Differential Pulse Polarographic Determination of Submicrogram Quantities of Carmustine and Related Compounds in Biological Samples

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Abstract □ A polarographic method was developed to determine the antineoplastic agent carmustine and other nitrosoureas, such as *N*-methyl-*N*-nitrosourea and *N*-cyclohexyl-*N*-nitrosourea, in biological fluids at levels well below 1 μg/ml or g. The stability of carmustine in different media was investigated to prevent losses during administration or assay. Examples of nitrosourea determination in biological samples are given.

Keyphrases □ Carmustine—differential pulse polarographic analysis in biological samples □ Nitrosoureas, various—differential pulse polarographic analyses in biological samples □ Polarography, differential pulse—analyses, carmustine and various nitrosoureas in biological samples □ Antineoplastics—carmustine, differential pulse polarographic analysis in biological samples

N-Alkyl-*N*-nitrosoureas and, in particular, carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea (I), are widely used antineoplastic agents. Unfortunately, their determination in biological samples is hampered by the low sensitivity of the currently used colorimetric method (1, 2). Garrett and Cusimano (3) investigated the polarographic behavior of various *N*-alkyl-*N*-nitrosoureas. Since that time, however, polarographic techniques have been improved considerably (4–6), and their basic sensitivity has been increased by a factor of at least 100, pushing the limits of applicability of polarography far below the microgram level (7, 8). Therefore, the possibility of using polarography for routine assays of I and other nitrosoureas in blood and tissues was investigated.

EXPERIMENTAL

Apparatus—Measurements were made on a modular polarograph[1] with a standard polarographic vessel[2]; for some measurements, the thermostated vessel[2] was used. These vessels require a minimum sample volume of 5 ml; a microcell[1] for 0.5–5 ml also was used with success.

Reagents—All reagents (potassium chloride, hydrochloric acid, citric acid, tartaric acid, ether, and *n*-pentane) were analytical grade. An interfering peak at E = 0.830 v, due to an impurity, appeared in some supplies of the citric acid used as a basic electrolyte; each original bottle had to be checked before use by running a blank polarogram. All solutions were prepared with redistilled water. Tridistilled, commercially supplied mercury was used for the dropping mercury electrode.

Compound I, *N*-cyclohexyl-*N*-nitrosourea (II), and *N*-methyl-*N*-nitrosourea (III) were obtained as lyophilized powders from two sources[3,4]. No difference in quality was observed between the different samples of I as long as the compound was stored in dry plastic bags (with silica gel inserts) at deep-freeze temperatures.

Extraction Procedures—Blood was prevented from clotting by 3.8% sodium citrate (nine parts of blood plus 1 part of citrate solution) and centrifuged (10 min at 3000 rpm) to separate plasma.

Procedure A—Plasma, 1 ml, was mixed with 1 ml of 0.2 *M* citric acid and extracted with 5 ml of *n*-pentane for 1 min. After separation of the two phases, 4 ml of the extract was evaporated directly in the thermostated polarographic vessel at 40° with 5 or 10 ml of 0.2 *M* citric acid.

Procedure B—Plasma, 1 ml, was mixed with 1 ml of 0.2 *M* citric acid and extracted three times with 4 ml of *n*-pentane for 3 min. A 10-ml aliquot of the combined extracts was then evaporated in a test tube with 0.2 *M* citric acid at 40° and submitted to polarography at room temperature.

Polarographic Measurements—Citric acid (0.2 *M*) was used as a basic electrolyte. Dissolved oxygen was removed by bubbling high purity nitrogen gas, passed first through redistilled water to remove all mechanical impurities. A three-electrode arrangement was adopted with a dropping mercury electrode, a platinum auxiliary electrode, and a saturated calomel electrode as reference. The mercury flow rate in the dropping mercury electrode was 1.25 mg/sec, and the height of the mercury column was 120 cm.

