ma or urine following administration of a single 50-mg oral dose to humans[11].

An *in vivo* study was conducted to confirm the applicability of the method. A plot of plasma levels of hydroflumethiazide following a single intravenous injection to a beagle dog is shown in Fig. 3. The curve shows a typical biphasic pattern, with a half-life of 10 min for the α-phase and of 90 min for the β-phase. The amount of drug excreted in a 24-hr urine collection was 8.6 mg or 57% of the dose. The reason for incomplete excretion is not known at present.

The analytical method was developed using human plasma and urine while the pilot experiment was conducted in dogs. However, dog plasma and urine were free of interfering impurities, as were the human fluids.

The procedure described in this paper is accurate and reproducible and has a high degree of sensitivity. It is quick and easy to carry out. One analyst can assay 20–25 plasma samples in a normal working day.

[11] Unpublished data.

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[x] To whom inquiries should be directed.

Diphenylhydantoin Microdetermination in Serum and Plasma by UV Spectrophotometry

JACK E. WALLACE [x] and HORACE E. HAMILTON

Abstract □ A modified UV spectrophotometric procedure for determining diphenylhydantoin in 0.1–0.2 ml of serum is described. Improvement of previously described techniques was accomplished by modifying the extraction conditions and refluxing assembly. Continuous extraction of the oxidized diphenylhydantoin provided the maximum yield of benzophenone, resulting in optimal sensitivity as well as reproducibility of analytical values. Reliable clinical assays are achieved on 0.1 ml of serum containing 0.5 μg of diphenylhydantoin. Recovery of the drug from biological fluids is approximately 94%.

Keyphrases □ Diphenylhydantion—microdetermination in serum and plasma, UV spectrophotometry □ Microdetermination—diphenylhydantoin in serum and plasma, UV spectrophotometry □ UV spectrophotometry—microdetermination of diphenylhydantoin in plasma and serum

The requirement for plasma levels of diphenylhydantoin in the clinical management of epileptic patients has been well documented (1–4). Earlier colorimetric and spectrophotometric methods for determining this common anticonvulsant were plagued by interference from other drugs, necessitating extensive separation techniques (5–7). Wallace *et al.* (8) developed the first sensitive and specific spectrophotometric determination for diphenylhydantoin not requiring preliminary separation from other drugs, a technique based upon the oxidation of the parent drug to benzophenone. Numerous modifications of that method have been published (4, 9–14), with a primary objective of determining the drug in as little as 0.4–2.0 ml of specimen (4, 12–14).

GLC techniques are available both for the determination of unchanged diphenylhydantoin (15–18) and derivatized diphenylhydantoin (19–25). GLC techniques are often complicated and usually demand a time interval exceeding that required for a spectrophotometric scan. Additionally, the lability of the hydantoins makes them highly susceptible to degradation at the injection temperature conditions of the chromatograph.

The present report describes two modifications of the spectrophotometric analysis: one utilizing conventional condensers and requiring 1 ml of plasma and the other utilizing a modified reflux condenser[1] and requiring only 0.1–0.2 ml plasma. The methods, in addition to providing a greater sensitivity than that achieved by available methods, eliminate the time-consuming evaporation of chloroform required in the Lee (4) and Saitoh (13) methods. Additionally, a far greater product stability than exists in the "bomb oxidation techniques" is obtained.

EXPERIMENTAL

Apparatus—Reflux condensers were mounted on a support[2]. The semimicrodetermination utilizes a conventional condenser[3]; the microdetermination utilizes a previously described "external cold finger" reflux condenser[1]. Heating mantles[4], 270 w, 500-ml ca-

[1] Unpublished data.
[2] Flexaframe.
[3] Allihn.
[4] Glas-Col.

