single, oral, convulsive doses of DDT, aldrin, dieldrin, or endrin. SGOT, SGPT, and SLDH levels in the treated animals were significantly increased above those seen in either the undosed or vehicle-treated controls.

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# Simultaneous Extraction of Tissue Norepinephrine and Serotonin


**BARRIE M. PHILLIPS, PAUL J. KRAUS, TONI L. HAMMES, and JERRY L. LEELING**



**Abstract** □ A new procedure for the extraction of tissue monoamines combines the homogenization and butanol extraction steps. In addition to the significant reduction in the time necessary for extraction which is characteristic of single-extraction procedures, greater extraction efficiencies are obtained. Hence, the absolute amount of amine available for spectrophotofluorometric determination is greater and the potential error arising from correction for losses during extraction is reduced. The procedure has been routinely employed in this laboratory for more than 2 years, and has proven to be reliable.



**Keyphrases** □ Serotonin, norepinephrine—simultaneous tissue extraction □ Tissue extraction—serotonin, norepinephrine, radioactive □ Scintillometry, liquid—analysis


Since the report of Shore and Olin (1) virtually all methods of tissue norepinephrine determination have incorporated extraction of the amine from a tissue homogenate prepared in dilute hydrochloric acid. This type of extraction was also used by Wiegand and Perry (2) to determine tissue epinephrine, serotonin, DOPA, and dopamine in addition to norepinephrine.

Shore and Olin (1) recognized that the efficiency of norepinephrine extraction into butanol from a hydrochloric acid homogenate was low. In addition, the homogenate–butanol extraction phase of the procedure was time consuming. Callingham and Cass (3) also observed that destruction of amine due to local overheating during homogenization in glass, and pipeting errors due to homogenate frothing were disadvantages inherent in this procedure. They avoided these problems by pulverizing the frozen tissue in a punch press. The pulverized tissue was then added to a salt-saturated butanol–0.01 *N* hydrochloric acid system for extraction. Hence, while losses of amine prior to extraction were minimized, total recovery of amine remained poor due to the unfavorable partition coefficient of catecholamines in the hydrochloric acid–butanol system. Chang (4) developed a procedure in which homogenization was performed in acidified butanol. This procedure had the advantage of simplicity by combining the homogenization and extraction steps, but the overall recovery of catecholamines, although improved over previous methods, was still poor. Fleming *et al.* (5) developed a procedure in which homogenization was performed in acetone, and the amines were then transferred into acidified butanol. While extraction efficiency was apparently improved and losses of amines were minimal, the procedure is somewhat complex and relatively time-consuming.

The present procedure was developed in an attempt to prevent the loss of monoamines due to factors observed by Callingham and Cass (3), to improve the

**Table I**—Extraction of Tissue Norepinephrine and Serotonin

| Tissue | Average Weight, g. | Percent Monoamine Extracted[a] — Norepinephrine-7-$^3$H: Present Method | Norepinephrine-7-$^3$H: From 0.01 *N* HCl Tissue Homogenates[c] | 5-Hydroxytryptamine-3-$^{14}$C[b]: Present Method | 5-Hydroxytryptamine-3-$^{14}$C[b]: From 0.01 *N* HCl Tissue Homogenates[c] |
|---|---|---|---|---|---|
| Adrenal | 0.036 | I 92.14 ± 0.90<br>II 93.96 ± 1.02 | 54.20 ± 0.62 | 99.12 ± 0.12 | 68.12 ± 1.56 |
| Spleen | 0.510 | I 83.55 ± 0.56<br>II 81.99 ± 0.85 | 51.03 ± 1.31 | 95.67 ± 0.89 | 61.99 ± 3.12 |
| Uterus | 0.250 | I 89.60 ± 1.08<br>II 86.72 ± 1.33 | 53.88 ± 1.15 | 94.29 ± 1.04 | 76.67 ± 2.76 |
| Heart | 0.620 | I 89.78 ± 2.86<br>II 84.12 ± 0.48 | 48.45 ± 1.87[d] | 96.01 ± 0.40 | — |
| Brain | 1.540 | I 80.24 ± 0.64<br>II 81.95 ± 0.57 | 48.70 ± 0.96 | 93.91 ± 0.51 | 61.22 ± 2.11 |
| Control[e] | 1.500 ml. | I 83.99 ± 0.81<br>II 85.31 ± 0.71 | 42.33 ± 2.81 | 103.10 ± 0.99 | 61.68 ± 1.42 |

[a] Mean (n = 6) percent of amine found in the final extract ± standard error. [b] According to Ansell and Beeson (6), the efficiency of extraction of serotonin from tissue by the method of Chang was about 65%. [c] Total homogenate volume 5.0 ml. for each tissue. [d] Efficiency of extraction of norepinephrine from heart by the method of Chang was 69.66 ± 0.59% (n = 30). [e] 0.05 *M* pH 7.4 phosphate buffer.

recovery of amines and to simplify the extraction process.

