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## Note

# Simultaneous determination of dantrolene and its metabolite, 5-hydroxydantrolene, in human plasma by high-performance liquid chromatography

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Dantrolene sodium, 1-{[5-(*p*-nitrophenyl)furfurylidene]amino}hydantoin sodium salt hydrate, is a muscle relaxant which acts directly on skeletal muscle [1—3]. Dantrolene sodium is metabolized in the liver principally by 5-hydroxylation of the hydantoin group (5-hydroxydantrolene) [4—7] and for a small part by reduction of the nitro group followed by acetylation (acetylated dantrolene) [4]. The major metabolite, 5-hydroxydantrolene, exhibits skeletal muscle relaxing in rats [8], and presumably has pharmacological activity in humans [9—10].

Many methods have been reported for the quantitation of dantrolene in biological fluids: differential pulse polarography [4], spectrophotofluorometry [5—11] and a colorimetric procedure [6]. Recently, the quantitation of dantrolene using high-performance liquid chromatography (HPLC) has been reported by Saxena and co-workers [12, 13] and Hackett and Dusci [14]. However, the major interest in these papers was the quantitation of dantrolene itself and they were not successful in the separate determination of 5-hydroxydantrolene.

Although Hollifield and Conklin's [5] method is one of the most reliable analytical procedures for determining 5-hydroxydantrolene specifically, in

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practice it is time-consuming for a chemist to analyze large numbers of samples at the same time because of the lengthy procedures involved. Furthermore, the method is not sufficient for determining low plasma levels of 5-hydroxydantrolene after administration of a clinical dose of dantrolene sodium in humans.

Therefore, a simple and sensitive method was required to deal with large numbers of clinical samples. This paper describes the simultaneous, simple and rapid quantitation of dantrolene and 5-hydroxydantrolene by HPLC in plasma after the administration of a clinical dose of dantrolene sodium to humans. The minimum detectable concentration of dantrolene and 5-hydroxydantrolene in human plasma was about 0.03 μg/ml each.

## EXPERIMENTAL

### *Chemicals*

Dantrolene sodium and 5-hydroxydantrolene were used as received (Morton-Norwich, New York, U.S.A.). All reagents used were of reagent grade supplied by Wako (Osaka, Japan).

### *Apparatus*

The HPLC system consisted of a solvent pump (Model 6000A, Waters Assoc.), a reversed-phase HPLC column (LiChrosorb RP-18, 10-μm particle size, 30 cm × 4 mm I.D.; E. Merck, Darmstadt, G.F.R.), and a variable-wavelength UV detector (Model UVIDEC-100-II, Japan Spectroscopic Co.) set at 310 nm. The plasma extracts were injected into the HPLC column. The mobile phase was prepared by mixing equal volumes of methanol and 0.1 *M* acetate buffer (pH 7.4) and this eluent was passed through the HPLC system at a rate of 1.0 ml/min.

### *Extraction procedure*

Plasma samples (0.5 ml) were placed in 10-ml ground-glass stoppered test-tubes, and 2 ml of 0.2 *M* acetate buffer solution (pH 4.0) and 50 μl of benzanilide methanol solution (25 μg/ml) were added. After shaking for 1 min, 5 ml of ethyl acetate were added and shaken for 10 min. The mixture was centrifuged at 1500 *g* for 10 min. Then 4.5 ml of the organic layer were transferred to a 10-ml test-tube and evaporated to dryness at 75°C under a gentle nitrogen stream. Then the residue was dissolved in 50 μl of methanol—0.1 *M* acetate buffer solution, pH 7.4 (50:50, v/v) and, after centrifugation (10 min at 1500 *g*), 10—20 μl of the upper layer were injected into the HPLC system. All determinations were performed by calculating the peak height ratios of each compound to the internal standard. A standard curve was run with each set of samples.

## RESULTS AND DISCUSSION

Hackett and Dusci [14] reported a simple protein-precipitation technique to determine dantrolene in human plasma using HPLC. However, the report failed to separate 5-hydroxydantrolene from dantrolene specifically in human

plasma, probably due to low recovery of 5-hydroxydantrolene under the extraction conditions and low separative ability of the eluent used.

In the present study, recoveries of dantrolene sodium and 5-hydroxydantrolene in the pH range 3.0—6.5 were measured. At lower pH, the recoveries of dantrolene and the metabolite were satisfactory but dantrolene suffered decomposition during the extraction procedure. Therefore, acetate buffer solution of pH 4.0 was selected for extraction of dantrolene and 5-hydroxydantrolene from plasma samples. Concerning the HPLC eluent, methanol—0.1 *M* acetate buffer gave satisfactory separation of the drug and the metabolite as demonstrated in Fig. 1. To increase the reliability of the assay method, an internal standard (benzanilide) was incorporated into the plasma sample.

Dantrolene and 5-hydroxydantrolene have maximum UV absorbance at about 400 nm in the mobile phase. At this wavelength, however, peaks (possibly derived from human plasma) interfering with dantrolene and 5-hydroxydantrolene appeared on the chromatogram. Therefore, various wavelengths were explored and more clearly separated peaks were obtained with the detector set at 310 nm. Fig. 1 shows the chromatograms of blank plasma, plasma spiked with dantrolene, 5-hydroxydantrolene and internal standard determined at 310 nm. No interference by the normal plasma components was noted, except a small peak at the retention time about 10 min. The separation of dantrolene, 5-hydroxydantrolene and the internal standard was found to be satisfactory.