Table I—Diphenylhydantoin Determination by Semimicroprocedure

Concentration[a], μg/ml	Absorbance[b,c]	Absorbance/ Concentration	Recovery[c], %
2.5	0.057 ± 0.009	0.0764	103.5 ± 15.8
5	0.105 ± 0.003	0.0700	94.9 ± 3.1
10	0.211 ± 0.003	0.0702	95.2 ± 1.0
20	0.421 ± 0.005	0.0701	95.0 ± 1.1
30	0.620 ± 0.018	0.0689	93.3 ± 2.7
40	0.810 ± 0.020	0.0675	91.4 ± 2.3
60	1.267 ± 0.036	0.0703	95.3 ± 2.7
80	1.655 ± 0.016	0.0691	93.6 ± 1.3
Mean		0.0703 ± 0.0044 (6.3%)	95.3 ± 6.0 (94.1 ± 2.0 over the range 5–80 μg/ml)

[a] Determinations performed on 1.0 ml plasma. [b] Absorbance at 247 nm, adjusted for mean plasma blank of 0.012. [c] Mean of triplicate determinations ± standard deviation.

pacity, were positioned upon magnetic stirrers beneath the condenser. Six reflux units were attached to a single variable transformer[5] which applied voltage to each heating mantle through the use of a multiple-electric outlet[6]. A similar outlet box applied line voltage to each of the six magnetic stirrers. A convenient reflux system may be achieved by mounting two sets of six reflux units each from a single support matrix, one set to the front and the other to the rear. The use of BB's or shot pellets as a heat transfer medium allows the heating mantles to be used with varying sizes of flasks.

Spectrophotometric measurements were performed on a ratio-recording spectrophotometer[7] equipped with a three-cell multiposition mount with microcell holder. Quartz microcells, 10 mm square × 25 mm, 0.4-ml capacity, were utilized for the microdeterminations; quartz cells, 10 mm square × 45 mm, 3.5-ml capacity, were utilized for the semimicrodeterminations.

Procedure—*Microdetermination*—Five milliliters of ethylene dichloride, 0.1–0.2 ml of plasma, serum, or whole blood, and 0.1 ml of 1 *N* HCl are pipetted into a 15-ml glass-stoppered tube. The tube is stoppered, shaken vigorously for 1 min, and, following removal of the stopper, centrifuged for 2 min at 2000 rpm. Then 4.5 ml of the ethylene dichloride layer is transferred to a second tube and similarly extracted into 2.5 ml of 5 *N* NaOH. Two milliliters of the sodium hydroxide extract, approximately 0.2 g of potassium permanganate, 1.0 ml of spectrograde *n*-heptane, and a 1.3-cm (0.5-in.) Teflon-coated magnetic stirring bar are placed in a 50-ml round-bottom flask fitted with a 24/40 ground-glass joint.

Semimicrodetermination—With 25 ml of ethylene dichloride, 1 ml of plasma or serum, and 0.5 ml of 1 *N* HCl, extraction and centrifugation are performed as already described in a 50-ml round-bottom centrifuge tube. The aqueous layer may be aspirated and the solvent decanted into a stoppered 50-ml graduated cylinder, or a known quantity, *i.e.*, 24 ml, may be transferred *via* pipet to a second 50-ml centrifuge tube. Ten milliliters of 5 *N* NaOH is added and the tubes are stoppered, shaken vigorously for 1–2 min, and subsequently centrifuged for 2 min at 2000 rpm. Nine milliliters of the aqueous layer is transferred to a 50-ml round-bottom flask, to which are added a magnetic stirring bar, 3 ml of *n*-heptane, and approximately 0.2 g of potassium permanganate.

The boiling flask is attached to the reflux condenser and secured into a heating mantle, which is supported by the top surface of a magnetic stirrer. After the mixture has refluxed with vigorous stirring for 25 min, optimally achieved with 40 v ac applied to the heating mantles, the heating mantles are removed and the flasks are allowed to cool for 5–10 min. Then 10–25 ml of water is poured into the flasks through the open end of the reflux columns.

The flasks are removed, sufficient water is added to float the heptane layer just into the neck of the flask, and the heptane layer is transferred to the quartz cells. The heptane layer is scanned spectrophotometrically over the 310–230-nm range, or the absorbance is measured at 247 nm. Since all standards and unknowns are treated identically, calculations are accomplished by application of a simple ratio:

$$\frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \text{concentration of standard} = \text{concentration of specimen} \quad \text{(Eq. 1)}$$

If different volumes of specimen and standard are used, or if different volumes of solvent are recovered, appropriate corrections must be made in the final calculation.

