetic acid (30%) was added to 0.5-ml samples of each solution. The samples were vortexed for 10 sec and centrifuged for 10 min at 2500 rpm.

A portion of the supernate (250 μl) was transferred to a 15-ml conical glass tube. The internal standard (25 μl) was added to the supernate, the sample was vortexed for 10 sec, and a portion of the supernate (90 μl) was injected. The absolute recoveries of both drugs at the 1:0, 3:1, 1:1, and 1:3 dilutions were determined by relating the peak area ratios of the drugs to the internal standard at these plasma dilutions to the peak area ratios obtained from the 0:1 dilution.

Rearrangement of III to I in Plasma—Citrated human plasma adjusted to pH 7.4 was used to prepare 250-μl samples of 30.5 μg of III/ml. Immediately after preparation, the samples were incubated for various periods at 37°. At the end of the incubation, 50 μl of 30% trichloroacetic acid was added to the sample. The sample then was vortexed for 10 sec and centrifuged for 10 min at 2500 rpm. A portion of the supernate (90 μl) was injected onto the high-pressure liquid chromatograph. The peak area ratio of III to the sum of III and I was determined at the various time intervals. The rate constants for the equilibrium between III and I were estimated by the NONLIN program employing Eq. 1 with unweighted data.

[11] The concentrations of I and III are expressed as chloramphenicol equivalents. The relationship is such that 1.0 mg of the acid form of either ester will yield 0.7635 mg of chloramphenicol upon hydrolysis.

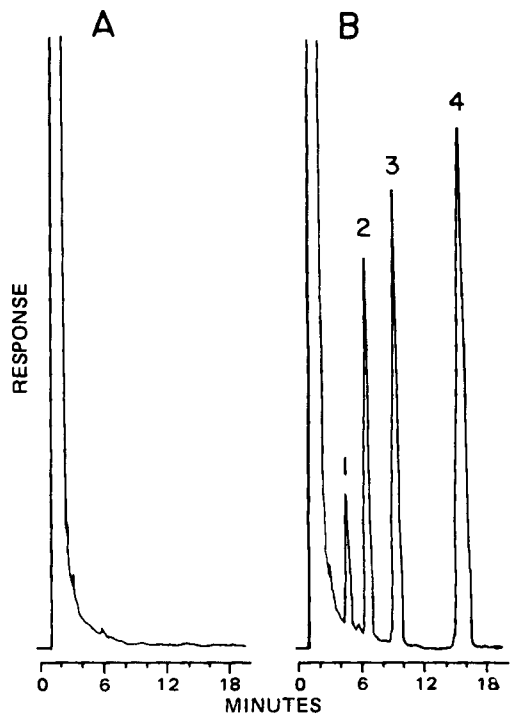

Figure 2—*Sample chromatogram for the assay in plasma. Key: A, blank plasma; B, spiked plasma; 1, I (4.4 μg/ml); 2, III (14.2 μg/ml); 3, chloramphenicol (13.7 μg/ml); and 4, benzocaine internal standard.*

RESULTS AND DISCUSSION

Determination of Equivalence of Molar Absorbance of I and III—The standard curve for the quantification of III also was used to quantitate I. Compound III is commercially available and is stable in crystalline form. Compound I, a pale-yellow oil at room temperature, is difficult to weigh accurately and transfer, rearranges to III under ambient conditions, and can be isolated only by preparative liquid chromatography or other suitable chromatographic methods. Although the technique of utilizing the standard curve for III to quantitate I and III offers numerous advantages, it is valid only if the molar absorptivities for I and III are equivalent for the specified instrument conditions.

The validity of this assumption was tested by measuring the concentrations of III and I as III rearranged to I. If the sum of the concentrations of I and III are constant utilizing a standard curve prepared from III to quantitate both I and III, then the molar absorptivities are equivalent. Figure 1 illustrates the rearrangement of III to I and the resulting equilibrium between the two compounds. The mean sum of the concentrations of I and III was $9.08 \pm 0.10 \times 10^{-5}$ *M* ($\pm SD$, $CV = 1.1\%$) for the 13 determinations. Since this value was constant within very narrow limits and showed no trend as the ratio of the concentration of the two compounds changed, no difference was detected in the molar absorptivities of I and III.

