(44.6% recovery) for the capsules. The mean difference of 130.8 mg of hydroxyethinamate in the 24-hr urine from the tablets and capsules was significant. However, this significant difference can be explained largely by the unusually low recovery of 77 mg of III from one subject. Without this subject, the average 24-hr urine recovery of III was 385 mg for the other 11 subjects. The mean difference of 105 mg of III recovered in the 24-hr urine from the two dosage forms was not significant.

The half-life of ethinamate in plasma was determined from a semilogarithmic regression analysis. The $t_{1/2}$ of ethinamate in plasma from these products was 1.9 ± 0.3 hr.

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Solubility and Ionization Characteristics of Phenytoin

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Abstract □ The solubility of phenytoin was determined in pH 7.4 and 5.4 phosphate buffers at five temperatures; in hydroalcoholic solutions, 0–4% methanol; and in pH 4.8–8.4 buffer solutions. From the temperature data, the enthalpy and entropy of solution of this nonideal system were calculated and were similar at both pH values. The data obtained from the buffer solutions were used to calculate the apparent dissociation constant, pKa′, of phenytoin as 8.06. A GLC method with on-column methylation was used to quantitate phenytoin with 5-(*p*-methylphenyl)-5-phenylhydantoin as an internal standard. The assay uses chloroform for extraction of the drug from aqueous solutions. The ratio of peak heights was adjusted for weights of aqueous and organic layers, and results were calculated in micrograms per gram of sample and mole fraction of phenytoin. Although hydroalcoholic solutions enhanced drug solubility, there is a potentially significant disadvantage in using alcohol for clinical studies.

Keyphrases □ Phenytoin—solubility and ionization characteristics in various buffers and hydroalcoholic solutions □ Solubility—phenytoin in various buffers and hydroalcoholic solutions □ Ionization characteristics—phenytoin in various buffers and hydroalcoholic solutions □ Anticonvulsants—phenytoin, solubility and ionization characteristics in various buffers and hydroalcoholic solutions

Because of its regulating effect on the bioelectric activity of the nervous system, phenytoin is used widely as an anticonvulsant and cardiac antiarrhythmic (1). The therapeutic activity of the drug appears to be related to the concentration of free unbound drug in the plasma (2, 3). The present paper reports some fundamental physicochemical properties of the drug which have considerable relevance for pharmacokinetic studies. A GLC method was devised to achieve acceptable sensitivity (<5% error) from 0.5 to 100 μg of phenytoin/ml.

The apparent dissociation constant, pKa′, was 8.31 when determined by UV spectrophotometry and 8.33 when determined by potentiometric titration in hydroalcoholic solutions (4). A graph of pKa′ values as a function of the ethanol concentrations yields the apparent pKa′ value in water (0% alcohol). A pKa′ of 9.2 was determined by titration in 50% methanol (5), in good agreement with that for 50% ethanol (4).

Phenytoin is very slightly soluble in alkali at 26° (5), *e.g.*, 165 μg/ml at pH 9.1 (borate buffer) and 1.52 mg/ml at pH 10, but it is practically insoluble in water, *e.g.*, 14 μg/ml at pH 7 and 20 μg/ml at pH 7.4 at 24° (6). However, it is sparingly soluble in alcohol, 19.4 mg/g (7) or 1 g in about 60 ml, and about one part in 30 ml of acetone (8). It is only slightly soluble in ether and chloroform (9).

Shortly after the introduction of phenytoin as an anticonvulsant in 1937 (10), the first analytical method, a titrimetric procedure, was developed for the determination of quantitative plasma phenytoin levels (11, 12), but this method lacked sensitivity in the clinical microgram range. Several spectrophotometric procedures (13–18) had considerably improved sensitivity.

