Table I—Intra- and Interassay Relative Standard Deviations for Two Concentrations of Chlorambucil and Isocyanate of Chlorambucil

Assay	Conc., μg/mL	Mean of Fluorescence	*RSD*, % Intra-assay	*RSD*, % Interassay
Chlorambucil				
1	2	11.9 ± 0.854	7.17	
2	2	11.6 ± 1.25	10.7	9.25
3	2	11.5 ± 1.08	9.39	
4	2	9.63 ± 0.479	4.97	
1	5	48.2 ± 1.41	2.92	
2	5	52.1 ± 1.93	3.70	8.79
3	5	58.0 ± 1.73	2.98	
4	5	57.8 ± 2.66	4.60	
Isocyanate of Chlorambucil				
1	2	20.3 ± 1.60	7.88	
2	2	20.3 ± 1.20	5.91	
3	2	13.6 ± 0.980	7.21	21.1
4	2	14.5 ± 1.17	8.07	
1	5	51.1 ± 1.65	3.23	
2	5	50.0 ± 1.73	3.46	
3	5	48.9 ± 1.03	2.11	5.25
4	5	45.2 ± 1.16	2.57	

limit of sensitivity of 0.1 μg/mL found with this method was 50-fold lower than the one obtained with the nitrobenzylpyridine method (5).

Reproducibility of the Assay—We found intra-assay *RSD* of 8.1 and 3.6%, respectively, at 2 and 5 μg/mL. The interassay *SD* was determined on four fluorescence measurements at 2 and 5 μg/mL and we found *SD* of 9.3 and 8.8%, respectively. The intra- and interassay relative standard deviations were also obtained with the isocyanate derivative of chlorambucil at the same concentrations (Table I).

Fluorophore Stability—Contrary to the nitrobenzylpyridine assay, the fluorescence could be read several hours after the photoactivation of the samples by UV. When the fluorophore produced was kept in the dark, it was stable for at least 24 h. We found a variation of only 5% in the fluorescence during the 24-h period at room temperature. This is an advantage over the nitrobenzylpyridine method, which yields a highly unstable chromophore.

DISCUSSION

The method presented above offers many advantages over the existing method for measuring alkylating activity. This fluorescence method is at least 50 times as sensitive as the nitrobenzylpyridine colorimetric procedure proposed by Balazs *et al.* (5). The interassay relative standard deviations given may represent the variations in the intensity of the UV lamp and the daily variations in the fluorometer light source; these deviations are much lower when a standard curve is used for each assay. This assay may be performed in protein-containing solutions, in contrast to the other assays available which require incubation at 85°C and cause protein precipitation.

Since alkylating activity is measured in this assay, it is essential that the assay is performed with an active alkylating agent; therefore, chlorambucil must be kept in a desiccator to prevent hydrolysis and formation of the hemimustard. The method proposed above measures the alkylating activity of chlorambucil; it should be useful for studying the metabolism of this compound in biological fluids.

REFERENCES

(1) O. Klatt, A. C. Griffin, and J. S. Stehlin, Jr., *Proc. Soc. Exp. Biol. Med.*, **104**, 629 (1960).

(2) H. G. Petering and G. J. Van Giessen, *J. Pharm. Sci.*, **52**, 1159 (1963).

(3) A. M. Agree and R. L. Meeker, *Talanta*, **13**, 1151 (1966).

(4) R. Truhaut, E. Delacoux, G. Brûlé, and C. Bohuon, *Clin. Chim. Acta*, **8**, 235 (1963).

(5) M. K. Balazs, C. A. Anderson, R. H. Iwamoto, and P. Lim, *J. Pharm. Sci.*, **59**, 563 (1970).

(6) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**(9), 1435 (1955).

(7) M. A. Chirigos and J. A. R. Mead, *Anal. Chem.*, **7**, 259 (1964).

(8) R. C. Gaudreault, M.Sc. Thesis, Université Laval, Québec, 1979.