The drop time was limited by an electronically controlled hammer; a drop time of 3 sec was optimal. The differential pulse technique was adopted with the pulse height, ΔE, of 100 mv. The sweep normally covered the interval from −250 to −750 mv, and the sweep rate in most cases was 2 mv/sec. The output signal was filtered by a resistance–capacitance filter with a time constant of 0.1 sec. In addition, a gated accumulator was inserted to suppress experimental noise.

RESULTS AND DISCUSSION

Polarographic Properties of Nitrosoureas—The collected data indicate that, qualitatively, I is to some extent representative of all *N*-alkyl-*N*-nitrosoureas and that reduction of I on the dropping mercury electrode is not a simple electrode process. A full interpretation was not attempted, but the data were used to develop a routine analytical method[5].

Figure 1 illustrates the behavior of I in 0.1 *M* KCl acidified to varying degrees with hydrochloric acid. At pH 2.5, there was a single reduction wave (A), whose half-wave potential ($E_{1/2}$), width, and height all depended on pH. At higher pH values, wave A was still present, but a second

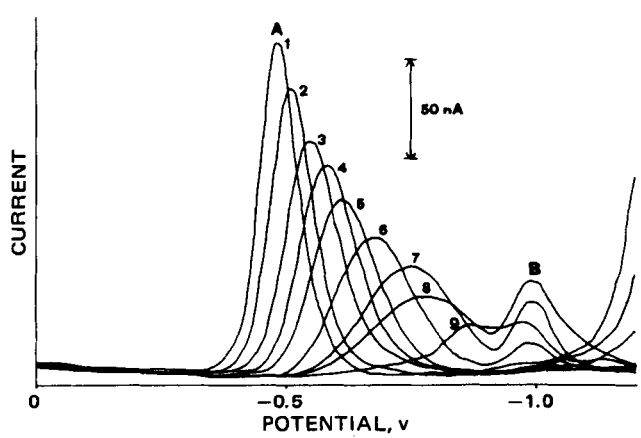

Figure 1—*Polarograms of I, showing dependence on acidity in the 0.1 M KCl–hydrochloric acid basic electrolyte. The concentration of I was* 2×10^{-5} M. *The experimental setup was as described in* Experimental, *except that the differential pulse height was 20 mv and the gated accumulator was not used. Key (pH values): 1, 1.56; 2, 2.00; 3, 2.58; 4, 3.00; 5, 3.50; 6, 4.02; 7, 4.52; 8, 5.02; and 9, 5.70.*

[1] Universal E 310, Bruker Physik AG, 7512 Karsruhe-Rheinstetten, Silberstreifen, West Germany.
[2] Metrohm, Herisau, Switzerland.
[3] National Cancer Institute, Bethesda, Md.
[4] Simes, Milan, Italy.
[5] A detailed analysis of the reduction process will appear elsewhere.

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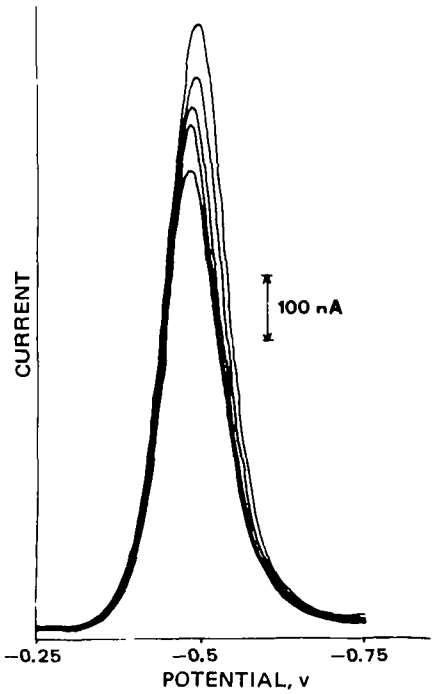

Figure 2—*Polarograms of I, showing dependence on citric acid concentration. The concentration of I was 2 × 10^{-5} M. The experimental setup was as described in* Experimental. *The concentrations of citric acid for the curves (from top to bottom) were 0.025, 0.05, 0.1, 0.2, and 0.5* M.

wave (B) appeared at somewhat more negative potentials. The height and width of wave B also depended on pH, but its position appeared to be nearly constant.

