Determination of Dantrolene in Biological Specimens Containing Drug-Related Metabolites

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Abstract □ A sensitive and specific method is described for the determination of dantrolene, a new skeletal muscle relaxant. Method specificity for dantrolene in the presence of dantrolene-related metabolites is based on a combination of solvent extractions, chromatography, and the fluorescence characteristics of dantrolene. The method is satisfactory for use with plasma, blood, urine, bile, tissues, and feces. Modifications of the dantrolene method are also provided for estimating two dantrolene-related metabolites in biological fluids.

Keyphrases □ Dantrolene—analysis, biological fluids containing metabolites □ Spectrophotofluorometry—analysis, dantrolene in biological fluids containing metabolites

Dantrolene sodium[1], 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin sodium hydrate, is a new muscle relaxant which apparently acts directly and selectively on skeletal muscle (1–3). During the early development of this drug, a spectrophotofluorometric procedure was developed for the determination of dantrolene, 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]-hydantoin, in blood and urine (4). Subsequent investigation revealed that dantrolene is metabolized by both nonreductive and reductive pathways, and a polarographic method was developed which determines the total nonreduced or reduced metabolites (5). The drug is reduced to 1-[[5-(*p*-aminophenyl)furfurylidene]amino]-hydantoin and subsequently acetylated to 1-[[5-(*p*-acetamidophenyl)furfurylidene]amino]hydantoin (5). A major nonreduced metabolite also was isolated which is currently designated as Metabolite A (5). Recently, it was established that Metabolite A interferes in the determination of dantrolene by the original fluorometric procedure.

A modification of the original procedure is now presented for determining dantrolene in biological specimens containing known drug-related metabolites. The method is satisfactory for use with plasma, blood, urine, bile, tissues, and feces. Methods for estimating two of the major metabolites, acetylated dantrolene and Metabolite A, in plasma, blood, and urine are also described.

EXPERIMENTAL

Reagents and Instrument—The reagents include *N,N*-dimethylformamide, reagent grade[2]; ammonium sulfate, special enzyme grade[3]; sodium hydroxide, analytical reagent[4]; hydrochloric acid, analytical reagent[5]; 1-hexanol, practical grade[6]; heptane, practical grade[7]; 1-nitropropane, practical grade[8]; ethyl acetate, spectroquality[9]; methanol, anhydrous, analytical reagent grade[10]; and Sephadex G-10[11].

A spectrophotofluorometer[12] with the following instrumental parameters was used to measure fluorescence. Slit arrangement for both excitation and fluorescence was the No. 3 position, the light path was adjusted with a No. 3 spacer, and sensitivity was No. 8. For dantrolene and Metabolite A measurements, the excitation wavelength was 395 nm. and the fluorescence wavelength was 530 nm. For acetylated dantrolene measurements, the excitation wavelength was 365 nm. and the fluorescence wavelength was 405 nm.

Standard Solutions—Fifty milligrams of crystalline dantrolene or Metabolite A is dissolved in 50 ml. of *N,N*-dimethylformamide. Ten milliliters of this solution is placed in a 100-ml. volumetric flask, 40 ml. of *N,N*-dimethylformamide is added, and the solution is diluted to volume with water to obtain a drug concentration of 100 mcg./ml. This solution is then diluted with water to obtain the required drug concentrations. Fifty milligrams of crystalline acetylated dantrolene is dissolved in 50 ml. of *N,N*-dimethylformamide to obtain a 1000-mcg./ml. solution. This solution is then diluted with water to obtain the required drug concentrations.