(9) R. C. Gaudreault, Ph.D. Thesis, Université Laval, Québec, 1983.

ACKNOWLEDGMENTS

This work was supported by the Fondation Dr. Georges Phénix with a studentship to L. G. Bernier and by the C.R.S.Q. with a studentship to R. C. Gaudreault. The technical assistance of Pierre De Grandpré is acknowledged.

Quantitation of Phenobarbital and Phenobarbital Sodium in Pharmaceutical Dosage Forms

V. DAS GUPTA

Received April 11, 1983, from the *Department of Pharmaceutics, University of Houston, Houston, TX 77030.* Accepted for publication July 29, 1983.

Abstract □ A reverse-phase high-performance liquid chromatographic method for the quantitation of phenobarbital and phenobarbital sodium in pharmaceutical dosage forms (elixir, injection, and tablets) was developed. The method is precise and accurate with percent relative standard deviations of 0.9 (without an internal standard) and 0.7 (with an internal standard) based on six injections. The method is stability indicating and is more sensitive than the revised USP–NF method. The products of decomposition showed two new peaks in the chromatogram.

Keyphrases □ Phenobarbital—quantitation with phenobarbital sodium in pharmaceutical dosage forms □ Phenobarbital sodium—quantitation with phenobarbital in pharmaceutical dosage forms

Phenobarbital (I) and phenobarbital sodium (II), extensively used as sedatives, are available in a variety of dosage forms, including capsules, elixir, injectable, tablets, *etc.* The literature for the quantitation of phenobarbital has been surveyed by Schmidt and Pennington (1). According to this report, investigations in which reverse-phase HPLC have been used without the addition of a counterion have not been successful. A reverse-phase HPLC assay procedure for phenobarbital with 0.01 M tetramethylammonium chloride as the counterion has been reported (1); however, the use of counterions usually shortens column life (2).

The revised USP–NF method for the quantitation of phenobarbital appeared in USP Supplement 3 (3), and use of a mobile phase of pH 4.5 is recommended.

The purpose of this investigation was to develop a stability-indicating assay method for the quantitation of phenobarbital and phenobarbital sodium in pharmaceutical dosage forms at neutral or weakly basic pH without the addition of a counterion to the mobile phase.

EXPERIMENTAL SECTION

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS quality and used without further purification. The USP-quality powders of phenobarbital[1] and phenobarbital sodium[1] were used as received.

[1] American Chemical and Drug Co., Los Angeles, Calif.

0022-3549/84/0800-1158$01.00/0

Table I—Assay Results of Dosage Forms

Type of Dosage Form	Active Ingredient	Percent of Label Claim Found 230 nm[a]	245 nm[b]
Elixir			
Lot 1	I (4 mg/mL)	99.0	99.0
Lot 2	I (4 mg/mL)	99.8	100.2
Lot 3	I (4 mg/mL)	99.2	99.3
Injection			
Lot 1	II (65 mg/mL)	100.8	100.8
Lot 2	II (130 mg/mL)	99.4	99.6
Lot 3	II (65 mg/mL)	100.0	100.1
Lot 4	II (130 mg/mL)	101.2	101.0
Tablets			
Lot 1	I (15 mg)	98.8	98.9
Lot 2	I (30 mg)	100.2	99.9
Lot 3	I (60 mg)	100.0	100.4
Lot 4	I (100 mg)	99.2	99.2
Lot 5	I (16.2 mg) With 10.8 mg of belladonna extract per tablet	100.1	100.5
Lot 6	I (30 mg)	98.8	99.1
	Recovery Data of Synthetic Mixtures		
Mixture			
1	I (60 mg/200 mg of lactose)	99.8	—[c]
2	II (80 mg/300 mg of lactose)	100.0	—[c]
3	I (4 mg/mL)[d]	100.9	—[c]
Decomposed sample			
1	~4 min boiling	78.2%	—[c]
2	~30 min boiling	15.4%	—[c]

[a] With internal standard. [b] Without internal standard. [c] Not determined at this wavelength. [d] In aqueous solution containing 15% ethanol (v/v) and 45% glycerol (v/v).

