Fluorescence Determination of Microconcentrations of Chlorambucil after Photoactivation

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Received March 9, 1983, from the *Department of Biochemistry, Faculty of Medicine and the ‡School of Pharmacy, Université Laval, Québec, G1K 7P4, Canada. Accepted for publication May 20, 1983.

Abstract □ A fluorescence assay is described which measures the alkylating activity of chlorambucil or its isocyanate derivative after photoactivation in the presence of dimethyl sulfoxide. This assay has a lower limit of sensitivity of 100 ng/mL and *RSD* of <10% for chlorambucil. The method requires <5 μg of alkylating agent and the fluorophore produced is stable for at least 24 h.

Keyphrases □ Chlorambucil—isocyanate derivative, alkylating activity, fluorescence assay □ Nitrogen mustards—chlorambucil and its isocyanate derivatives, alkylating activity, fluorescence assay

Bifunctional alkylating agents are used extensively in the chemotherapeutic treatment of neoplastic and autoimmune diseases. Several colorimetric methods for the determination of specific nitrogen mustards have been described (1–5). These methods are based on the first report by Epstein *et al.* (6) who first showed the reaction of alkylating agents with nitrobenzylpyridine. A fluorometric method has also been described (7) which has a greater sensitivity, but it was not applicable to chlorambucil.

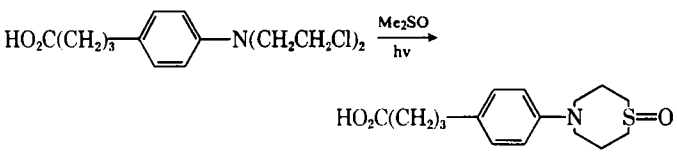
Gaudreault (8) reported that chlorambucil dissolved in dimethyl sulfoxide and exposed to UV light yielded a mixture of products. The fluorescence of the mixture was proportional to the amount of chlorambucil present. Perhydrothiazocine was the most important fluorophore; its structure was determined by mass spectrometry (8).

We describe here the use of this reaction (Scheme I) for the quantitative determination of chlorambucil. This method is sensitive while requiring little material.

EXPERIMENTAL SECTION

Chlorambucil[1] was dissolved in dimethyl sulfoxide[2]. The isocyanate of chlorambucil, 3-{4-[bis(2-chloroethyl)amino]phenyl}propyl-isocyanate, was produced in-house using a literature method (9).

A 200-W UV lamp[3] supplied by a power unit[4] was used for the photoactivation. A UV meter[5] was used to check the UV lamp periodically. Quartz cuvettes[6] (10-mm path length) were placed circularly at 10 cm around the lamp to expose the samples; the fluorescence was then read in the same cuvettes. A spectrophotofluorometer[7] was used to measure the fluorescence of the samples at 434 nm for emission after an excitation at 358 nm.



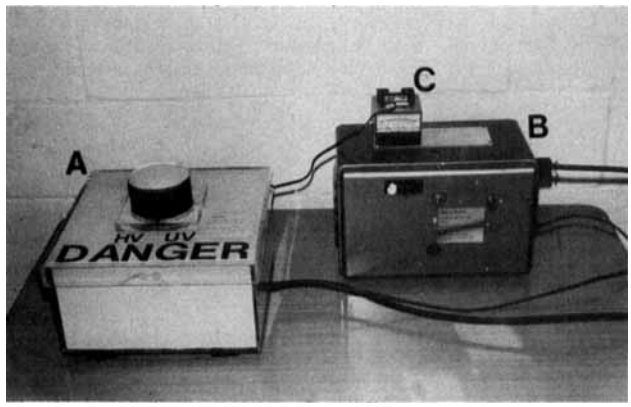
Scheme I

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Spectrophotometric grade; Aldrich Chemical Co., Milwaukee, Wis.
[3] Super Pressure Mercury Lamp HBO; OSRAM Gmb, Berlin-Munich, F.R.G.
[4] Model HB200 Mercury Burner Power Unit; Otto Watzka & Co., Montreal.
[5] Model J-225; Blak-Ray, San Gabriel, Calif.
[6] Fluorescence Open Cuves; Fisher Scientific Co.
[7] Model 4-0202; Aminco-Bowmann, Silver Spring, Md.

Chlorambucil (1 mg/mL) was dissolved in dimethyl sulfoxide and stored at −20°C until use. Standards from 0.1 to 20 μg/mL were freshly prepared by dilution of the stock solution with dimethyl sulfoxide. Two milliliters of the standard was placed in quartz cuvettes and the background fluorescence was read. Samples were placed for 30 min in the UV illuminator (Fig. 1). They were then allowed to cool for 20 min and the fluorescence was read again. The difference in the fluorescence was plotted against the concentration of chlorambucil. The method was also applied to the isocyanate of chlorambucil.

RESULTS

Standard Curves—Following the assay procedure described above, we found a linear relationship between the fluorescence and chlorambucil concentrations varying from 0.1 to 1 μg/mL ($r = 0.986$) and from 1 to 20 μg/mL ($r = 0.999$). We also found a very good linear relationship using the isocyanate of chlorambucil ($r = 0.999$) for concentrations varying from 1 to 10 μg/mL. The lower



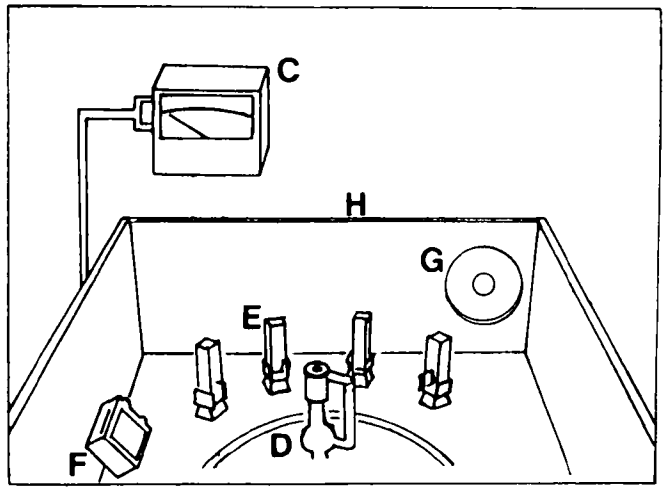


Figure 1—*UV illuminator for the photoactivation of chlorambucil. Key: (A) illuminator; (B) power unit; (C) UV meter; (D) UV lamp; (E) quartz cuvettes; (F) UV detector; (G) ventilator; (H) fiberglass box covered on the inside surface with reflecting material.*

0022-3549/84/0800-1157$01.00/0

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.

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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

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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.

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