The most recent development in phenytoin assay procedures involves the application of GLC, a highly specific and sensitive analytical technique (19, 20). A procedure using trimethylsilyl derivatives of phenytoin and its metabolic products is sensitive, but these products are unstable in the presence of moisture (21, 22). The quaternary ammonium base, trimethylanilinium hydroxide (I), was used successfully as a methylating agent (23) to convert the hydantoins to nonpolar, volatile derivatives, using the injection port of a gas chromatograph as the reaction chamber. This flash-heater methylation reaction is complete; the 1,3-dimethylhydantoin products are stable (24) and were identified by mass spectrometry as the sole products of this reaction (25).

EXPERIMENTAL

Materials—Phenytoin (5,5-diphenylhydantoin) and 5-(*p*-methylphenyl)-5-phenylhydantoin (II) were used as obtained (99+%)[1]. The melting point and IR spectrum of phenytoin were determined as an index of purity (9). Chloroform[2] and all other chemicals employed were reagent grade.

[1] Aldrich Chemical Co., Milwaukee, WI 53233.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.

Table I—Average Solubility for Six Samples of Phenytoin in Buffers at 25°

pH	Solubility, μg/ml (*SD*)	S/S^0
8.3	56.8 (2.8)	3.08
8.2	44.7 (2.2)	2.43
8.0	32.4 (2.0)	1.76
7.8	27.6 (1.8)	1.50
7.5	21.9 (1.4)	1.19
7.1	20.3 (0.9)	1.10
4.9	18.4 (0.8)	—

Phenytoin Extraction—Phenytoin and II, as an internal standard, were extracted from aqueous samples in chloroform and measured by a modification of several methods using flash-heater methylation (24, 26, 27). To 1 ml of an aqueous sample in a 50-ml glass-stoppered centrifuge tube was added 5 ml of chloroform containing 2 μg of II/ml. The mixture was shaken[3] for 15 min and centrifuged[4] at 1000 rpm for 6 min to break any emulsion. As much of the organic (lower) phase as possible was removed by pipet and placed in a 15-ml ground-glass-stoppered centrifuge tube.

This mixture was dried under a nitrogen stream on a 60° water bath. Twenty-five microliters[5] of a solution of I, 0.5 *M* in methanol[6], was added to the dry residue, and 1 μl of the extract was injected into the gas chromatograph. The centrifuge tubes and aqueous and organic solvents were weighed[7] to permit calculation of mole fractions from the microgram per gram concentrations.

Phenytoin Analysis—A gas chromatograph[8] fitted with dual hydrogen flame-ionization detectors was used. Each U-shaped borosilicate glass column (183 cm × 2 mm i.d.) was packed with 3% phenyl methyl silicone[9] coated on acid-washed, dimethyldichlorosilane-treated, high performance flux-calcined diatomite support[10], 100–200 mesh. Small silanized glass wool plugs were inserted in both ends of the columns after packing by the vibrator method.

Heat conditioning was accomplished by connecting the columns to the injector inlet only with a nitrogen purge of about 20 ml/min and maintaining a temperature of 325° for at least 24 hr (the recommended maximum is 350° for the packing). During periods of inactivity, the column oven was set at 160° with a small nitrogen flow of about 5–10 ml/min.

For routine analysis of phenytoin, the on-column injectors were sealed with high temperature septa[11] and maintained at 250°. The same temperature was chosen for the detectors. The column was operated isothermally at 230° using a nitrogen carrier gas flow rate of about 30 ml/min (65 psi) and a hydrogen and air flame, 30 ml/min (20 psi) and 300 ml/min (40 psi), respectively. The electrometer output was monitored with a 1-mv recorder[12] with a chart speed of 0.254 cm (0.1 in.)/min at a range of 10^{-11} amp/mv, and the signal was usually attenuated by a factor of 2–64.

Under these operating conditions, a 1-μl injection[5] of an extracted standard solution of 10 μg/ml each of phenytoin and II produced peak height responses of about one-half to three-fourths full scale. Retention times of the methylated phenytoin and II were 8 and 12 min, respectively. There was no trap for any trimethylamine eluted from the GLC column since the entire effluent passed through the flame detector.