## METHODS

With the exception of the *n*-butanol (practical grade) all chemicals are reagent grade. All glassware is washed in detergent and then soaked in 100° nitric acid. Water is glass-distilled.

Whole organs or tissue samples are removed rapidly from rats killed by cervical dislocation and frozen on dry ice; the tissues are generally covered with dry ice to facilitate rapid freezing. After weighing, the tissue is transferred to a No. 16-207 50-ml. homogenizing flask (Virtis) containing 50 ml. of washed (1) butanol at 5° and 2 g. of sodium chloride; the flask is packed in ice. After 30 to 60 sec., the tissue is homogenized for 4 min. at a fixed fraction (64% in the present case) of full speed on the homogenizer,[1] using a single-number 16-108 blade on the macroshaft. If homogenization is initiated immediately, large clumps of the frozen tissue, which resist further homogenization, may be produced.

The resulting mixture is then transferred to a 50-ml. round-bottom centrifuge tube and centrifuged[2] at 2,500 r.p.m. for 5 min. Forty milliliters of the supernatant fluid, which corresponds to the extract obtained in the 0.01 *N* hydrochloric acid tissue homogenate–butanol extraction procedure, is transferred into a 200-ml. centrifuge bottle containing 4.0 ml. 0.01 *N* hydrochloric acid and 80 ml. washed (1) *n*-heptane. The bottle is then closed with a rubber stopper lined with a film (Mylar). The resulting mixture is agitated at 150 excursions per minute on a platform shaker[3] for 5 min. and then centrifuged at 2,000 r.p.m. for 5 min. After aspiration of the bulk of the organic layer, the remaining liquid is quantitatively transferred into a 15-ml. graduated conical centrifuge tube and centrifuged at 2,000 r.p.m. for 5 min. After measurement of the volume of extract recovered, and aspiration of the remaining organic layer, monoamines are determined in the aqueous extract as described by Shore and Olin (1) and Wiegand and Perry (2).

Efficiency of monoamine extraction was investigated by the addition of 10 or 20 µl. of a 0.01 *N* hydrochloric acid solution containing either 95000 DPM DL-norepinephrine-7-$^3$H (50 mc./m*M*)[4] or 52000 DPM 5-hydroxytryptamine-3′-$^{14}$C (10 mc./m*M*)[5] to the homogenizing flask just prior to the start of homogenization. Aliquots of the *n*-butanol and final acid extracts were transferred to vials containing 14 ml. of a scintillation mixture consisting of naphthalene 80 g./l., 2,5-diphenyloxazole 10 g./l., and 1,4-di-[2-(5-phenyloxazolyl)]benzene 0.5 g./l., in a 1:3:3 mixture of xylene, dioxane, and ethylene glycol monoethyl ether and counted in a liquid scintillation spectrometer for a period sufficient to yield less than 2% counting error. Internal standards were employed to determine percent counting efficiency.

For purposes of comparison, homogenates of additional tissues were prepared as described by Shore and Olin (1). Aliquots of radioactive amines were added to the homogenates, and the efficiency of monoamine extraction from the 0.01 *N* hydrochloric acid homogenate was determined as outlined above.

Stability of norepinephrine to the chemical and enzymatic environment encountered during the extraction procedure was in-

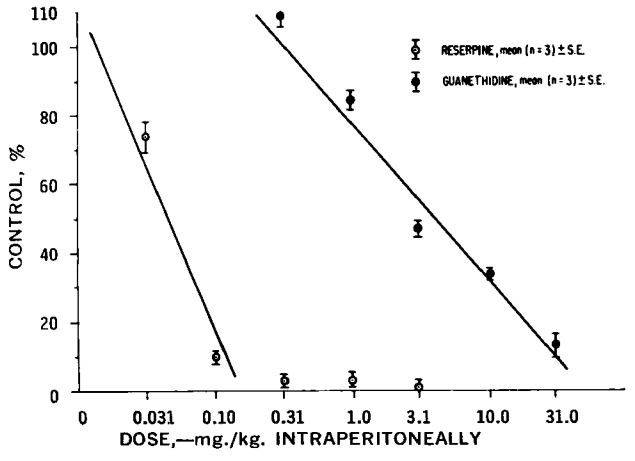


**Figure 1**—*Effect of reserpine and guanethidine on total heart norepinephrine in male rats 8 hr. after administration.*