Apparatus—The chromatograph[2] was equipped with a multiple-wavelength detector[3], a recorder[4], and a digital integrator[5]. A semipolar column[6] (30 cm × 4 mm i.d.) was used. The mobile phase contained 35% (v/v) of methanol in 0.02 M aqueous ammonium acetate. The flow rate was 2.0 mL/min, and the temperature was ambient. The sensitivity was set at either 0.2 (245 nm) or 0.1 (230 nm), and the chart speed was 30.5 cm/h.

Preparation of Stock and Standard Solutions—The stock solutions of phenobarbital (2.0 mg/mL in methanol), phenobarbital sodium (2.0 mg/mL in water) and the internal standard, mezlocillin sodium[7] (10.0 mg/mL in water) were prepared fresh daily. The standard solutions were prepared by diluting the appropriate quantities of stock solutions with water, methanol, or a mixture of both, as needed. All standard solutions of phenobarbital sodium were diluted with water. The standard solution of phenobarbital for assays at 230 nm contained 10% methanol (final concentration, v/v) in water, and pure methanol was used for diluting all solutions for assays at 245 nm. No internal standard was used for assays at 245 nm due to very poor absorbance at this wavelength. For assays at 230 nm, the concentration of the internal standard was 600.0 μg/mL.

Preparation of Assay Solutions—For assay at 230 nm, an appropriate quantity (3.75 mL) of phenobarbital elixir USP (4.0 mg/mL) was mixed with 10 mL of methanol and 6.0 mL of stock solution of the internal standard and brought to volume with water (100 mL). For assay at 245 nm, 6.25 mL of elixir was diluted to 25.0 mL with methanol.

Injections—An appropriate quantity of the injection of phenobarbital sodium was diluted with water to contain either 0.15 or 1.0 mg/mL of the drug for assays at 230 and 245 nm, respectively. The solution for assay at 230 nm also contained 0.6 mg/mL of mezlocillin sodium (internal standard).

Tablets—An appropriate quantity of the fine powder representing 200.0 mg of the phenobarbital powder was thoroughly mixed with 80 mL of methanol and shaken for 5 min. The mixture was brought to volume (100 mL) with methanol and filtered[8]. The first 15 mL of the filtrate was rejected and then collected for assay. For assays at 230 nm, 7.5 mL of the clear filtrate was mixed with 2.5 mL of methanol and 6.0 mL of the internal standard stock solution and brought to volume (100 mL) with water. For assays at 245 nm, a 12.5-mL quantity of the clear filtrate was diluted to 25 mL with methanol.

Assay Solutions of Decomposed Phenobarbital Sodium—A 30.0-mg sample of phenobarbital sodium powder was mixed with ~2 mL of ~1 M NaOH solution in a 150-mL beaker. The mixture was boiled on a hot plate for (*a*) ~4 min and (*b*) ~30 min; additional water was added as needed, and the mixture was cooled to room temperature and neutralized (pH ~6–8) with ~4 mL of ~0.5 M HCl. A 12.0-mL quantity of the stock solution of the internal standard was added, and the mixture was brought to volume (200 mL) with water. These solutions were assayed only at 230 nm.

Chromatographic Procedure—A 20-μL aliquot of the assay solution was injected into the chromatograph at the conditions described (solutions with internal standard, 230 nm; others, 245 nm). For comparison, an identical volume of the standard solution containing an identical concentration of the drug was injected after the assay solution was eluted.

Calculations—Since the peak heights (ratio of the peak heights when internal standard was added) were directly related to concentrations, the results were calculated by the equation:

$$100 \times \frac{R_{pha}}{R_{phs}} = \text{percent of the label claim}$$

where R_{pha} is the peak height (ratio of the peak height of drug to internal standard for assays at 230 nm) of the assay solution and R_{phs} is the peak height of the standard solution.