The assumption that the molar absorptivities of I and III are equivalent may not be valid for a different mobile phase or if the effluent is monitored at a different wavelength, although studies demonstrated that the molar absorptivities also were equivalent when the mobile phase pH was adjusted to 4.0 or 7.0. The technique also assumes that neither I nor III forms any other compounds during the sampling interval. The only other compound that could be detected was chloramphenicol, which formed slowly and amounted to only 0.19×10^{-5} *M* at the 230-min sample. This process was considered negligible for the purpose of evaluating the equivalence of the molar absorptivities of III and I.

Quantification in Biological Fluids—A typical chromatogram for the assay in plasma is presented in Fig. 2. Changes in the mobile phase pH had a dramatic effect on the retention times of III and I. Both I and III eluted before chloramphenicol when the mobile phase pH was 5.7 and after chloramphenicol when the mobile phase pH was 4.0. A summary of sample standard curves for chloramphenicol and III in plasma is presented in Table I. Three determinations were performed at each concentration. The intercepts for both standard curves were not significantly different from zero. Linear regression analysis of four-point standard curves for 14 patients demonstrated excellent correlation ($r^2 = 0.991$–1.000 for chloramphenicol and $r^2 = 0.998$–1.000 for III).

Table I—Standard Curves for Chloramphenicol and III

	Chloramphenicol					III				
Concentration, μg/ml	100	40	20	10	1	152.71	76.35	30.54	7.64	0.76
Mean peak area ratio[a]	4.152	1.698	0.874	0.439	0.043	4.816	2.555	1.058	0.265	0.028
CV, %	2.32	1.42	1.02	1.32	0.46	0.26	1.19	0.73	4.15	6.91
Slope[b]			0.0417					0.0320		

[a] Drug to standard. [b] Forced through the origin.

Table II—Stability of III in Plasma Expressed as Percent Converted to Chloramphenicol

Plasma pH	Temperature	Hours 0.5	1.0	2.0	6.0	12.0	24.0
4.0	0°	0	0.1	0.5	0.6	0.9	0.8
4.0	25°	0.4	0.5	1.2	1.4	2.5	4.3
7.4	0°	0.3	0.3	0.5	2.3	4.1	5.3
7.4	25°	0.4	0.7	1.0	2.6	3.5	5.4
7.4	37°	0.8	2.1	1.8	10.8	24.1	27.8

Table III—Stability of III in Plasma Expressed as Percent of I[a]

Plasma pH	Temperature	Hours 0.5	1.0	2.0	6.0	12.0	24.0
4.0	0°	0	0	0	0	0	0
4.0	25°	0	1.7	3.4	4.3	5.0	6.4
7.4	0°	2.8	4.1	5.2	14.8	28.8	32.1
7.4	25°	31.9	32.0	30.8	33.7	33.3	29.8
7.4	37°	30.1	30.8	31.4	30.3	32.1	30.9

[a] (I)/[(I) + (III)].

Potential assay interference was investigated by adding the following drugs to plasma: phenytoin, aspirin, penicillin, ampicillin, cefazolin, cimetidine, oxacillin, theophylline, phenobarbital, isoniazid, gentamicin, ethambutol, probenecid, sulfamethoxazole, and trimethoprim. These compounds did not produce a chromatographic peak or did not have the same retention time as chloramphenicol. Trimethoprim interfered with the quantification of I.

Stability of III in Plasma—Tables II and III illustrate the effects of pH and temperature on the stability of III in plasma. The recovery was 61.3 ± 1.8 μg/ml (±*SD*) for these determinations. The results at physiological conditions confirm findings by previous investigators (1) that chloramphenicol succinate is not rapidly hydrolyzed by plasma esterases to chloramphenicol.

Since rearrangement of III to I proceeded rapidly at pH 7.4 and 25°, it was necessary to acidify plasma to pH 4.0 before preparing a standard curve for III. Optimal conditions to prevent the rearrangement involve placing plasma in an ice bath and acidifying the plasma to pH 4.0. The addition of 20 μl of 4 *N* HCl/ml of plasma is sufficient to lower the pH of plasma to ~4.0.