Calibration—Standard solutions were prepared from dry chemicals without further purification. The melting point and IR spectra[13] were determined for phenytoin and were acceptable evidence of purity. Stock solutions of phenytoin, 1 mg/ml, and II, 250 μg/ml, were prepared in anhydrous methanol and maintained in a freezer at −15° for no more than 6 months. No apparent loss of activity was noted over this time.

Standard solutions of phenytoin were freshly prepared from aliquots of the stock solution and were evaporated to dryness under nitrogen at room temperature. These samples were redissolved in 1% (w/v) methanol in pH 7.4 aqueous buffer, so that the ionic strength of the final solution was 0.16. Multiple samples were extracted in less than 1 hr. A 2-μg/ml solution of II in chloroform, the extracting solvent for phenytoin, was maintained at −15°; unused portions were discarded after 30 days.

[3] Wrist-action shaker, Burrell Corp., Pittsburgh, Pa.
[4] Damon/IEC HT centrifuge, Damon Corp., Needham Heights, Mass.
[5] Hamilton Co., Reno, Nev.
[6] Southwestern Analytical Chem., Austin, Tex.
[7] H5 Mettler balance, Mettler Instrument Corp., Hightstown, N.J.
[8] Model 2100, Varian Aerograph, Los Altos, CA 94002.
[9] OV-17, Varian Aerograph, Los Altos, CA 94002.
[10] HP Chromosorb W, Varian Aerograph, Los Altos, CA 94002.
[11] HT9, Applied Science Laboratories, State College, Pa.
[12] Model A-25, Varian Aerograph, Los Altos, CA 94002.
[13] Model 457 grating IR spectrophotometer, Beckman Instruments, Fullerton, Calif.

Table II—Average Solubility[a] for Six Samples of Phenytoin in Buffered (pH 7.4) Methanol at 25 and 37°

Temperature	Methanol, % (w/v)				
	0	0.5	1.0	2.0	4.0
25°	20.6 (1.3)	21.2 (1.0)	21.8 (0.8)	24.2 (0.5)	27.7 (0.3)
37°	30.6 (0.9)	32.2 (0.8)	34.2 (0.6)	36.4 (0.3)	38.6 (0.3)

[a] Values are micrograms per milliliter with the standard deviation given in parentheses.

A series of standard solutions, 1–50 μg/ml, were extracted, completely dried, and kept in stoppered centrifuge tubes at room temperature for up to 6 months without any apparent loss of sample activity. These dried standards permitted ready calibration of the gas chromatograph. Several sets of standard solutions were analyzed; the chromatograms were linear with respect to peak height ratios of phenytoin to II up to 100 μg/ml, with reproducibility of less than 5% error. A single 5-ml extraction of a 100-μg/ml phenytoin solution was examined for completeness of the extraction procedure. Samples of the separate aqueous and chloroform layers were evaporated to dryness, and direct comparison demonstrated a >99% extraction.

Solubility—Methanolic solutions for the determination of phenytoin solubility were prepared using pH 7.4 phosphate buffer with an ionic strength of 0.16. Determination of the apparent pH values[14] for these 0.5, 1, 2, and 4% methanol solutions demonstrated no significant pH shifts from the pH measured in the buffer alone.

A series of pH 5.6–8.4 phosphate buffers with an ionic strength of 0.16 were prepared for the determination of the solubility and the dissociation constant of phenytoin at 25°. Similarly prepared solutions at pH 5.4 and 7.4 were used to determine the change in phenytoin solubility with temperature over 25–50°.

The saturated solubility of phenytoin in alcohols and buffers was attained using a previously described procedure (28). The solute, in an amount in excess of its solubility (about 100 mg), was placed in polytef[15]-lined screw-capped glass vials with each solvent system. The vials were closed tightly and rotated at 28 rpm in a large constant-temperature (±0.2°) water bath[16] maintained successively at 50, 44, 37, 30, and 25°. After at least 24 hr of continuous rotation, a time sufficient to obtain saturated solutions at 25°, each sample vial was removed in succession for assay.

