Liquid Chromatography in Pharmaceutical Analysis VII: Determination of Dantrolene Sodium in Biological Fluids

S. J. SAXENA, I. L. HONIGBERG ˣ, J. T. STEWART, and J. J. VALLNER

Abstract □ Dantrolene sodium can be determined in plasma and urine samples by high-pressure liquid chromatography without interference from its two major metabolites, the hydroxy and acetamido compounds. The minimum detectability of the drug using this procedure is 8 ng. The complete assay including extraction, evaporation, and separation steps can be performed in approximately 70 min with an accuracy of 1–3%.

Keyphrases □ Dantrolene sodium—high-pressure liquid chromatographic analysis, biological fluids □ High-pressure liquid chromatography—analysis, dantrolene sodium, biological fluids □ Relaxants, skeletal muscle—dantrolene sodium, high-pressure liquid chromatographic analysis, biological fluids

Dantrolene sodium, 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin sodium salt hydrate, first reported by Snyder *et al.* (1), is a peripherally acting skeletal muscle relaxant indicated in controlling the manifestations of clinical spasticity resulting from serious chronic disorders such as spinal cord injury, stroke, cerebral palsy, and multiple sclerosis (2).

Analytical methods previously reported for dantrolene sodium include spectrophotofluorometry (3, 4), differential pulse polarography (5), and a qualitative colorimetric procedure (6). In continuing efforts to apply high-pressure liquid chromatography (HPLC) in pharmaceutical analysis (7–9), an HPLC procedure for the determination of dantrolene sodium in a dosage form was reported (9). This procedure has been adapted to the determination of dantrolene sodium in human plasma and urine in the presence of its two major metabolites, the hydroxy and acetamido compounds (5, 10).

The HPLC procedure described here separates and simultaneously quantitates the drug with no interference from the metabolites. The fluorometric and the polarographic methods require a differential analytical technique to measure the metabolites in the presence of the drug. The complete HPLC assay can be performed in approximately 70 min, including denaturing the proteins, extraction of the drug and its metabolites, chromatographic separation, and quantification. Biological determinations by HPLC were compared with the spectrophotofluorometric method. A preliminary *in vitro* experiment to determine the amount of dantrolene sodium bound to blood cells was also performed.

EXPERIMENTAL

Reagents and Chemicals—Micronized dantrolene sodium[1] powder was used. Reagent grade solvents and chemicals were used as received; solvents constituting the mobile phase, carbon tetrachloride and dimethylformamide, were filtered through a 0.5-μm filter[2] prior to use. The pH of a 0.5 *M* aqueous solution of dimethylformamide was consistently 6.8 ± 0.1.

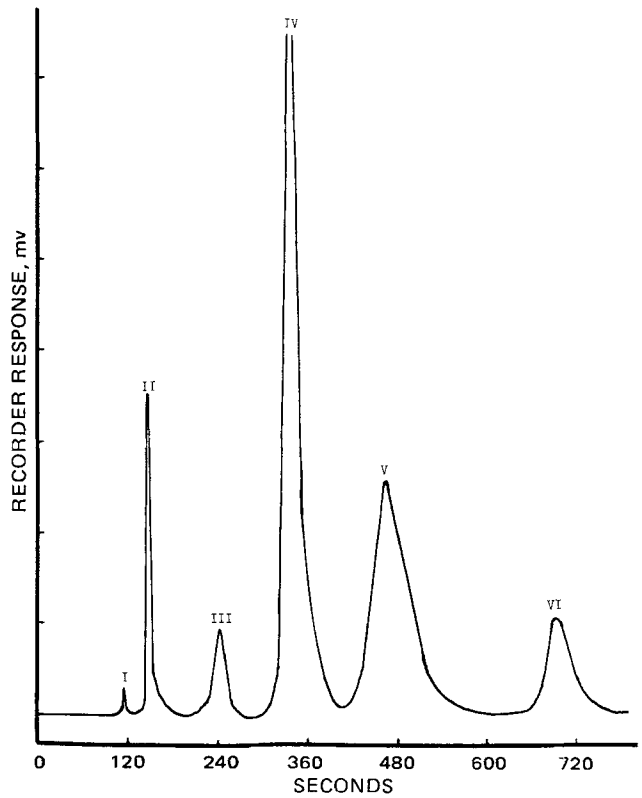

Figure 1—*Illustrative liquid chromatogram of dantrolene, its known metabolites, and furil (internal standard) in butanol–chloroform at a flow rate of 2.0 ml/min. The mobile phase was carbon tetrachloride–dimethylformamide (90:10). Key: I, solvent front; II, furil; III, impurity in dantrolene sodium; IV, dantrolene; V, hydroxy metabolite; and VI, acetamido metabolite.*

Mobile Phase—Carbon tetrachloride–dimethylformamide (90:10) was used as the mobile phase. The mixture was degassed before use.