Both III and I were isolated from the plasma and urine of human subjects. Preparing biological samples to study the rearrangement of III *in vivo* or to study independently the pharmacokinetics of I and III poses certain problems. The best method of plasma preparation involves immediately placing blood samples on ice and separating the plasma in a refrigerated centrifuge. The samples then should be acidified as described previously while the plasma is still cooled to 0°.

Although I and III possess different chemical structures, the usefulness of studying the pharmacokinetics of these compounds independently is mitigated by the fact that the two compounds are in rapid equilibrium. Doses of chloramphenicol succinate contain approximately the same fraction of I that appears in human urine and plasma. An analysis of 13 doses of chloramphenicol succinate[12] prepared in a hospital pharmacy by dilution in 50 ml of 5% dextrose injection revealed that 20.0 ± 1.7% (±*SD*) of the dose existed as I immediately prior to administration. Doses were administered between 1 and 24 hr after preparation.

Effect of Plasma Concentration on Drug Recovery for Assay—The absolute recoveries for chloramphenicol and III were 63.0 ± 2.4 and 49.2 ± 2.3% (±*SD*), respectively, when the samples were prepared with 30% trichloroacetic acid. These relatively low recoveries were the result of drug binding to the denatured protein. The effect of protein concentration on the absolute recovery of chloramphenicol and III is illustrated in Fig. 3.

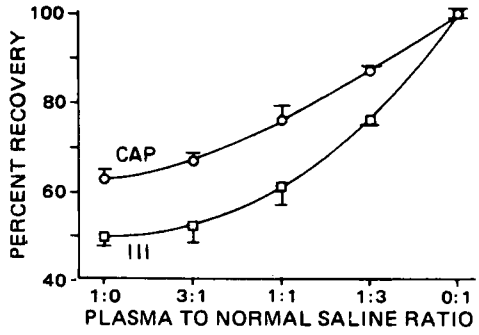

Figure 3—*Effect of protein concentration on drug recovery for assay for various ratios of plasma to normal saline (CAP = chloramphenicol).*

Table IV—Kinetic Parameters for the Rearrangement of III to I

Medium	k_f, min^{-1}	k_r, min^{-1}	$\frac{0.693}{k_f + k_r}$, min	K_{eq}	r^2
Buffer	0.0097	0.0297	17.6	0.327	0.990
Plasma	0.0285	0.0681	7.2	0.418	0.996

Although the absolute recovery for the trichloroacetic acid method is less than optimal, it is adequate for assaying chloramphenicol in the therapeutic range. It offers the advantage of minimal sample preparation time and produces cleaner solutions for injection than does organic solvent extraction. The major disadvantage to the trichloroacetic acid method is that the standard curve should be prepared from the plasma of the individual patient. This approach is preferable because quantitative and qualitative differences in the plasma protein composition might affect the absolute recovery of the compounds assayed.

Kinetics of Rearrangement of III to I—The kinetics of the rearrangement of III to I were studied *in vitro* in a phosphate buffer and plasma at physiological pH and temperature (Table IV). The apparent K_{eq} in the buffer was different from that in plasma (Fig. 4).

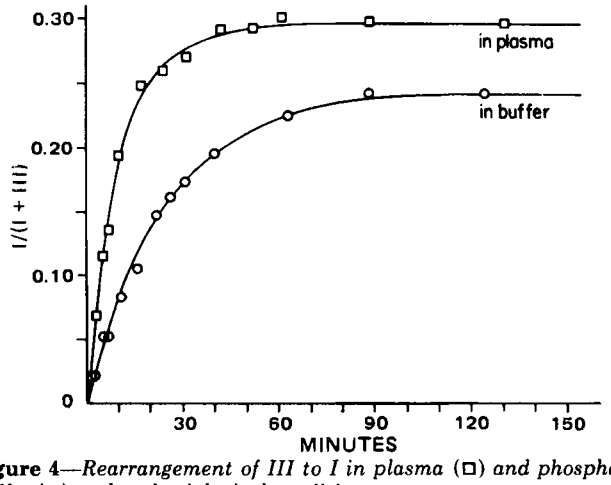

Figure 4—*Rearrangement of III to I in plasma (□) and phosphate buffer (○) under physiological conditions.*

[12] Chloromycetin Sodium Succinate, Parke-Davis Co.