The exterior of the vial was quickly dried, and the cap was unscrewed carefully to prevent water contamination. A 2-ml sample of the saturated solution was drawn into a glass syringe and filtered into a small glass vial through a cellulose acetate filter with a pore size of 0.22 μm[17]. A 1-ml sample of this filtrate was placed in a tared 50-ml centrifuge tube, the total weight was recorded, and the filtrate was extracted. All glass syringes, pipets, and filter apparatus was prewarmed to prevent drug precipitation.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to phenytoin by the height of the peak due to II. A peak height adjustment was made using the weights of the aqueous and chloroform layers. Calibration curves were constructed from the results of extracted standards by plotting the concentration of phenytoin in micrograms per gram against the respective adjusted peak height ratios.

RESULTS AND DISCUSSION

Phenytoin is more soluble in alkaline solutions (Table I). The total solubility, S, can be expressed as a function of the hydrogen-ion concentration, solubility of the unionized form, S^0, and the dissociation constant, K_a:

$$S = S^0 + P^- = S^0 + K_a S^0/[\mathrm{H}^+] \quad \text{(Eq. 1)}$$

Krebs and Speakman (29, 30) transformed this equation into the more customary pH and pKa notation:

$$\log(S/S^0 - 1) = \mathrm{pH} - \mathrm{pKa} \quad \text{(Eq. 2)}$$

[14] Expandomatic SS-2, Beckman Instruments, Fullerton, Calif.
[15] Teflon (du Pont).
[16] Portatemp, Precision Scientific, Chicago, Ill.
[17] Millipore Corp., Bedford, Mass.

Table III—Average Solubility for Six Samples of Phenytoin as a Function of Temperature

pH	Temperature °C	°K	$1/T$, °K^{-1}	Solubility, μg/g (*SD*)	Mole Fraction × 10^6
5.4	25.4	298.5	0.00335	19.4 (1.0)	1.4
	30.2	303.3	0.00330	23.9 (1.7)	1.7
	37.2	310.3	0.00323	30.1 (2.1)	2.2
	44.7	317.8	0.00315	43.4 (2.3)	3.1
	50.3	323.4	0.00309	50.4 (2.8)	3.6
7.4	25.2	298.3	0.00335	20.5 (1.1)	1.5
	30.1	303.2	0.00330	24.6 (1.7)	1.8
	37.1	310.2	0.00322	31.0 (2.2)	2.2
	44.1	317.2	0.00315	45.6 (2.5)	3.3
	49.7	322.8	0.00310	55.1 (3.0)	4.0

so that a plot of pH against the logarithmic term gives a straight line intersecting the pH axis at the pKa. The value of the pKa is described by this method as an apparent dissociation constant, pKa′, since the activity coefficients are unknown. From the data plotted in Fig. 1, a value of 8.06 for the pKa′ was obtained. This pKa′ differs slightly (8.3) from that reported by Agarwal and Blake (4), but their methods and materials differed and the temperature was not specified.

Table II contains the data for the solubility of phenytoin in methanolic solutions at 25 and 37° and shows the increase in phenytoin solubility with increasing alcohol concentration. The data also show the expected increase in solubility with temperature for all concentrations in methanol. The phenytoin could not be redissolved easily or quickly after extraction without using methanol. The reasons for this finding are not fully apparent, but one possible mechanism is solvate formation by phenytoin and methanol. A 1% (w/v) methanol solution in buffer was chosen for all studies to ensure a phenytoin concentration in the range of up to 20 μg/ml.

The solubility of phenytoin is quite low in aqueous buffered solutions (μ = 0.16), and the results at 25° (Table I) agree with those of Glazko and Chang (6). Several studies with phenytoin, *e.g.*, protein binding, reported concentrations of the drug exceeding the aqueous solubility and, in fact, used hydroalcoholic solutions of the drug without considering the effects of the alcohol on the binding process (31–33).