Comparison of Oxidation Techniques—For evaluation of the nonreflux oxidation (13), 4 ml of 5 *N* NaOH containing diphenylhydantoin and 0.2 g of potassium permanganate were alternately placed into a 15-ml tube having a ground-glass stopper or into a 5-ml long-stem glass ampul which was then hermetically sealed. The stoppered tube or sealed ampul was immersed in a boiling water bath for 5–25 min, after which it was removed and cooled to room temperature. Then the contents were extracted with 2 ml heptane and scanned spectrophotometrically.

RESULTS AND DISCUSSION

Both the semimicro- and microprocedures provide quantitative

Table II—Diphenylhydantoin Determination by Microprocedure

Concentration[a], μg/ml	Absorbance[b]	Absorbance/ Concentration	Recovery, %
5	0.050 ± 0.008	0.0694	94.4
10	0.099 ± 0.002	0.0688	93.5
20	0.196 ± 0.002	0.0680	92.6
30	0.302 ± 0.012	0.0699	95.1
40	0.392 ± 0.003	0.0680	92.6
60	0.598 ± 0.007	0.0692	94.1
80	0.793 ± 0.017	0.0688	93.7
Mean		0.0689 ± 0.0027	93.7 ± 3.6

[a] Triplicate plasma determinations performed on 0.2-ml specimens. [b] Absorbance at 247 nm ± standard deviation, adjusted for mean plasma blank of 0.022.

Table III—Reproducibility Achieved with Different Volumes of Heptane in Reflux

Volume of Heptane, ml	250-ml Flask[a,b], Standard Condenser	50-ml Flask[a,b], Standard Condenser	50-ml Flask[a,c], External Cold Finger
0.50	—[d]	—[d]	11.6
0.75	—[d]	—[d]	3.8
1.00	5.6	6.4	2.0
3.00	0.8	0.5	—[d]

[a] Percent relative standard deviation, mean of triplicate determinations. [b] Nine milliliters of 5 *N* NaOH, 10 μg diphenylhydantoin/ml heptane. [c] Two milliliters of 5 *N* NaOH, 10 μg diphenylhydantoin/ml heptane. [d] Not determined.

[5] Staco.
[6] CRC Multi-lectric.
[7] Beckman Acta CIII.

Table IV—Comparison of Reflux and Nonreflux Oxidation Techniques

Oxidation Technique[a]	Absorbance/ Concentration[b], μg/ml
Sealed ampul, 5 min	0.0051 ± 0.0008
Sealed ampul, 25 min	0.0377 ± 0.0004
Glass-stoppered tube, 5 min	0.0536 ± 0.0022
Glass-stoppered tube, 25 min	0.0393 ± 0.0053
Reflux[c], 25 min	0.0770 ± 0.0021

[a] Twenty micrograms diphenylhydantoin either refluxed in the presence of alkaline permanganate and heptane or incubated in alkaline permanganate and subsequently extracted with heptane. [b] Mean of triplicate determinations. [c] Semimicroreflux; *i.e.*, 3 ml heptane, Allihn condenser.

recovery of diphenylhydantoin at subtherapeutic through toxic levels from small volumes of plasma (Tables I and II, respectively). Wallace *et al.* (8) previously demonstrated that the alkaline permanganate oxidation, utilized in conjunction with an acidic extraction, is a highly specific determination for diphenylhydantoin, free of interference from other drugs, drug metabolites, or naturally occurring constituents. Previous spectrophotometric determinations for diphenylhydantoin utilized large boiling flasks and solvent volumes for reflux, resulting in an extensive dilution of the benzophenone product. Use of 50-ml boiling flasks with the condenser[3] requires only 3 ml of heptane, which results in a significant increase in sensitivity. However, a further decrease in the solvent volume to 2 ml, when using the standard condenser, more than doubles the coefficient of variation that is achieved with 3 ml of solvent, apparently due to an insufficient amount of liquid solvent available for the continuous extraction of benzophenone.

Utilization of an "external cold finger" reflux condenser induces a more rapid condensation of solvent vapors, which permits the utilization of smaller volumes of solvent and results in a enhanced sensitivity (Table III). The cold finger condenser was developed for working with extremely small amounts of refluxing solvent[1]. It essentially involves the projection of the cooling surface of the condenser to a point only 2.54 cm (1 in.) above the boiling liquid.