Analysis of Dantrolene in Plasma, Blood, and Urine—To 0.5 ml. of biological fluid, add 2.0 ml. of water and then 2.0 ml. of a saturated ammonium sulfate solution and mix thoroughly. Mix the solution with 5.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging the sample at 2000 r.p.m. for 10 min. Transfer 4.5 ml. of the hexanol–heptane extract (top layer) to a test tube and add 2.5 ml. of 0.2 *N* sodium hydroxide. Shake the tube for 2 min. and centrifuge at 2000 r.p.m. for 10 min. Remove 2.0 ml. of the sodium hydroxide extract (bottom layer) and place on a hydrated Sephadex G-10 column (0.6 cm. wide × 3.5 cm. long). The column is prepared with previously hydrated Sephadex. After the extract passes through the Sephadex, wash the column with 4.0 ml. of 0.2 *N* sodium hydroxide and discard the eluate. Dantrolene is eluted with 4.0 ml. of a 5% *N,N*-dimethylformamide water solution. Addition of 1.0 ml. of 0.3 *N* hydrochloric acid to the *N,N*-dimethylformamide eluate adjusts the pH to 1–2. Mix the solution with 4.0 ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min.

Then remove a 3.0-ml. aliquot of the nitropropane–heptane extract (top layer) for fluorometric measurement. Set the spectrophotofluorometer at 30 fluorescence units with a 4-mcg. aqueous dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from the biological specimens are then measured, and the amount of dantrolene is calculated from aqueous dantrolene standards. When the fluorescence obtained with dantrolene exceeds 30 fluorescence units, quenching occurs. Under these conditions the solvent extract is diluted with additional nitropropane–heptane (1:1) containing 2.7% hexanol until a value of 30 fluorescence units or less is obtained.

Estimation of Metabolite A in Plasma, Blood, and Urine—To 0.5 ml. of biological fluid, add 2.0 ml. of water and then 2.0 ml. of a saturated ammonium sulfate solution and mix thoroughly. Mix the solution with 5.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging the sample at 2000 r.p.m. for 10 min. Transfer 4.5 ml. of the solvent extract (top layer) to a test tube and add 2.5 ml. of 0.2 *N* sodium hydroxide. Shake the tube vigorously for 2 min. and centrifuge at 2000 r.p.m. for 10 min. Remove 2.0 ml. of the sodium hydroxide extract and transfer to a test tube. Addition of 3.0 ml. of 0.3 *N* hydrochloric acid adjusts the pH to 1–2. Mix the solution with 4.0

[1] Dantrium, Eaton Laboratories.
[2] Matheson, Coleman and Bell (DX 1730).
[3] Schwarz/Mann (1946).
[4] Mallinckrodt Chemical Works (7708).
[5] Mallinckrodt Chemical Works (2612).
[6] J. T. Baker Chemical Co. (N-229).
[7] Matheson, Coleman and Bell (HX-80).
[8] Matheson, Coleman and Bell (NX-705).
[9] Matheson, Coleman and Bell (EX-245).
[10] Mallinckrodt Chemical Works (3016).
[11] Pharmacia Fine Chemicals.
[12] Baird Atomic Fluorispec, model SF-1.

Table I—Recovery of Dantrolene from Biological Fluids

Fluid	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.05	0.10	0.50	1.0	2.0	4.0	Percent Recovery[b], Mean ± SD
Water	0.2	0.3	0.7	3.4	7.4	14.8	29.8	—
Dog blood	0.2	0.3	0.7	3.4	7.3	14.8	29.8	99.7 ± 0.6
Dog plasma	0.2	0.3	0.7	3.3	7.2	14.5	30.2	98.8 ± 1.7
Dog urine	0.2	0.3	0.7	3.5	6.7	15.4	29.8	99.5 ± 4.7
Human blood	0.2	0.3	0.7	3.4	7.1	14.4	29.8	98.8 ± 1.8
Human plasma	0.2	0.3	0.6	3.4	6.9	14.4	29.8	96.0 ± 5.6
Human urine	0.2	0.3	0.6	3.3	6.7	14.4	29.8	95.1 ± 5.7

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene standards.

ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and then centrifuging at 2000 r.p.m. for 10 min.