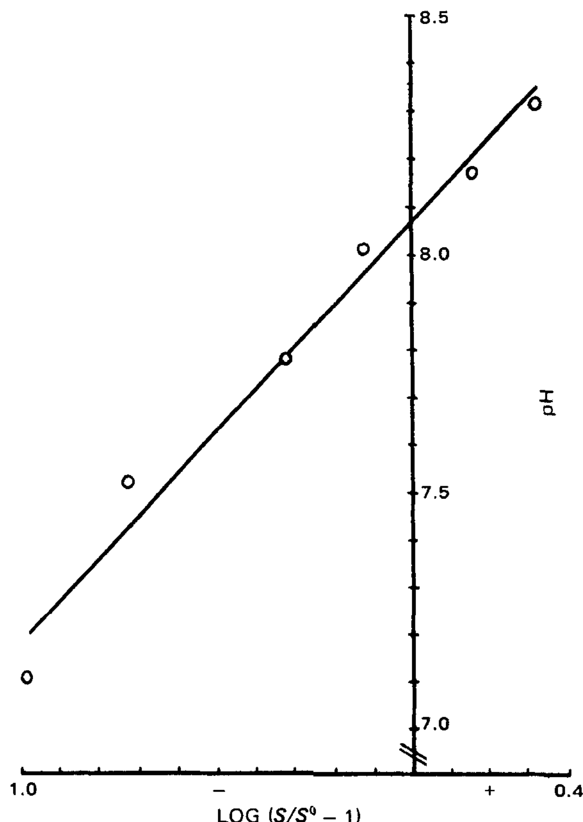

Figure 1—*Plot of log*(S/S^0 – 1) *for phenytoin as a function of pH,* r = *0.977. The intercept on the pH axis is the apparent pKa.*

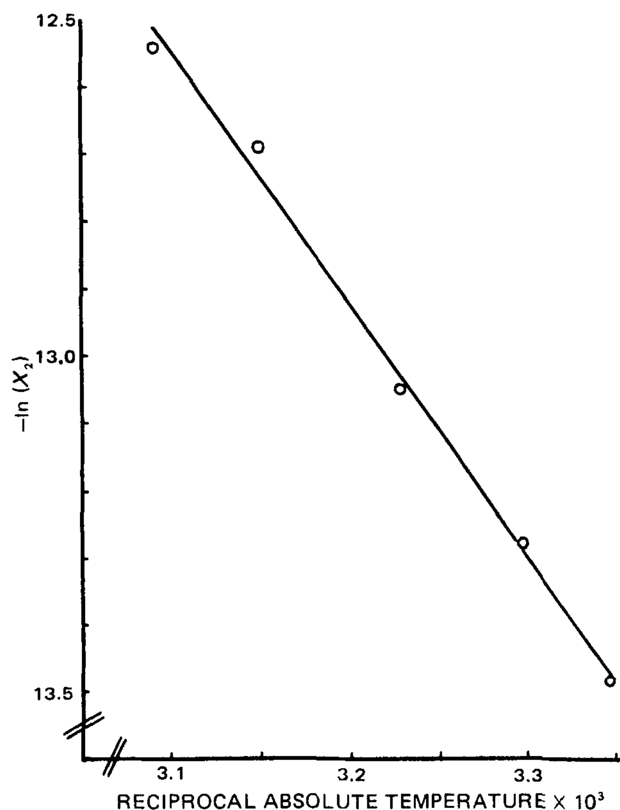

Figure 2—*Van't Hoff plot for solubility of phenytoin at pH 5.4,* r = *0.995.*

The data for the phenytoin solubilities at pH 5.4 and 7.4 at five temperatures (Table III) are expressed as micrograms per gram and were converted to mole fractions from the appropriate specific gravity of the solutions. Specific gravities were determined by a pycnometer; in all cases, the difference in weight per milliliter between samples and reference was less than 1%. This procedure allowed for the determination of thermodynamic relationships that do not require corrections for the cratic effect, *i.e.*, volume changes on mixing (34). Values for the enthalpy and entropy of solution were determined from the slope and intercept of the plot of log mole fraction solubility against the reciprocal absolute temperature, as shown in Fig. 2 for pH 5.4 (35).