Internal Standard Solution—Powdered furil[3] was dissolved in 1-butanol–chloroform (30:70) to give a stock internal standard solution of 400 μg/ml. This solution was filtered through a 0.5-μm filter[4] prior to use.

Standard Solution for Calibration Curve—A stock solution of dantrolene sodium (5 μg/ml) was prepared by dissolving powdered dantrolene sodium in 0.9% sodium chloride solution.

Calibration Curve—Into individual 60-ml separators were placed accurately pipetted volumes of 1.0, 2.0, 3.0, and 4.0 ml of the dantrolene sodium stock solution. The volume of each solution was adjusted to 7.0 ml with 0.9% sodium chloride solution. Then 40 ml of water-saturated 1-butanol–chloroform (30:70) and 2 ml of 0.1 *N* HCl were added. The solutions were shaken to partition the dantrolene acid into the organic layer. Then solid ammonium sulfate, about 1 g/ml of aqueous solution, was added in small portions to each solution and gently shaken for 5 min.

The solutions were set aside for 5 min, and then the organic layer (lower) was collected. The aqueous layer was shaken with another 40 ml of 1-butanol–chloroform, and the wash was combined with the previous

[1] Courtesy of Eaton Laboratories, Norwich, N.Y.
[2] Catalog No. FHLP04700, Millipore Corp., Bedford Mass.
[3] Catalog No. 2040, Eastman Organic Chemicals, Rochester, N.Y.
[4] Catalog No. LSWF01300, Millipore Corp., Bedford, Mass.

Table I—Calibration Data for Standard Drug Solutions

Initial Concentration of Dantrolene Sodium, μg/ml	Observed Results[a]
0.7143	2.0689 ± 0.0114
1.4286	3.9922 ± 0.0595
2.1429	6.1423 ± 0.0318
2.8571	8.0774 ± 0.0974
Slope = 0.4035	
Intercept = 0.0263	
$r \pm s_{yx}$[b] = 0.9998 ± 0.0495	

[a] D/IS ratio is based on three replicate injections of standard solution. Table value shows the mean ± *SD*. D/IS is the ratio of the integrated area of the drug at some concentration to the integrated area of furil at 50 μg/ml. [b] *r* is the correlation coefficient calculated from linear regression analysis, and s_{yx} is the sample standard deviation, also from regression analysis.

organic layer. The combined organic layer was evaporated to dryness in a hot water bath (~70°) under a stream of nitrogen. The residue was dissolved in 500 μl of 1-butanol–chloroform, and 500 μl of the internal standard solution was added. The solution was then filtered through a 0.5-μm filter, and 40 μl was injected into the liquid chromatograph.

Dantrolene Sodium in Human Plasma and Urine—To 3.0 ml of biological fluid was added 10 μg of dantrolene sodium. This solution was treated in the same manner as the standard solutions described under *Calibration Curve*. A control blank using 0.9% sodium chloride solution instead of the biological fluid was also included. Replicate injections of 40 μl were made for each sample.

***In Vitro* Binding of Dantrolene Sodium to Whole Blood Cells**—Dantrolene sodium (10 μg) was added to each of three 5-ml samples of whole blood; these samples were gently shaken and set aside for equilibration at room temperature for 5 min. The samples were then centrifuged at 1000 rpm for 20 min. The supernatant plasma from each tube was transferred to separate separators. A volume of 0.9% sodium chloride solution equal to the volume of the plasma removed was added to each packed cell sample, and the mixture was shaken gently and centrifuged again at 1000 rpm for 20 min.

The supernates from each tube were transferred to the respective separators, and the wash procedure was repeated once again. The plasma and washings in each separator were extracted and analyzed in the same manner as outlined under *Dantrolene Sodium in Human Plasma and Urine*. Injections of 40 μl were made.

Chromatographic Conditions—A liquid chromatograph equipped with a pump[5], a variable wavelength detector[6], an integrator with a digital printout[7], and a column containing a porous silica packing[8] was used. The degassed mobile phase was pumped through the column at 2.0 ml/min (1800 psig) at room temperature (23 ± 2°) until a stable baseline was obtained. Replicate 40-μl injections of samples and standard solutions were made using a 100-μl syringe[9]. A record of drug elution from the column as peaks on a chromatogram was provided by a chart recorder. The peaks were detected at 375 nm. The solute was measured by digital integration of the peak area in each case.