Then remove a 3.0-ml. aliquot of the nitropropane–heptane extract (top layer) for fluorometric measurement. The spectrophotofluorometer is set at 30 fluorescence units using a 4-mcg. aqueous dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from the biological samples are then measured. If a fluorescence greater than 30 units is encountered, quenching occurs. Then the solvent extract is diluted with nitropropane–heptane (1:1) containing 2.7% hexanol until 30 fluorescence units or less is obtained.

To obtain an estimate of Metabolite A in biological fluids, aliquots of each sample must be analyzed by this procedure and by the procedure described for dantrolene. The amount of fluorescence attributed to Metabolite A is based on the difference in fluorescence units found between the two methods. The estimated amount of Metabolite A present is calculated from aqueous Metabolite A standards.

Analysis of Dantrolene in Bile, Tissues, and Feces—The dantrolene method was slightly modified since large amounts of dantrolene and drug-related metabolites were anticipated in bile, tissues, and feces. Homogenize 0.5 g. of tissue or feces in 2.0 ml. of a saturated ammonium sulfate solution in a Potter-Elvehjem glass homogenizer[13]. Add 1.0 ml. of water to the homogenate, mix, and transfer the homogenate to a test tube. Then rinse the homogenizer tube and pestle with 3.5 ml. of water which is added to the homogenate. Bile samples are processed by mixing 0.25 ml. of bile with 2.0 ml. of a saturated ammonium sulfate solution and then adding 4.5 ml. of distilled water.

Mix the homogenate or bile solution with 10.0 ml. of a hexanol–heptane admixture (4:1) by shaking the tube vigorously for 1 min. and then centrifuging at 2000 r.p.m. for 10 min. Remove 9 ml. of the solvent extract (top layer) and place in a test tube. Add 5 ml. of 0.2 *N* sodium hydroxide, shake the tube vigorously for 2 min., and centrifuge at 2000 r.p.m. for 10 min. Remove 4.0 ml. of the sodium hydroxide extract (bottom layer) and place on a hydrated Sephadex G-10 column (0.8 cm. wide × 9.0 cm. long), prepared as described earlier. After the extract passes through the Sephadex, wash the column with succeeding 0.2 *N* sodium hydroxide aliquots of 2.0, 5.0, and 4.0 ml. Then wash the column with 5.0 ml. of a 20% methanol–water solution and discard the eluate. Dantrolene is eluted from the column with 8.0 ml. of a 10% *N,N*-dimethylformamide–water solution. Addition of 2.0 ml. of 0.3 *N* hydrochloric acid to the *N,N*-dimethylformamide eluate adjusts the pH to 1–2. Mix the solution with 4.0 ml. of a nitropropane–heptane admixture (1:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min.

Then remove 3 ml. of the nitropropane–heptane extract (top layer) for fluorometric measurement. Fluorometric measurement is handled exactly as described previously for dantrolene, with the exception that necessary dilutions to avoid quenching are made with nitropropane–heptane (1:1) containing 4.3% hexanol.

Analysis of Acetylated Dantrolene in Plasma, Blood, and Urine—Acetylated dantrolene is determined in biological fluids by slightly modifying the procedure used to determine dantrolene in tissues. Samples are processed by mixing 0.5 ml. of biological fluid with 2.0 ml. of a saturated ammonium sulfate solution and then with 4.5 ml. of distilled water. The dantrolene tissue procedure is then followed as described through the step involving acidification of the *N,N*-dimethylformamide eluate.

The acidified *N,N*-dimethylformamide eluate from the Sephadex column is mixed with 3.5 ml. of an ethyl acetate–nitropropane admixture (9:1) by shaking the tube vigorously for 1 min. and centrifuging at 2000 r.p.m. for 10 min. A 2.5-ml. aliquot of the ethyl acetate–nitropropane extract (top layer) is then removed for fluorometric measurement. The spectrophotofluorometer is set at 3 fluorescence units with a 3-mcg. aqueous acetylated dantrolene standard subjected to the analytical procedure. The fluorescences of the solvent extracts from biological samples are then measured, and the amount of acetylated dantrolene is calculated from aqueous acetylated dantrolene standards. When the fluorescence obtained with acetylated dantrolene exceeds 3 fluorescence units, quenching occurs. Under such conditions the solvent extract is diluted with ethyl acetate–nitropropane (9:1) until a value of 3 fluorescence units or less is obtained.