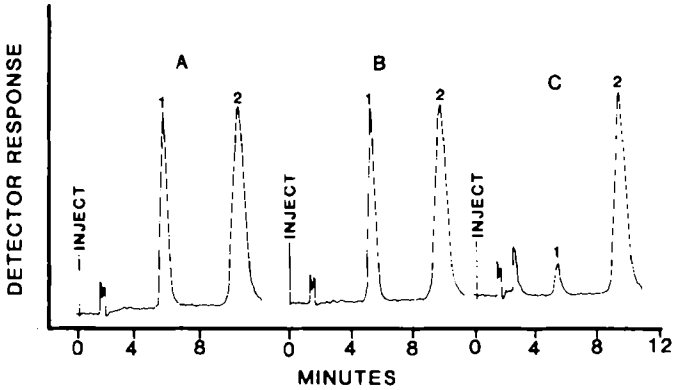

Figure 1—*Typical chromatograms at 230 nm. Peaks 1 and 2 are from phenobarbital sodium (II) and mezlocillin sodium (internal standard), respectively. Key: (A) standard solution; (B) injection containing 130 mg/mL of II; (C) decomposed solution (~30 min boiling, see text).*

[2] Model ALC 202 equipped with a U6K universal injector; Waters Associates, Milford, Mass.
[3] Spectroflow monitor SF770; Schoeffel Instrument Corp., Ramsey, N.J.
[4] Omniscribe 5213-12; Houston Instruments, Austin, Tex.
[5] Autolab minigrator; Spectra-Physics, Santa Clara, Calif.
[6] μ-Bondapak phenyl (catalog no. 27198); Waters Associates, Milford, Mass.
[7] Miles Pharmaceutical, West Haven, Conn.
[8] Whatman no. 1 filter paper.

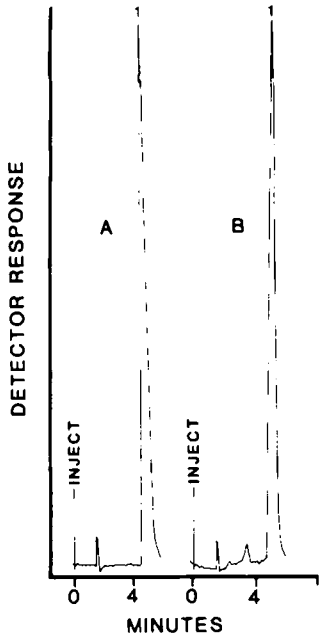

Figure 2—*Typical chromatograms at 245 nm. Peak 1 is from phenobarbital sodium (II). Key: (A) standard solution; (B) injection containing 130 mg/mL of II.*

RESULTS AND DISCUSSION

The assay results indicate (Table I) that the proposed HPLC method can be adopted for the quantitation of phenobarbital and phenobarbital sodium in pharmaceutical dosage forms. The method is accurate and precise. The percent relative standard deviations based on six injections were 0.9 and 0.7 at 245 and 230 nm (with internal standard), respectively. The internal standard was not used for assays at 245 nm due to poor absorption of mezlocillin at this wavelength. However, the results by both assay methods were similar (Table I). The concentrations *versus* peak heights were directly related in the 7- to 21-μg range at 245 nm. The ratio of peak heights (drug to internal standard) were directly related to drug concentrations at the 1.5- to 4.5-μg range at 230 nm. The method is ~1.7 times more sensitive at 230 than at 245 nm, as determined by using a standard solution (300 μg/mL in water) and a sensitivity of 0.1. Moreover, the method is at least two times more sensitive than the USP-NF method (3), as determined by UV spectrometry with the two mobile phases. The elixirs and injections can be simply diluted and assayed for phenobarbital and phenobarbital sodium. A simple preliminary extraction procedure is required to assay phenobarbital in tablets. There was no interference from excipients present in the tablets and the red colorant of the elixir (Table I).