A very tentative interpretation might be that there are two forms (A and B) of I in an equilibrium of the type B + H^+ = A. Form A, prevailing at low pH, is reduced at the mercury electrode in a one-electron process involving H^+ ions. Form B is reduced in a one-electron process that does not involve H^+ ions. For analytical purposes, a single sharp peak at a well-defined position is preferable. The results, therefore, indicate the necessity of a suitable acid buffer.

The choice of buffer was complicated by the fact that the compound behaved somewhat differently when weak acids, such as citric, were used as the basic electrolyte (Fig. 2). Then there was just one peak at about −500 mv, and the pH dependence of the peak parameters was very small. The width of the peak considerably exceeded the value characteristic for a one-electron process, thus indicating a complex electrochemical mechanism. The dependence of the current (I_{500}) on the square root of the height of the mercury column ($\sqrt{h}$) was linear. For a 2 × 10^{-5} *M* solution, the least-squares fit of the data obtained for column heights ranging from 30 to 120 cm gave the relation $I(nA) = 92.978 \cdot \sqrt{h}$ cm − 193.048; the correlation coefficient was $r = 0.998$.

When analyzing low concentrations of I (10^{-7} *M*), the choice of the basic electrolyte was critical for another reason. The buffer itself gave a relatively high background because of the capacitive component of the current, and this background current should be as flat as possible throughout the range of potentials at which the test drug peak appears. The background currents of a number of basic electrolytes were recorded in the range of potentials from −250 to −750 mv at very high sensitivity, and the differences were marked.

The final choice was 0.2 *M* citric acid (an acceptable alternative was tartaric acid). Figure 3 shows the polarograms of I at concentrations ranging from 5 × 10^{-8} to 5 × 10^{-7} *M*. The measurements were made in 5 ml of the basic electrolyte so the smallest quantity measured was about 50 ng. By using a microcell, this figure can be reduced by a factor of about 10. Assays at these levels must be done with the utmost care to avoid impurities (*e.g.*, reagents, water, mercury, and glassware) and any sources of noise (*e.g.*, bad contacts).

At low concentrations, the background current complicates quantitative evaluation of the data. Therefore, a method based on measurements of the current values I_{400}, I_{500}, and I_{600} at potentials of −400, −500, and −600 mv, respectively, was employed.

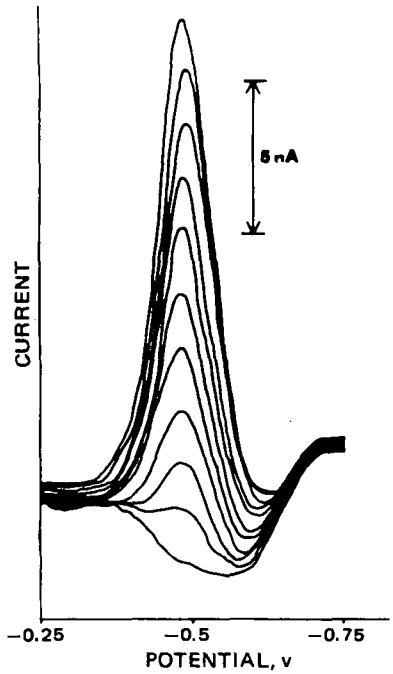

Figure 3—*Calibration curves of I in 0.2* M *citric acid under conditions described in* Experimental. *The concentrations (from bottom to top) were 0 (basic electrolyte), 5 × 10^{-8}, 10^{-7}, 1.5 × 10^{-7}, 2 × 10^{-7}, 2.5 × 10^{-7}, 3 × 10^{-7}, 3.5 × 10^{-7}, 4 × 10^{-7}, 4.5 × 10^{-7}, and 5 × 10^{-7}* M.