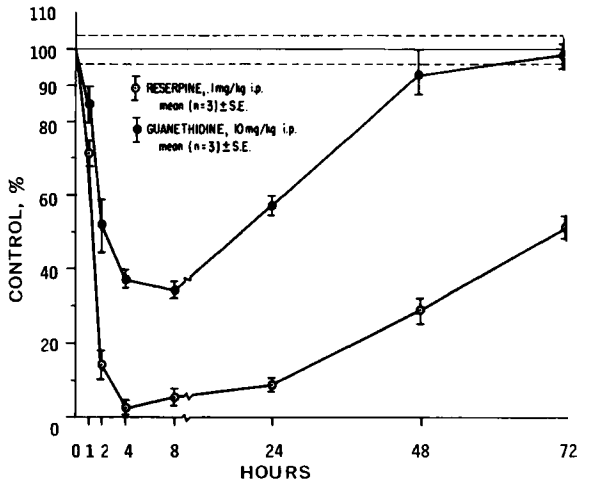


**Figure 2**—*Effect of reserpine and guanethidine on total heart norepinephrine in male rats.*

[1] Virtis 45, The Virtis Co., Inc., Gardiner, N. Y.
[2] International Centrifuge, Universal Model U. V.
[3] Eberbach Corp. reciprocating shaker.
[4] New England Nuclear.
[5] Nuclear-Chicago.

vestigated in a separate experiment. Five hundred nanograms nonradioactive norepinephrine and 50000 DPM norepinephrine-7-$^3$H were added, just prior to extraction in the homogenizer, to hearts obtained from rats dosed intraperitoneally 8 hr. previously with 3.1 mg./kg. reserpine. Six similar hearts, which received no added norepinephrine, were carried through the procedure and served as blanks for the spectrophotofluorometric determinations.

## RESULTS AND DISCUSSION

Determination in the final 0.01 *N* hydrochloric acid extract of the two materials added to the reserpine-depleted hearts, and correction of nonradioactive norepinephrine values for percent extraction observed with norepinephrine-7-$^3$H, revealed that 102.4 ± 7.45% of the added norepinephrine was recovered.

Results of extraction efficiency studies are summarized in Table I. These findings indicate that norepinephrine extraction efficiency is increased 60 to 100%, while serotonin extraction efficiency is increased 25 to 55%. Serotonin extraction is nearly complete in all tissues, while norepinephrine extraction values are somewhat lower and appear to be partially dependent on the total weight of tissue extracted. That the extraction efficiency values are reproducible is indicated by the results obtained in two separate experiments with norepinephrine-7-$^3$H.

In this laboratory, screening is routinely carried out with these extraction efficiency values (yielding results similar to those illustrated in Figs. 1 and 2). For greater precision, or in cases where the weight of tissue employed may vary, it is advisable to add radioactive amine to some of the samples to serve as an internal standard.

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# Consecutive First-Order Kinetic Consideration of Hydrocortisone Hemisuccinate


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**Abstract** □ The degradation of hydrocortisone hemisuccinate has been studied at 70° and at pH's of 6.9, 7.2, and 7.6. An expected first-order consecutive reaction was found to be operative and it is presumed that ester hydrolysis occurs by way of an intramolecular attack. The blue tetrazolium assay confirmed that the production of a species devoid of the 17-dihydroxyacetone side chain occurred subsequent to the formation of the steroid alcohol.



**Keyphrases** □ Hydrocortisone hemisuccinate—degradation kinetics □ Degradation, hydrocortisone hemisuccinate—first-order reaction □ Colorimetric analysis—spectrophotometer □ Blue tetrazolium—color reagent


The availability of a quantitative analytical method is essential to the formulator or scientist for the implementation of predictive kinetics. The application of various environmental parameters, such as temperature, permits the rapid approximation of relevant rates and produces data which aid in the mechanistic interpretation of chemical reactions. Thus, it is often found that dosage forms and their physical and chemical properties, such as solubility or buffer content, are predicated upon the nature of the active moieties and the rates at which decomposition occurs.

The hydrolytic pathway of the 21-hydrocortisone hemiester of succinic acid has been investigated under varied environmental conditions, such as pH and temperature (1, 2). The pH profile indicates that hydrolysis is due to a specific acid catalysis in the pH range of 1.0 to about 2.5 and specific hydroxyl catalysis from approximately 7.6 to 10.0. In the intermediate range, the compound may be subjected to an intramolecular attack of the anion on the ester carbonyl carbon or specific hydroxyl-ion catalysis of the undissociated hemiester.

Steroidal alcohols containing the 17-dihydroxyacetone side chain have been kinetically described under varying conditions of pH, temperature, buffer media, and oxygen deprivation. The rate of disappearance of the dihydroxyacetone function of prednisolone was investigated in solutions of varying hydroxyl-ion concentration, both in the presence and absence of air (3).

The oxygen-deprived system produced neutral and acidic steroidal components at a rate which exceeded that of the oxidative system. In addition, it was assumed that the oxidative degradations did not produce the neutral component. The effect of trace metal content in aqueous solutions of prednisolone has also been studied (4) and shown to significantly increase the production of the steroid devoid of the 17-dihydroxyacetone function.

Sequestering agents were demonstrated to reduce the degradation rate under similar conditions of pH and temperature. Hydrocortisone (cortisol) has also been shown to decompose to fractions devoid of the 17-dihy-