Simultaneous Spectrophotometric Determination of Diphenylhydantoin and Phenobarbital in Biologic Specimens

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A rapid, accurate method for determining diphenylhydantoin and phenobarbital in a single biologic sample is described. Separation of the two drugs after their initial chloroform extraction from the specimen is not required. After extraction, diphenylhydantoin is converted to benzophenone by permanganate oxidation. Phenobarbital is determined by differential absorption between pH values of 12.0 and 10.5. Final quantitation in each case is carried out by ultraviolet spectrophotometry. Barbiturates do not interfere with the diphenylhydantoin analysis or vice versa. The method is sensitive enough for detecting subtherapeutic amounts of each compound with a high degree of specificity.

The ability to rapidly determine therapeutic concentrations of diphenylhydantoin and phenobarbital when concomitantly present in small amounts of biologic specimens has long been needed in laboratory medicine. A requirement exists for concentrations of the drugs in blood during the treatment of numerous types of neurologic disorders. Determination of the two drugs has been described in several reports (1–3). Gas chromatographic methods (4, 5) are available, but they are time-consuming when applied to routine screening situations. Sandberg et al. (5) recently described a gas chromatographic technic for analysis of serum diphenylhydantoin levels. The method requires conversion of the drug to methoxydiphenylhydantoin by reacting the residue from a chloroform extract with ethereal diazomethane, a compound of known toxicity and explosive characteristics. The analysis lacks an element of specificity which is inherent in gas chromatographic technics.

Procedures which utilize ultraviolet spectrophotometry for determining unconverted diphenylhydantoin in the presence of phenobarbital...

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are sensitive, yet require elaborate extraction technics. In our hands, they give incomplete separation of the two drugs which results in a significant degree of variability in the final results. Procedures for determining diphenylhydantoin by means of oxidation to benzophenone (6–8) have been reported. With these methods it has been established that phenobarbital, which is oxidized to nonultraviolet-absorbing compound(s), does not interfere with the ultraviolet determination of diphenylhydantoin. As far as this author can determine, the reliability of analyzing phenobarbital by ultraviolet spectroscopy in the presence of hydantoin derivatives has not been examined previously.

Combination of the Goldbaum procedure for analyzing barbiturates (9) with the permanganate oxidation procedure for determining diphenylhydantoin (8) has resulted in an analytic scheme which permits simultaneous determination of both drugs in small amounts of biologic materials. Separation or extensive purification of the two drugs from a chloroform extract of the biologic specimen is not required.

**Methods and Materials**

**Reagents**

*Ammonium chloride buffer* Dissolve 16 g of NH₄Cl in 100 ml of distilled water.

*Sodium hydroxide, 0.45 N* Add 1.8 g of NaOH (J. T. Baker Chemical Co., No. 3726 or equivalent) to 1 L of distilled water. The NaOH solution is adjusted so that when 0.1 ml of 16% NH₄Cl is added to 0.6 ml of the base the resulting pH is 10.5 ± 0.2.

*Hydrochloric acid, 0.5 N* Pipet 42 ml of conc. HCl into 1 L of distilled water.

*Alkaline permanganate reagent* Carefully dissolve 28 g of NaOH, low in carbonate (J. T. Baker Chemical Co., No. 3726 or equivalent), in 100 ml of distilled water. After the solution has cooled, add 1 g of potassium permanganate (Mallinckrodt Chemical Works, No. 7068 or equivalent).


**Apparatus**

Any spectrophotometer capable of accurate measurements at 245 mµ is adequate for use in the determination. In this study a Beckman DK-2A ratio-recording spectrophotometer was used for the ultraviolet absorption measurements. The cells used throughout the study were 10 mm in path length.
Procedure

The volumes of 5–8 ml of whole blood, serum, plasma, or urine are adjusted to a pH between 6.0 and 7.5 by the dropwise addition of either 0.5 N HCl or 0.45 N NaOH, and placed in a 250-ml separatory funnel to which is added 100 ml of chloroform. This mixture is shaken vigorously for 3 min. The chloroform layer is removed by filtration through Whatman No. 541 or equivalent filter paper into a graduated cylinder, and the volume of recovered chloroform is recorded for use in the final calculations. To the chloroform is added 5 ml of 0.45 N NaOH, and the mixture is shaken for 3 min. After separation, the NaOH layer is removed. Care should be taken to recover as much of the alkaline layer as possible. In each of two microcuvets (Scientific Glass Apparatus Co., Inc., No. S-4821-2, Type IS or equivalent) of 1.0-ml capacity is placed 0.6 ml of the aqueous layer. To one cuvet is added 0.1 ml of 0.45 N NaOH, and to the other, 0.1 ml of 16% NH₄Cl. The difference in absorbance between the two solutions at 260 mμ is used to calculate the phenobarbital concentration (10).