The literature does not apparently contain any previous reports on the thermodynamic functions for phenytoin. The enthalpy of fusion was determined[18], using samples of phenytoin from this laboratory, to be approximately 47.5 kjoules/mole (11.3 kcal/mole) by differential scanning calorimetry. The entropy of fusion was calculated as 84 joules/kelvin (20 cal/mole kelvin) using the melting point of 294° found for this sample.

The enthalpy of solution is positive, as would be expected for nonelectrolytes with low aqueous solubility. The entropy of solution is negative, since the highly ordered crystal is dispensed into smaller (disordered) units in solution, a process accompanied by an entropy increase. However, the nonpolar phenyl groups of the drug may change the structure of water surrounding these groups (36) into a more ordered arrangement, accounting for a negative entropic effect that outweighs the positive entropy associated with the breakdown of the crystal lattice. Thermodynamic functions derived from the system at pH 5.4 and 7.4 are: enthalpy, 31.3 (7.5) and 33.6 (9.0) kjoules/mole (kcal/mole), respectively; and entropy, −7.1 (−1.7) and −1.4 (−0.3) joules/mole kelvin (cal/mole kelvin), respectively. The acid was essentially unionized (>99%) at pH 5.4, while the data at pH 7.4 (>80% unionized) represent both ionized and unionized species.

Investigators must have a clear understanding of the physicochemical characteristics of phenytoin. Because of its limited solubility, hydroalcoholic solutions have been used in the past to broaden the experimental range of concentrations. Alcohol would affect *in vitro* binding experiments; studies intended to be of clinical relevance should be conducted at physiologic temperature, pH, and ionic strength in aqueous buffer.

[18] At Upjohn Laboratories.

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Determination of Acenocoumarol in Plasma and Urine by Double Radioisotope Derivative Analysis

Y. Le ROUX ˣ and J. RICHARD

Abstract □ Acenocoumarol, to which a ^{14}C-labeled internal standard has been added, is extracted at pH 4 into ethyl acetate–heptane (20:80 v/v), back-extracted into aqueous sodium hydroxide solution after solvent washing with a pH 7 buffer, and reextracted after acidification in the solvent mixture. It is then acetylated with ^{3}H-acetic anhydride. The acenocoumarol acetyl derivative is separated from the metabolite derivatives by TLC, and its radioactivity is measured. The method is specific and sensitive to a concentration of 8 ng/ml.

Keyphrases □ Acenocoumarol—double radioisotope derivative analysis, plasma and urine □ Radiochemistry—double radioisotope derivative analysis of acenocoumarol in plasma and urine □ Anticoagulants—acenocoumarol, double radioisotope derivative analysis in plasma and urine

Acenocoumarol (I) is an oral coumarin anticoagulant[1]. It is analyzed in biological fluids using the O'Reilly *et al.* (1) method. This technique was originally developed for the determination of another coumarin anticoagulant, warfarin, which was then administered in much higher doses than acenocoumarol. It is a simple UV absorption assay, performed after solvent extraction; its sensitivity is limited to 1 μg/ml, and its specificity has not been verified. Nevertheless, it has been used for the determination of acenocoumarol (2). Two other methods, neither of which is more sensitive, also were reported (3, 4). Recently, a fluorometric assay after TLC was reported that is more sensitive (0.2 μg/ml) (5).

The metabolism of acenocoumarol was only recently elucidated[2]. Several metabolites were found: 3-[α-(4′-aminophenyl)-β-acetylethyl]-4-hydroxycoumarin (III), 3-[α-(4′-acetamidophenyl) -β- acetylethyl] -4- hydroxycoumarin (IV), two diastereoisomers of the alcohol 3-[α-(4′-nitrophenyl)-γ-hydroxybutyl]-4-hydroxycoumarin (V), 3-[α-(4′-nitrophenyl)-β-acetylethyl] -4,7- dihydroxycoumarin (VI), and 3-[α-(4′-nitrophenyl)-β-acetylethyl]-4,6-dihydroxycoumarin (VII).

All attempts to elaborate a GLC procedure for aceno-

[1] Sintrom, Ciba-Geigy.

[2] W. Dieterle *et al.*, to be published.