Determination of Dantrolene Sodium by Fluorescence—Spiked samples of urine and blood plasma, each containing 10 μg of dantrolene sodium, were assayed by a previously reported fluorometric method (9). The extraction procedure was identical to the one used for analysis by HPLC except that the final residue, after evaporation, was diluted to 10 ml with 1-butanol–chloroform (30:70). The fluorescence of each solution was measured in a spectrophotofluorometer[10] with the following operating parameters: excitation and emission slit widths, 5 nm; sensitivity, 3.0; excitation wavelength, 395 nm; and emission wavelength, 575 nm.

With the fine sensitivity control, the recorder was set to read full scale (100 units) for a 1-butanol–chloroform solution obtained from a 0.9% sodium chloride control containing 10 μg of dantrolene sodium, which was carried through the same extraction procedure as the biological fluids. Measurements for the biological fluid extracts were made relative to this arbitrary setting.

[5] Waters Associates liquid chromatograph, model ALC/GPC 201, equipped with an M-6000 pump.
[6] Perkin-Elmer model LC-55 variable wavelength UV–visible detector.
[7] Infotronics integrator model CRS-204.
[8] Waters packed <10-μm μPorasil column, 4 mm i.d. × 30 cm.
[9] Model B-110, Precision Sampling Corp., Baton Rouge, La.
[10] Perkin-Elmer fluorescence spectrophotometer model MPF-4.

Table II—Determination of Dantrolene Sodium in Human Plasma and Urine

Sample	Amount of Dantrolene Sodium Added, μg	HPLC, % Found[a]	Fluorometric, % Found[a]
Urine	10	91.27 ± 0.47[b]	90.94 ± 1.32[b]
Plasma	10	82.67 ± 1.09	81.68 ± 0.92

[a] Based on triplicate determinations of three individual samples. [b] Confidence limits at $p = 0.05$.

RESULTS AND DISCUSSION

Dantrolene sodium is converted to dantrolene acid in acidic media and can be extracted into an organic solvent (9). In biological fluids, an additional protein precipitation step was necessary. Precipitation was accomplished by the use of 1 g of ammonium sulfate/ml of aqueous solution. In addition, the smaller concentrations of dantrolene sodium in biological fluids necessitated the concentration of the organic extract from biological fluids prior to the HPLC analysis.

Dantrolene was eluted within 6 min (Fig. 1). Figure 1 illustrates the elution and separation of the two major metabolites of dantrolene, the hydroxy and acetamido compounds. These metabolites were well-separated from the parent drug and internal standard. The presence of the metabolites had no significant effect on the ratio of the drug to the internal standard (D/IS), even when present in equal concentrations. All components were detectable at the 375-nm wavelength used in this procedure. Figure 2 shows a typical chromatogram for dantrolene sodium from a spiked plasma sample.

The area under the curve for each peak on the chromatogram was digitally integrated. The D/IS ratio was calculated for each dantrolene concentration. A linear regression analysis of the data at the four standard concentrations of dantrolene sodium gave the results shown in Table I.

To determine the percent accuracy of the procedure, two known concentrations of dantrolene sodium were treated as unknowns and their

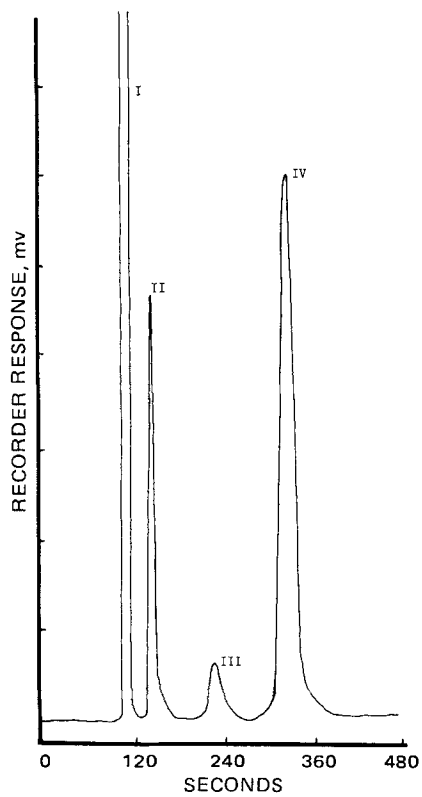

Figure 2—*Liquid chromatogram of dantrolene from a spiked plasma sample of dantrolene sodium at a flow rate of 2.0 ml/min. The mobile phase was carbon tetrachloride–dimethylformamide (90:10). Key: I, unidentified plasma components at solvent front; II, furil (internal standard); III, impurity in dantrolene sodium; and IV, dantrolene.*