RESULTS

Analysis of Dantrolene—When subjected to the procedure, dantrolene standards in water, biological fluids, and tissues exhibit linear standard curves from 0.1 to 4.0 mcg. The recoveries of added dantrolene from dog and human biological fluids (plasma, blood, and urine) are presented in Table I. Average recoveries of added dantrolene from rat and monkey biological fluids (plasma, blood, and urine) were 100.9% ± 4.3 *SD* and 99.8% ± 0.4 *SD*, respectively, over a range of 0.1–4.0 mcg. The recoveries of added dantrolene from dog bile and selected tissues are presented in Table II. An average recovery of added dantrolene from selected monkey tissues of 97.1% ± 5.5 *SD* was obtained over a range of 0.1–4.0 mcg. When 4.0 mcg. of dantrolene was added to dog feces, a recovery of 100.2% ± 2.8 *SD* was obtained. Agreement between the aqueous and reference (fluids, tissues, and feces) dantrolene standards makes it unnecessary to analyze reference dantrolene standards with each set of biological samples.

As shown by the results in Table I, the lowest dantrolene level detectable in biological fluids with accuracy is 0.1 mcg./ml., using a 0.5-ml. specimen. However, recent investigation revealed that a

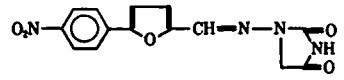
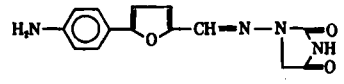
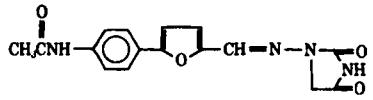
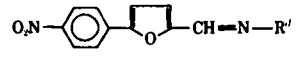
Table II—Recovery of Dantrolene from Dog Bile and Tissues

Fluid or Tissue	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.1	1.0	4.0	Percent Recovery[b], Mean ± SD
Water	0.2	0.7	6.6	29.6	—
Bile	0.3	0.7	6.3	29.4	98.0 ± 2.6
Liver	0.2	0.7	6.7	29.4	100.5 ± 1.7
Kidney	0.2	0.6	6.6	28.8	97.5 ± 4.7
Lung	0.2	0.7	6.7	29.8	100.9 ± 1.2
Muscle	0.2	0.6	6.2	29.4	93.2 ± 6.2
Heart	0.2	0.6	6.7	29.4	98.7 ± 5.1
Spleen	0.2	0.7	6.5	29.4	99.7 ± 1.1
Fat	0.2	0.6	6.5	29.1	95.9 ± 5.9
Brain	0.2	0.6	6.3	28.8	94.7 ± 5.3
				Mean	98.0 ± 3.7

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene standards.

[13] Kontes, size c, K-88550.

Table III—Fluorescence of Dantrolene and Related Metabolites

Compound[a]	Structure	Fluorescence Units[b]
Dantrolene		74.8
Amino-dantrolene		2.4
Acetylated dantrolene		ND[c]
Metabolite A		2.3

[a] Each compound (10 mcg.) was subjected to the method specific for dantrolene. [b] Control-corrected fluorescence units. [c] ND = none detectable. [d] *Reference 5.*