It was demonstrated previously (14) that, within wide limits, the amount of potassium permanganate (0.1–0.4 g) and the molarity of the sodium hydroxide (5–11 *M*) are not critical for the quantitative oxidation of diphenylhydantoin. For the present micro- and semimicromodification, the amount of potassium permanganate should exceed 0.05 g and the concentration of the alkali should exceed 5.0 *M*.

Certain investigators (13) employed a nonreflux alkaline permanganate oxidation of diphenylhydantoin, in which the aqueous layer is extracted with solvent subsequent to, rather than concurrent with, the oxidative reactions. Comparative studies in this laboratory indicate that the observed absorbance–concentration ratio is significantly less in the nonreflux techniques, and a marked decrease in absorbance is observed if the time of incubation is prolonged only slightly past optimum at temperatures exceeding 90° (Table IV). It was suggested (13) that these observations were a result of the volatile nature of the product, benzophenone, and that the losses occurred through the spaces between the ground-glass joint and the glass stopper of the tubes.

A decreased yield of benzophenone was achieved if the magnetic stirrers were temporarily turned off during reflux. When considering also the high boiling point of benzophenone, it is apparent that the decreased sensitivity obtained in the nonreflux technique is a consequence of overoxidation of benzophenone (product degradation), rather than its volatility.

A comparison of the absorbance–concentration values obtained with the nonreflux technique, utilizing both glass-stoppered tubes and hermetically sealed ampuls (to prohibit any benzophenone volatility), with those achieved by the reflux technique further indicates that the decreased sensitivity of the nonreflux procedure is not a consequence of the volatility of benzophenone (Table IV). The sensitivity achieved with the ampul and tube samples at 5 min of incubation was 72 and 70%, respectively, of that achieved by the reflux procedure; both the ampul and tube samples exhibited a similar decrease in sensitivity with increasing duration of incubation.

Although the nonreflux technique does not provide the sensitivity obtained through reflux, it does offer the advantages of not requiring the more expensive reflux apparatus and of achieving its optimum sensitivity in less time than that required for the reflux technique. Thus, for laboratories having a limited budget and not possessing reflux apparatus, the nonreflux technique may be the most appropriate method. But for those laboratories possessing the necessary apparatus, the enhanced sensitivity and reproducibility achieved by the reflux method more than offset the slight additional time of analysis. The utilization of the cold finger condenser makes for a micromethod that is unequaled in sensitivity by the sealed oxidation techniques.

Table V—Diphenylhydantoin Levels in Patients' Plasma Level (Micrograms per Milliliter)

Patient	Micromethod[a]	Macromethod[b]
1	24.5, 25.3	25.1
2	19.3, 21.5	20.0
3	3.5, 5.6	—[c]
4	28.2, 29.1	28.9
5	0.6, 1.1	3.5
6	8.9, 10.0	8.7
7	30.3, 30.5	29.0
8	13.2, 13.5	13.2
9	0, 0.5	0
10	18.4, 19.1	19.4
11	8.5, 10.3	11.2
12	30.4, 32.5	28.9
13	22.5, 24.0	—[c]
14	0, 0	4.2

Mean difference between duplicate determinations—±1.0 μg/ml

Mean difference between macro- and microdetermination—±1.3 μg/ml

[a] Microdeterminations performed in duplicate in this laboratory. [b] Macromethod (14) performed in a public hospital laboratory in single determinations. [c] Sample size insufficient for macrodetermination.

To demonstrate the clinical application and effectiveness of the proposed techniques, 14 patient specimens were analyzed in duplicate by the microprocedure (Table V). The mean difference between duplicate determinations was 1.0 μg/ml, the mean concentration being 15.4 μg/ml. The diphenylhydantoin levels had been obtained on 12 of the specimens (single determination only) at a public hospital laboratory utilizing an earlier modification of the Wallace *et al.* (14) method; the mean difference between levels in this study and the hospital levels was 1.3 μg/ml.

The proposed micromethod provides the most sensitive, reliable, and reproducible values of any available spectrophotometric method capable of determining diphenylhydantoin in 0.1 ml of serum. The limit of accurate quantitation for 0.1–2.0-ml specimens is 0.5 μg/specimen.

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* To whom inquiries should be directed.