The following equation is used for both the sample, *S*, and a blank containing only basic electrolyte, S_0:

$$S \text{ or } S_0 = I_{500} - 0.5 \times (I_{400} + I_{600}) \qquad \text{(Eq. 1)}$$

The difference $s = (S - S_0)$ is then used in empirical calibration graphs; *s* is directly proportional to the concentration of I even at the lowest detectable level. The slope depends, of course, on the experimental setup (capillary, height of the mercury reservoir, drop time, and temperature) so a new calibration is necessary each time any relevant parameter is changed; $s = (K)$ (concentration). In Fig. 3, $k = 0.0355$ amp/mole × $liter^{-1}$. When a calibration graph is not available, the method of standard addition can always be used; but even in this case, the quantity *s* should be used if the concentrations involved are close to the detection limit. Figure 4 gives the polarographic curves of some *N*-alkyl-*N*-nitrosoureas in 0.2 *M* citric acid at a concentration of 10^{-6} *M*. While all nitrosoureas are polarographically active and give rise to peaks between −0.4 and −0.8 v, differences exist between different members of this group.

Thus, assays of II can be made using the same techniques as for I, with the additional advantage of higher sensitivity. On the other hand, the

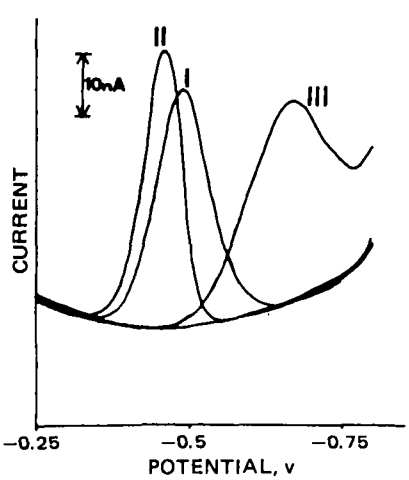

Figure 4—*Polarograms of 10^{-6}* M *I, II, and III in 0.2* M *citric acid under conditions described in* Experimental. *The bottom line is the basic electrolyte.*

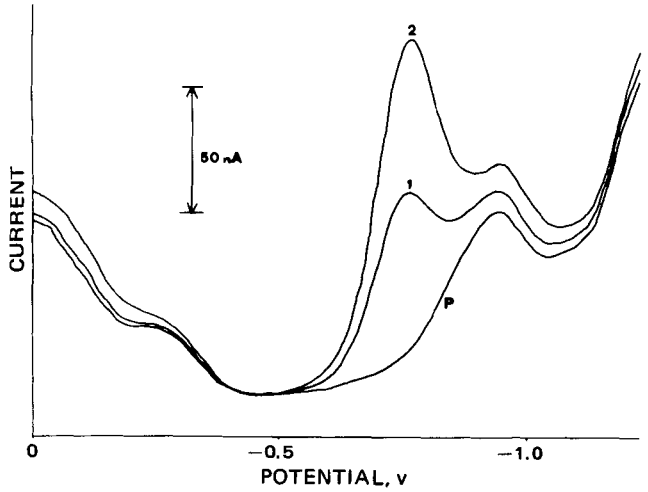

Figure 5—*Polarograms of rat plasma (P) containing 10 (1) and 20 (2) μg of I/ml. The sample was prepared by adding 1 ml of plasma to 9 ml of 0.2* M *citric acid. The conditions were as described in* Experimental, *except that the pulse height was 50 mv and the accumulator was not used.*

peak of III is much broader than that of I and appears at more negative potentials where the reproducibility of the background current is lower because of common impurities in the citric acid. As a result, the sensitivity of III assays is expected to be lower than with I unless the technique is suitably modified.