The apparent K_{eq} in plasma may be greater because I is more extensively protein bound. It also may result from the use of trichloroacetic acid as a protein precipitant.

CONCLUSIONS

Chloramphenicol-3-monosuccinate (III) exists in rapid equilibrium with chloramphenicol-1-monosuccinate (I) under physiological conditions and in intravenous solutions prepared for administration. The analytical technique developed permits the quantification of chloramphenicol, I, and III in biological fluids and reconstituted commercial preparations. The methodology may be adapted for certifying chloramphenicol sodium succinate and offers advantages over the current FDA spectrophotometric method, which does not differentiate between these compounds. The analysis of I was simplified by demonstrating that I and III have similar molar absorptivities.

The results of previous investigators who reported that chloramphenicol succinate is not rapidly hydrolyzed by plasma esterases were confirmed. A rapid analytical technique is offered that will allow future studies of the effects of renal and hepatic disease on the pharmacokinetics and bioavailability of chloramphenicol sodium succinate.

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Solubility and Partitioning I: Solubility of Nonelectrolytes in Water

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Abstract □ On the basis of a semiempirical analysis, an equation was obtained that enables the estimation of the aqueous solubility of either liquid or crystalline organic nonelectrolytes:

$$\log S_w \approx -1.00 \log PC - 1.11 \frac{\Delta S_f(MP - 25)}{1364} + 0.54$$

where log PC and ΔS_f are estimated from the chemical structure and MP is either known or experimentally determined. Analysis of this equation provides a means of assessing the role of crystal structure [as reflected by the melting point (MP) and the entropy of fusion (ΔS_f)] and of the activity coefficient [as reflected by the octanol–water partition coefficient (PC)] in controlling the aqueous solubility of a drug. Techniques are also provided for estimating the entropy of fusion of organic compounds.

Keyphrases □ Solubility—nonelectrolytes in water, estimation techniques □ Nonelectrolytes—estimation of solubility in water □ Aqueous solubility—nonelectrolytes, estimation techniques

Aqueous solubility has long been recognized as a key factor in controlling drug efficacy. Before an orally administered drug can become available to its receptor, it first must dissolve in the GI fluid. Both the dissolution rate and the maximum amount of drug that can be dissolved are governed by the solubility of the drug in the medium (1).

The design of orally active drugs must account for the effects of structural modifications on solubility. The lack of sufficient aqueous solubility often causes a drug to appear inactive or less active than some reference compound of a series. Aqueous solubility is a key factor in the design of parenteral and ophthalmic formulations, and it also is important in controlling taste. For these reasons, some appreciation of the relationship between aqueous solubility and chemical structure is needed.

THEORETICAL

In spite of the tremendous importance of aqueous solubility in pharmacy and other applied chemical disciplines, it is a poorly understood phenomenon. There are no generally useful guidelines for estimating the solubility of a substance in water from a consideration of its structure and physical properties. One reason that solubility of crystalline compounds has successfully defied attempts to make it predictable is that it is not a simple equilibrium but rather a combination of equilibria.

This report attempts to provide some guidelines for understanding the factors that govern aqueous solubility and for estimating the aqueous solubility of nonelectrolytes. Subsequent reports will deal with the estimation of the solubility of weak electrolytes.

Factors Influencing Aqueous Solubility—The aqueous solubility of a drug is governed by three major factors: (*a*) the entropy of mixing; (*b*) the difference between the drug–water (DW) adhesive interactions and the sum of the drug–drug (DD) and water–water (WW) cohesive interactions; and (*c*) the additional drug–drug interactions associated with the lattice energy of crystalline drugs, which are designated $\overline{DD}$ and are not applicable to liquids.

The entropy of mixing can be thought of as a force that favors complete

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