Table IV—Recovery of Dantrolene or Metabolite A from Biological Fluids

Fluid	Fluorescence Units[a] — Dantrolene, mcg. — Control	0.05	0.10	0.50	1.0	2.0	4.0	Percent Recovery[b], Mean ± *SD*	Fluorescence Units[a] — Metabolite A, mcg. — 0.10	0.50	1.0	4.0	Percent Recovery[b], Mean ± *SD*
Water	0.2	0.3	0.7	3.5	7.4	14.8	29.8	—	0.4	2.1	4.4	19.8	—
Dog blood	0.2	0.3	0.6	3.4	7.1	15.1	29.7	96.6 ± 5.6	0.4	2.2	4.3	19.8	100.7 ± 3.0
Dog plasma	0.2	0.3	0.7	3.6	7.2	15.5	30.5	101.1 ± 2.8	0.4	2.1	4.3	19.8	99.5 ± 1.0
Dog urine	0.3	0.3	0.7	3.4	6.8	15.0	29.7	98.1 ± 3.3	0.4	2.2	4.2	20.7	101.0 ± 4.5
Human blood	0.2	0.3	0.7	3.4	7.4	15.1	29.8	99.8 ± 1.6	0.4	2.0	3.9	19.8	95.7 ± 5.7
Human plasma	0.2	0.3	0.7	3.4	7.0	14.7	29.8	98.3 ± 2.4	0.4	2.2	3.9	20.8	99.5 ± 8.0
Human urine	0.2	0.3	0.6	3.3	7.0	14.7	29.1	95.0 ± 5.0	0.4	2.2	4.2	19.8	100.0 ± 4.1

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous dantrolene or Metabolite A standards.

concentration as low as 0.02 mcg./ml. is also detectable. Method sensitivity for dantrolene is 0.2 mcg./g. in tissues (0.5-g. specimen) and 0.4 mcg./ml. in bile (0.25-ml. specimen).

Method Specificity—The specificity of the method for the determination of dantrolene is based on a combination of solvent extractions, chromatography, and the fluorescence characteristics of dantrolene in the nitropropane–heptane admixture (1:1). As shown by the results in Table III, appreciable fluorescence is not obtained when known dantrolene-related metabolites are subjected to the method specific for the determination of dantrolene.

Analysis of Metabolite A—The dantrolene metabolite A is estimated in biological fluids by using a combination of the method specific for dantrolene and a method capable of measuring both dantrolene and Metabolite A simultaneously. Subjection of either dantrolene or Metabolite A standards in water, plasma, blood, and urine to this procedure yields linear curves for both dantrolene and Metabolite A from 0.1 to 4.0 mcg. Recoveries of either added dantrolene or Metabolite A from biological fluids as determined by this procedure are presented in Table IV. The procedure has a sensitivity of 0.2 mcg./ml. (0.5-ml. specimen) for Metabolite A.

Analysis of Acetylated Dantrolene—The acetylated dantrolene metabolite is determined in biological fluids by slightly modifying the method specific for the determination of dantrolene in tissues. When acetylated dantrolene standards in water, plasma, blood, and urine are subjected to this modified method, linear curves are obtained from 0.2 to 3.0 mcg. Recoveries of added acetylated dantrolene from biological fluids as determined by this method are shown in Table V. Method sensitivity is 0.4 mcg./ml. for a 0.5-ml. specimen. Neither dantrolene nor the dantrolene-related metabolites, amino-dantrolene and Metabolite A, interfere in the analysis of acetylated dantrolene by this procedure.

Table V—Recovery of Acetylated Dantrolene from Biological Fluids

Fluid	Fluorescence Units[a] — Acetylated Dantrolene, mcg. — Control	0.2	0.5	1.0	3.0	Percent Recovery[b], Mean ± *SD*
Water	0.4	0.2	0.5	0.8	2.6	—
Human blood	0.5	0.2	0.5	0.8	2.6	100.0 ± 0.0
Human plasma	0.4	0.2	0.5	0.8	2.6	103.0 ± 6.0
Human urine	0.4	0.2	0.5	0.8	2.6	100.0 ± 0.0

[a] Control-corrected data based on a mean from at least three samples. [b] Based on aqueous acetylated dantrolene standards.