Stability of I in Various Solutions—Nitrosoureas decompose easily unless the pH values are kept sufficiently low (2, 9, 10). The stability of I in various experimental circumstances was examined to establish reliable time limits for its clinical administration and assay. The pharmaceutical preparation[4] of I was dissolved directly in the unbuffered physiological saline or 5% glucose used for infusion. The pH values of the resulting solution differed considerably from case to case; in several experiments, the pH values remained slightly above 7 and I decomposed completely in less than 1 hr. Therefore, in clinical applications involving infusions of I, pH differences in the unbuffered infusion solutions may be responsible for broad variations in drug delivery and poor reproducibility of the treatment.

On the other hand, no measurable decay was observed in animal plasma buffered with sodium citrate and kept for several weeks at −20°. The half-life of I in citric acid (0.2 *M*) at 0° was about 270 hr; at 20°, it was 16.9 hr. Very slow decay of the compound was observed in ethanol solution acidified by a drop of 1 *M* citric acid per 10 ml; the half-life of I at 20° was between 250 and 300 hr.

Routine Assays of I in Biological Samples—Figure 5 shows the polarogram of 1 ml of plasma, with and without addition of I, measured in 10 ml of 0.2 *M* citric acid. Surprisingly, the peak of I was at −750 mv rather than at the expected −500 mv. A possible explanation is complex formation; preferential binding of I to some plasma proteins has, in fact, been reported (2). However, assay of I in untreated plasma is hindered by the background produced by the plasma itself, which limits the usefulness of the method to concentrations exceeding about 5 μg/ml of plasma. Moreover, it is difficult to eliminate oxygen from the plasma because of foam formation. Foaming may be avoided by denaturing the plasma proteins with 5% $HClO_4$ and removing them by filtration prior to polarography.

The background from blank plasma treated with perchloric acid was, however, exactly the same as when the plasma was not denatured, indicating that it was not due to proteins. The nitrosourea added to the plasma before treatment was no longer detectable in the filtrate, while I added to the filtrate after removal of the proteins gave a peak at its normal position of −500 mv. This result confirms that I forms a strong complex with plasma proteins but, at the same time, precludes any possibility of using denaturation for assay purposes.

An excellent method for solving these problems is to extract the plasma with an apolar organic solvent such as ether or *n*-pentane. After separation of the phases, an aliquot of the organic phase is evaporated in a vessel containing the basic electrolyte (possibly directly in the polarographic cell). There is no detectable background due to the plasma itself. With I, the extraction yield depends on the efficiency and time of shaking.

A simple 1-min extraction (Procedure A) gave yields in the range of 85–90% for ether or *n*-pentane. The standard error of determination of parallel samples extracted with *n*-pentane did not exceed 3.3% within the concentration range studied; the calibration curve was linear. *n*-Pentane is preferred because ether usually contains traces of polarographically active peroxides that interfere with the determination of I. Ether also dissolves considerable amounts of water (7%), which end up in the basic electrolyte and may represent a complication if the final volume is to be kept small (microcell).

Extraction of whole blood directly also was attempted. While the extraction yields were essentially the same as for plasma, difficulties were encountered because of lipids in the blood cells. These were extracted and eventually led to colloid impurities in the solution to be analyzed polarographically. The colloid particles caused severe noise on the dropping mercury electrode unless they were first removed by filtration. The technique finally adopted is simple and quantitative. As described in *Experimental* (*Procedure B*), it consists of removal of the blood cells by centrifugation, triple extraction by *n*-pentane, and evaporation of the organic extract in a vessel containing a known amount of the 0.2 *M* citric acid used as the basic electrolyte.