Antitumor Activity and Cardiac Stimulatory Effects of Constituents of *Anthopleura elegantissima*

R. J. QUINN *, MIDORI KASHIWAGI ‡, TED R. NORTON ‡ x, S. SHIBATA ‡, M. KUCHII ‡, and R. E. MOORE *

Abstract □ A crude aqueous ethanolic extract of the sea anemone, *Anthopleura elegantissima* (Brandt), displayed activity against two experimental mouse tumors (P-388 lymphocytic leukemia and Ehrlich ascites tumor). In addition, it displayed a positive inotropic effect on isolated rat atria. Preliminary separations established that the three biological activities are due to three different constituents of this marine organism.

Keyphrases □ *Anthopleura elegantissima* (Brandt)—separation of constituents, screened for antitumor activity and cardiac stimulatory effects □ Sea anemone—separation of constituents, screened for antitumor activity and cardiac stimulatory effects □ Antitumor activity—constituents of *A. elegantissima* screened □ Cardiac stimulatory activity—constituents of *A. elegantissima* screened

The antitumor activity of a crude extract of the sea anemone, *Anthopleura xanthogrammica* (Brandt) (1), and the cardiac stimulatory effects of extracts of both *A. xanthogrammica* and *A. elegantissima* (2) have been reported. An aqueous ethanolic extract of *A. elegantissima*[1] has been found active against P-388 lymphocytic leukemia (P-388) and Ehrlich ascites tumor in mice and produces a positive inotropic effect on isolated rabbit auricles (Table I). These three effects are due to three different constituents obtained in a preliminary fractionation of the crude extract.

Gel permeation chromatography using Resin 1[2] separates the P-388 active material, which is not retarded, from the Ehrlich ascites and heart stimulant substances, which are retarded. The P-388 active material is further purified using Resin 2[3] and the Ehrlich ascites and heart stimulant substances are separated from each other using cellulose chromatography.

Taurine was also isolated from the *A. elegantissima* extract. Taurine was first isolated from a marine organism from the red algae *Ptilota pectinata, Porphyra umbilicalis,* and *Gelidium cartilagineum* (3). *N,N*-Dimethyltaurine was isolated previously from the red alga *Furcellaria fastigiata* (4), while both the *N*-methyl- and *N,N*-dimethyltaurines were shown to be constituents of the sponge *Calyx nereis* (5); the trimethyl derivative, taurobetaine, was found in the gorgonian *Briareum asbestinum* (6).

RESULTS AND DISCUSSION

A typical chromatogram, monitored at 280 nm, of the crude extract on Resin 1 is shown in Fig. 1. Fraction A (V_e/V_0 0.86–1.50) was active against P-388, Fraction C (V_e/V_0 1.73–1.97) displayed a strong positive inotropic effect on isolated rat atria (2) and some inhibition of Ehrlich ascites, and Fraction D (V_e/V_0 1.97–2.25) inhibited Ehrlich ascites tumor.

Further gel permeation chromatography of Fraction A was achieved on Resin 2 (Fig. 2). The P-388 active constituent separated from the large amount of 280-nm absorbing material at V_e/V_0 0.83–1.31 but was distributed over a large region (Fraction H, V_e/V_0 1.31–2.35). In comparison to the crude extract, Fraction H showed a marked improvement in activity against P-388.

Fraction C was separated further by cellulose chromatography (Fig. 3). The Ehrlich ascites active material was eluted almost at

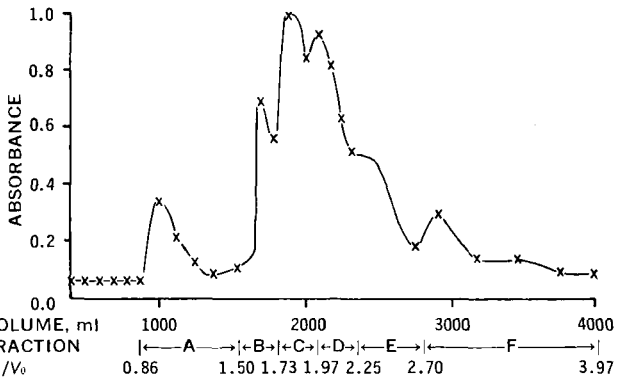

Figure 1—*Chromatography of crude extract on Resin 1.*

[1] The authors thank Dr. Cadet Hand, Director, Bodega Marine Laboratory, Bodega Bay, Calif., for supplying a collection of the animals.
[2] Sephadex G-25.
[3] Sephadex G-75.