DISCUSSION

The described method is sensitive and specific for the determination of dantrolene in biological fluids and tissues. Essentially, the procedure consists of the extraction of dantrolene and two major dantrolene-related metabolites, Metabolite A and acetylated dantrolene, from biological specimens into a solvent admixture and then into a dilute alkaline solution. Metabolite A is then separated and removed from dantrolene and acetylated dantrolene by column chromatography. After both dantrolene and acetylated dantrolene are eluted, dantrolene is extracted into a solvent admixture and measured fluorometrically.

Apparently, blood dantrolene concentrations are related to the resultant skeletal muscle relaxation observed in man after oral administration of dantrolene sodium (6). Since dantrolene-related metabolites also are present in the blood under these conditions (5), a method specific for dantrolene is required to evaluate accurately the drug-induced pharmacological effect in relation to the concurrent blood dantrolene concentration. This method specificity is also necessary for investigating urinary dantrolene excretion since greater amounts of both Metabolite A and acetylated dantrolene than of dantrolene are found in human urine after oral administration of dantrolene sodium (5).

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DRUG STANDARDS

Determination of Liothyronine and Thyroxine in Dried Thyroid by GLC

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Abstract—The analysis reported here was successful in the quantitative determination of liothyronine and thyroxine in driedthyroid. Three basic steps were employed: (*a*) hydrolysis of the dried thyroid to release the iodoamino acids from their protein linkage, (*b*) separation of liothyronine and thyroxine from the interfering substances by extraction and column chromatography, and (*c*) detection and quantitation by GLC.

Keyphrases □ Thyroid, dried—separation, GLC analysis, liothyronine and thyroxine □ Liothyronine and thyroxine—separation, GLC analysis in dried thyroid □ Thyroxine and liothyronine—separation, GLC analysis in dried thyroid □ GLC—analysis, liothyronine and thyroxine in dried thyroid

The thyroid gland contains a number of iodoamino acids, of which the principal hormones secreted are thyroxine and liothyronine. Small amounts of 3,3′,5′-triiodothyronine, diiodotyrosine, and monoiodotyrosine are also present (1). However, biological activity appears to be present only in thyroxine and liothyronine, with little or no activity in 3,3′,5′-triiodothyronine (2, 3). The original method of standardization was done by the determination of total organic iodine (4) or by the "thyronine" iodine content determination of Harington and Randall (5). Although this method is highly nonspecific, it is still being utilized, with slight modifications, as the official USP XVIII method.

GLC appeared to hold a great deal of promise in the determination of thyroid hormones. The results would be specific and would differentiate between the compounds found useful in therapy, namely liothyronine and thyroxine. Other compounds of lesser activity such as 3,3′,5′-triiodothyronine would also be separated and quantitatively determined.

The high molecular weight and the lack of volatility necessitated the formation of a volatile derivative. Derivatives prepared by Stouffer *et al.* (6) and Richards and Mason (7) required a two-step reaction. Zimmerer and Grady (8) used a one-step method to form methyl derivatives by means of diazomethane, but their derivatives eluted as a pair of peaks for both liothyronine and thyroxine and this did not appear suitable for our approach. A one-step derivatization method using a silylating agent appeared to be the method of choice. Although Zimmerer and Grady (8) noted the difficulty in obtaining reproducible silylation, this was not noticed in our work when suitable precautions were taken. Since thyroxine and liothyronine are found bound in a peptide linkage to thyroglobulin, which is present in the colloid material in the follicles of the thyroid gland (1), it is necessary to break this peptide linkage either chemically or enzymatically to obtain the free iodoamino acids prior to GC.

Some authors used alkaline hydrolysis to break the peptide linkage (9–11) while others used various enzymes to achieve the same result (12–15). Generally, the method involved the refluxing of dried thyroid with various bases or the utilization of a number of proteolytic enzymes. The hydrolysates, obtained by either method, were extracted with 1-butanol.

However, the quantitation of liothyronine and thyroxine in the 1-butanol extract by GLC was not feasible due to the presence of interfering substances