Disappearance of I from Rat and Mouse Plasma—Male Sprague–Dawley rats[6], 226 ± 3 g, or male Albino Swiss mice CD-1, 20.9 ± 0.6 g, received 10 mg of I/g iv. Groups of animals were killed 2, 5, 10, 20, 30, 60, 90, and 120 min after I administration. Blood was collected (nine parts of blood plus 1 part of 3.8% sodium citrate) and centrifuged at 3000 rpm for 10 min. Prepared plasma was frozen at −20° for further analysis. Before *n*-pentane extraction, plasma samples were thawed at 37° for 5 min. Extraction Procedure B was used to determine the remaining drug. The data are reported in Table I, and kinetic analyses of these data are reported in Table II.

Table I—Disappearance of I from Rat and Mouse Plasma after Administration of 10 mg of the Drug/kg iv

Minutes	Concentration of I, μg/ml of Plasma (Mean ± *SE*) Rat	Mouse
2	2.36 ± 0.41	2.41 ± 0.38
5	1.70 ± 0.18	1.02 ± 0.15
10	0.66 ± 0.04	0.16 ± 0.04
20	0.38 ± 0.02	0.08
30	0.22 ± 0.04	<0.02
60	<0.02	<0.02

Table II—Kinetic Analysis[a] of the Data Reported in Tables I and III

Animal Species	Half-Life, $t_{1/2}$, min	Correlation[b] Coefficient, *r*	Initial Concentration, μg/ml	Volume Distribution, V_d, liters/kg
Rat	8.33 ± 0.14	0.990	2.25 ± 0.22	4.43
Mouse	2.17	0.984	4.57	2.19
Rat liver microsomes	49.74	0.999	40.0	

[a] Standard error is given wherever available. [b] Decay followed first-order kinetics in all cases.

Table III—Disappearance of I after Incubation with Rat Liver Microsomes

Minutes	Concentration of I, μg/ml of Incubation Medium
2	32.1
5	30.1
10	29.6
15	25.8
20	22.4
30	21.3
40	18.8
50	15.5
60	14.5

[6] CD-COBS, Charles River, Italy.

Disappearance of I after Incubation with Rat Liver Microsomes—Rat liver microsomes were prepared according to the standardized technique described previously (11). The incubation medium contained 0.93 μmole of I, 1.5 μmoles of NADP, 50 μmoles of glucose 6-phosphate, 0.7 international unit of glucose 6-phosphate dehydrogenase, 25 μmoles of magnesium chloride, 0.08 *M* phosphate buffer (pH 7.0), and 30 mg of microsomes in a total volume of 5 ml. Samples (1.0 ml) of the incubation medium were taken at different time intervals, acidified with citric acid (0.1 ml of 1 *M* solution), and immediately extracted with 5 ml of *n*-pentane for 10 min at room temperature. The results are reported in Tables II and III.

REFERENCES

(1) T. L. Loo and R. L. Dion, *J. Pharm. Sci.*, **54**, 809 (1965).

(2) T. L. Loo, R. L. Dion, R. L. Dixon, and D. P. Rall, *ibid.*, **55**, 492 (1966).

(3) E. R. Garrett and A. G. Cusimano, *ibid.*, **55**, 702 (1966).

(4) J. B. Flato, *Anal. Chem.* **44**, 75 A (1972).

(5) P. J. Elving, in "Electroanalytical Chemistry," H. W. Nurnberg, Ed., Wiley, London, England, 1974, p. 197.

(6) H. Hoffman and J. Volke, in *ibid.*, p. 287.

(7) A. M. Brooks, J. A. F. De Silva, and R. M. Hackman, *Am. Lab.*, **1974**, 23.

(8) D. J. Pietrzyk, *Anal. Chem.*, **46**, 52 R (1974).

(9) E. R. Garrett, S. Goto, and J. F. Stubbins, *J. Pharm. Sci.*, **54**, 119 (1965).

(10) J. A. Montgomery, R. James, G. S. McCaleb, and T. P. Johnston, *J. Med. Chem.*, **10**, 668 (1967).