Table III—Recovery Data of Dantrolene Sodium[a]

Sample	D/IS Ratio[b]	Recovery[c], %
Water	4.0368 ± 0.2148	99.4
Plasma	3.3620 ± 0.1916	82.7
Whole blood	2.7903 ± 0.0312	68.5

[a] Ten micrograms of dantrolene sodium was added to each sample. [b] Based on triplicate injections of three individual samples. [c] Calculated from linear regression analysis data.

concentrations were calculated from the constants (slope and intercept) obtained from the linear regression analysis. The concentration was calculated from the equation: D/IS = (slope × concentration) + intercept, using a programmable calculator[11]. Accuracy was 1–3% for the overall procedure.

The results for the spiked biological samples obtained from HPLC and fluorometry are shown in Table II. There was no significant difference between the values obtained by the two techniques at $p = 0.01$.

Recovery data of dantrolene sodium from water, plasma, and whole blood are shown in Table III. Extraction of the drug from spiked plasma accounted for approximately 82% of the added dantrolene. In the whole blood investigations, about 31.5% of the drug was not found in the plasma portion. Blood cells may account for about 15% binding of the dantrolene.

In summary, this procedure is useful for the determination of drug in the presence of its two major metabolites with a minimum detectability of 8 ng.

[11] Olivetti-Underwood Programma 101.

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Polynitro Aromatic Compounds in Analytical Chemistry I: Reaction with Ouabain and Digitoxin

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Abstract □ By the use of NMR spectroscopy, the highly colored reaction products formed by ouabain or digitoxin with 1,3,5-trinitrobenzene or 2,4,6-trinitroanisole in the presence of alkali (as used for the determination of these glycosides) are shown to be Meisenheimer complexes. The complexes are produced by attachment of a carbon of the butenolide ring to an aromatic carbon of the nitro compound with formation of a charge-delocalized cyclohexadienate anion.

Keyphrases □ Nitro aromatic compounds—1,3,5-trinitrobenzene and 2,4,6-trinitroanisole, NMR spectral characterization of products □ Ouabain—reaction with 1,3,5-trinitrobenzene or 2,4,6-trinitroanisole, NMR spectral characterization of products □ Digitoxin—reaction with 1,3,5-trinitrobenzene or 2,4,6-trinitroanisole, NMR spectral characterization of products □ NMR spectroscopy—characterization of products of reaction of ouabain and digitoxin with 1,3,5-trinitrobenzene or 2,4,6-trinitroanisole □ Cardiotonic agents—ouabain and digitoxin, reaction with 1,3,5-trinitrobenzene or 2,4,6-trinitroanisole, NMR spectral characterization of products

The most recent review article on the chemical estimation of ouabain and digitoxin was published in 1949 by Canbäck (1). In 1950, the same author (2) reported further results on the reaction of these cardiotonic glycosides with dinitro aromatic compounds in the presence of alkali. Canbäck attributed the intense color produced to the transfer of a proton from the butenolide ring of the glycoside to a ring carbon of the nitro compound. Later, Kimura (3) investigated the reaction between digitoxin and picric acid and proposed that the colored product resulted from the addition of the butenolide ring to one of the two unsubstituted carbons of the picric acid.

In the present work, NMR studies established that ouabain and digitoxin give Meisenheimer complexes (charge-delocalized cyclohexadienate anions) with both 1,3,5-trinitrobenzene and 2,4,6-trinitroanisole in dimethyl sulfoxide as the solvent. Comparison of the visible spectra of the products with those of materials produced in aqueous methanol (which more nearly approximates the normal assay medium) suggested that the colored components are the same in the two cases.

EXPERIMENTAL[1]

Visible Spectra—Approximately 3×10^{-5} *M* solutions of ouabain and digitoxin were used in a 1-cm cell. Excess 2,4,6-trinitroanisole and sodium hydroxide were added, and the spectra were measured against a reagent blank. The positions of the absorption maxima were: ouabain–trini-

[1] Visible spectra were obtained using a Cary model 15 UV-visible spectrophotometer. NMR spectra were run on a Jeolco MH-60-II spectrometer.