Of the remaining aqueous alkaline extract, 3 ml is placed in a 250-ml round-bottom flask, which is then connected to a vacuum rotary assembly. With vacuum applied, the flask is rotated in a water bath at 50–60° until the volume is reduced to less than 1 ml; this is done to remove traces of chloroform. With a good vacuum this operation should require only 5 min. The flask is removed from the assembly, and 20 ml of KMnO₄ reagent and 5 ml of spectro-quality n-heptane are added.

The contents of the flask subsequently are refluxed by utilization of a water-cooled condenser and Glas-Col heating mantle (Glas-Col Apparatus Co., Inc., Terre Taute, Ind.) for 30 min. with constant magnetic stirring. After cooling, the n-heptane is removed from the permanganate solution by means of a transfer pipet. This is done with no difficulty since the two solutions will separate within the pipet. Decantation of the mixture from the reaction flask may result in contamination of the n-heptane with residual matter on the connecting area of the flask. The n-heptane is transferred to a cuvet and read in the spectrophotometer at 220–340 mμ against a blank solution of n-heptane. For the most accurate quantitative, as well as qualitative, results, the blank should be prepared from an equivalent amount of biologic sample known to contain no diphenylhydantoin. Typical values from 10 ml of oxalated whole blood which contains no diphenylhydantoin should not exceed an absorbance of 0.04. Serum and plasma give slightly lower absorbance values for blanks than does whole blood. If analysis at a single wavelength is required, determine the absorption at 247 mμ. A reference
curve for the diphenylhydantoin portion of the analysis is prepared from aqueous solutions of the drug oxidized directly by the permanganate reagent in the presence of n-heptane. For phenobarbital a reference curve is also established from aqueous solutions of the drug.

Results and Discussion

Ultraviolet absorption spectrums of diphenylhydantoin and its oxidized reaction product are shown in Fig. 1. The capability of the oxidation technic to determine diphenylhydantoin between 0 and 20 µg/ml is illustrated in Table 1. The same table shows similar data for the

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Fig. 1. Ultraviolet absorption spectrums of diphenylhydantoin in water and of the diphenylhydantoin reaction product in n-heptane, both corresponding to a diphenylhydantoin concentration of 10 µg/ml.
analysis of phenobarbital at 260 m. The specificity of the permanganate oxidation for determination of diphenylhydantoin has been described in a previous report (8). The extent to which diphenylhydantoin interferes with phenobarbital, when the latter is determined by difference in the absorption at 260 m, has not been reported in the scientific literature. To establish this relationship, two matched cuvets containing diphenylhydantoin solutions of equivalent concentration were adjusted to pH 12.0 and 10.5 and used as sample and reference cells, respectively. It was observed that concentrations of diphenylhydantoin up to 100 μg/ml afford less than 0.01 differential absorbance at 260 m. Figure 2 demonstrates the lack of interference by diphenylhydantoin in the analysis of phenobarbital and vice versa. In fact, high levels of diphenylhydantoin do not affect the determination of phenobarbital (Fig. 3), nor do large amounts of phenobarbital influence the absorbance.

Fig. 2. Ultraviolet absorption spectrums of diphenylhydantoin product (solid line) and phenobarbital (broken line) after extraction of the drugs from whole blood. From patient taking oral therapeutic doses of both drugs. Whole blood levels: phenobarbital 16.4 μg/ml; diphenylhydantoin 15.8 μg/ml.
tion curve of the diphenylhydantoin product (Fig. 4). The reproducibility of results was excellent (Table 2). With each drug, recoveries from whole blood were better than 90%.

The sensitivity of the method is such that as little as 4–5 ml of blood is adequate for determination of therapeutic levels of both compounds. Other acid-extractable drugs, with the exception of salicylates, exhibit no interference. The extraction of salicylates can be avoided by carefully adjusting the biologic specimen to a pH of 7.0–7.5 prior to the initial chloroform extraction. In contrast to diphenylhydantoin and phenobarbital, salicylates are not extracted into chloroform from aqueous solutions that are slightly basic.

In the opinion of the author, the method proposed in this report has significant advantages over other procedures for the simultaneous determination of diphenylhydantoin and phenobarbital. The proposed
Fig. 4. Ultraviolet absorption spectrums of diphenylhydantoin product (solid line) and phenobarbital (broken line) after extraction of the drugs from whole blood. From patient taking oral therapeutic doses of both drugs. Whole blood levels: phenobarbital 26.6 μg/ml; diphenylhydantoin 4.2 μg/ml.

Table 2. Recovery of Diphenylhydantoin and Phenobarbital Added to Whole Blood

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Average recovery:

- Diphenylhydantoin: 95.9%
- Phenobarbital: 90.6%

* Ten milliliters of blood assayed in each determination.
procedure is simple, specific, and readily applicable to the routine analysis of the two drugs. Elaborate extraction techniques necessary in all previously reported methods are not required. Consistent and reproducible results are obtained after repeated analysis of the same specimen.

References