(11) D. L. Cinti, P. Moldeus, and J. B. Schenkman, *Biochem. Pharmacol.*, **21**, 3249 (1972).

Automated Potentiometric Procedure for Studying Dissolution Kinetics of Acidic Drugs under Sink Conditions

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Abstract □ An automated potentiometric procedure was used for studying *in vitro* dissolution kinetics of acidic drugs. Theoretical considerations indicated that the pH-stat method could be used to establish approximate sink conditions or, possibly, a perfect sink. Data obtained from dissolution studies using the pH-stat method were compared with data obtained from known sink and nonsink conditions. These comparisons indicated that the pH-stat method can be used to establish a sink condition for dissolution studies. The effective diffusion layer thicknesses for benzoic and salicylic acids dissolving in water were determined, and a theoretical dissolution rate was calculated utilizing these values. The close agreement between the experimental dissolution rates obtained under pH-stat conditions and the theoretical dissolution rates indicated that perfect sink conditions were established under the experimental conditions used.

Keyphrases □ Dissolution kinetics, *in vitro*—acidic drugs, automated potentiometric study, pH-stat method used to establish sink conditions □ Potentiometry, automated—study of *in vitro* dissolution kinetics of acidic drugs, pH-stat method used to establish sink conditions □ pH-stat method—used to establish sink conditions for *in vitro* automated potentiometric study of dissolution kinetics of acidic drugs

Recognition that the availability for GI absorption of drugs in oral solid dosage forms is often reflected by *in vitro* dissolution rates has stimulated the development and use of numerous dissolution rate tests for tablets and capsules. Various types of apparatus have been used to determine dissolution rates of drugs, most of which have been critically reviewed (1–3).

Emphasis has been placed on the need for dissolution systems to be operated under sink conditions, where there is no effective buildup of drug concentration (4, 5). It was suggested that unless sink conditions are embodied in the design of *in vitro* dissolution rate methods, the *in vitro* results may bear little relationship to *in vivo* observations (6). It was also suggested that all dissolution systems should be operated under sink conditions and that systems that do not meet this requirement should either be modified accordingly or discarded (7).

The utility of automatic titration at a stat-pH for studying *in vitro* dissolution rates of acidic and basic drugs was demonstrated previously (8, 9). A pH-stat procedure was used in evaluating antacid products (10, 11). It was thought that the pH-stat method might be used to establish approximate sink conditions or, possibly, a perfect sink during dissolution rate testing.

THEORY

The Noyes–Whitney (12) equation describes the dissolution process:

$$\frac{dW}{dt} = kS(C_{sat} - C_{soln}) \qquad \text{(Eq. 1)}$$

where dW/dt is the dissolution rate, k is a constant, S is the surface area of the dissolving solid, C_{sat} is the concentration of a saturated solution, and C_{soln} is the drug concentration in the solvent at time t. As the C_{soln} increases, the equation predicts that dW/dt decreases. When $C_{sat} \gg C_{soln}$, a sink condition exists; if a buildup of C_{soln} can be prevented, then a sink condition will be maintained.

The total solubility of a weak acid, HA, is the sum of the concentration of the unionized [HA] and the ionized acid [A^-]:

$$C_{sat} = [HA] + [A^-] \qquad \text{(Eq. 2)}$$

and the concentration, C_t(soln), at any time before saturation is:

$$C_t(\text{soln}) = [HA]_t + [A^-]_t \qquad \text{(Eq. 3)}$$

The solubility of the ionized species, A^-, in water is generally much greater than that of the unionized acid, HA. Therefore, for dissolution phenomena involving relatively small quantities of acidic drugs in solution, the concentration of the unionized acid determines the concentration buildup. For example, the solubility of salicylic acid is 1.0 g in 460 ml of water while the solubility of sodium salicylate is 1.0 g in 0.9 ml of water (13).

In dissolution, the sequence of events in Schemes I and II occurs for

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