The decomposed samples of phenobarbital sodium showed a new peak (peak 1, Fig. 1C) in the chromatogram, and the potency of the drug remaining intact was only 15.4% after ~30 min of boiling (see above). After ~4 min of boiling, there were two small peaks (side by side) in the chromatogram at about the same location as peak 1, Fig. 1C. The scheme of decomposition has been reported previously in the literature (4). It is interesting to point out that there were two additional peaks when the same lot of phenobarbital was assayed at 245 nm *versus* only one peak when assayed at 230 nm. It is perhaps due to the facts that (*a*) the concentration of excipients was 3.33 times higher (also considering AUFS of 0.2 at 245 nm and 0.1 at 230 nm) at 245 *versus* 230 nm and (*b*) there was poor or no absorption from one of the excipients at 230 nm. Furthermore, the same lot of phenobarbital sodium injection showed a small additional peak (Fig. 2B) when assayed at 245 nm *versus* almost none (Fig. 1B) when assayed at 230 nm.

REFERENCES

(1) W. F. Schmidt and L. J. Pennington, *J. Pharm. Sci.*, **71**, 954 (1982).

(2) R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, **15**, 413 (1977).

(3) Third Supplement to USP XX and to NF XV, The U.S. Pharmacopeial Convention, Rockville, Md., 1982, pp. 216–217.

(4) K. A. Connors, G. L. Amidon, and L. Kennon, "Chemical Stability of Pharmaceuticals—A Handbook for Pharmacists," Wiley, New York, N.Y., 1979, p. 280.

Mesophase Formation During Cholesterol Gallstone Dissolution in Human Bile: Effect of Bile Acid Composition

C. C. SU ‡, W. I. HIGUCHI *x, I. T. GILMORE §, R. G. DANZINGER ¶, and A. F. HOFMANN §

Received February 14, 1983, from the **College of Pharmacy, University of Utah, Salt Lake City, UT 84112*, ‡*College of Pharmacy, University of Michigan, Ann Arbor, MI 48105*, §*University of California at San Diego, San Diego, CA 92103, and* ¶*St. Boniface General Hospital, Winnipeg, Manitoba, Canada.* Accepted for publication July 8, 1983.

Abstract □ Duodenal bile obtained from patients with gallstones who were acutely infused with chenodeoxycholic acid, ursodeoxycholic acid, or cholic acid were examined for the propensity toward the formation of a liquid crystalline mesomorphic phase when cholesterol gallstones were incubated in these bile acids. Bile taken from patients infused with ursodeoxycholic acid was found to be enriched in ursodeoxycholic acid; mesophase formation was detected in these samples but not in bile from patients infused with chenodeoxycholic acid or cholic acid.

Keyphrases □ Cholesterol—gallstone dissolution in human bile, effect of bile acid composition □ Gallstone dissolution—human bile, effect of bile acid composition □ Bile acids—mesophase formation during cholesterol gallstone dissolution

Clinical investigations have shown that ursodeoxycholic acid is as effective or more effective than chenodeoxycholic acid in gallstone dissolution. From the physicochemical standpoint, these results are interesting, because ursodeoxycholic acid (1) and its conjugates are generally not as effective as chenodeoxycholic acid and its conjugates in solubilizing cholesterol in the bile acid or the bile acid–lecithin solution (2). We have recently suggested (2, 3) that during *in vitro* dissolution of cholesterol monohydrate in the form of powder, compressed disks, single crystals, or in gallstones, a transition from the micellar phase to the liquid crystalline phase occurs when the dissolution media contain lecithin and predominantly ursodeoxycholic acid conjugates, but not chenodeoxycholic acid or cholic acid conjugates.

It was shown that mesophase formation and subsequent dispersion may contribute to the total dissolution/disintegration of cholesterol in the solid phase. To determine whether mesophase formation would occur during the *in vitro* disso-

0022-3549/84/0800-1160$01.00/0