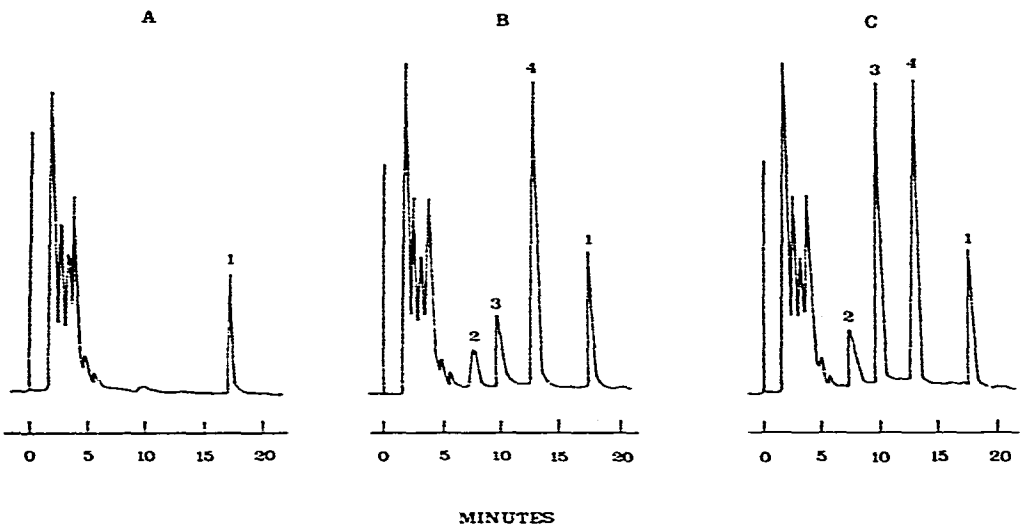


Fig. 1. Illustrative HPLC separation of dantrolene, 5-hydroxydantrolene and benzanilide (internal standard). The mobile phase was methanol—0.1 *M* acetate buffer, pH 7.4 (50:50, v/v); 10 μl of each of the samples below were applied to the column. (A) Human plasma blank; (B) plasma extract from a sample spiked with dantrolene (injected quantity of dantrolene, 0.02 μg), 5-hydroxydantrolene (injected quantity of 5-hydroxydantrolene, 0.02 μg) and benzanilide (injected quantity of benzanilide, 0.50 μg); and (C) a plasma sample obtained from a volunteer 6 h after oral administration of 50 mg of dantrolene sodium. Peaks: 1, unidentified plasma component; 2, 5-hydroxydantrolene; 3, dantrolene; 4, benzanilide (internal standard).

Recoveries of added dantrolene and 5-hydroxydantrolene from plasma are shown in Table I. The calibration curves for dantrolene and 5-hydroxydantrolene were determined by adding known quantities of dantrolene and 5-hydroxydantrolene to reference plasma samples. Statistical analysis of the data by linear regression indicated excellent linearity with correlation coefficients of 0.9999 and 0.9998, slopes of 1.179 and 0.969, and intercepts of 0.106 and −0.014 in the range of 0.031—1.000 μg/ml of plasma for dantrolene and 5-hydroxydantrolene, respectively.

TABLE I

VALUES OF PLASMA CALIBRATION CURVE POINTS AND RECOVERIES OF DANTROLENE AND 5-HYDROXYDANTROLENE FROM HUMAN PLASMA

$n$ = 3 in all cases.

| Sample | Concentration added (μg/ml) | Peak height ratio between sample and internal standard | Recovery (%) |
|---|---|---|---|
| Dantrolene | 0.031 | 0.143 ± 0.007* | – |
| | 0.063 | 0.180 ± 0.008 | 83.2 ± 4.1* |
| | 0.125 | 0.253 ± 0.008 | – |
| | 0.250 | 0.399 ± 0.009 | – |
| | 0.500 | 0.696 ± 0.008 | 87.0 ± 0.8 |
| | 1.000 | 1.285 ± 0.008 | – |
| 5-Hydroxydantrolene | 0.031 | 0.016 ± 0.001 | – |
| | 0.063 | 0.055 ± 0.004 | 66.1 ± 4.8 |
| | 0.125 | 0.104 ± 0.002 | – |
| | 0.250 | 0.228 ± 0.003 | – |
| | 0.500 | 0.458 ± 0.004 | 70.1 ± 0.6 |
| | 1.000 | 0.960 ± 0.005 | – |

*Mean ± S.E.

Plasma levels of dantrolene and 5-hydroxydantrolene after oral administrations of 50 mg of dantrolene sodium to six volunteers are shown in Fig. 2 as a function of time. Peak plasma levels of dantrolene of 0.50—0.95 μg/ml were attained at 4—8 h after administration, and peak plasma levels of 5-hydroxydantrolene of 0.11—0.30 μg/ml were attained at 6—8 h after administration. Plasma levels of 5-hydroxydantrolene were found to be 10—30% of those of simultaneously determined dantrolene.

Dantrolene is also metabolized to acetylated dantrolene, in which the nitro group of dantrolene is acetylated [4]. Acetylated dantrolene (which was found in urine, but not in plasma [7]) has a retention time of about 3 min. But since the peak of acetylated dantrolene was interfered with by the urine components, this metabolite could not be determined by this method.

The major advantages of the proposed method are its simplicity and rapidity. Both dantrolene and 5-hydroxydantrolene are determined in a single extraction procedure. Moreover, the method is sufficiently sensitive to determine 5-hydroxydantrolene in human plasma which contains 0.03—1.00 μg/ml of

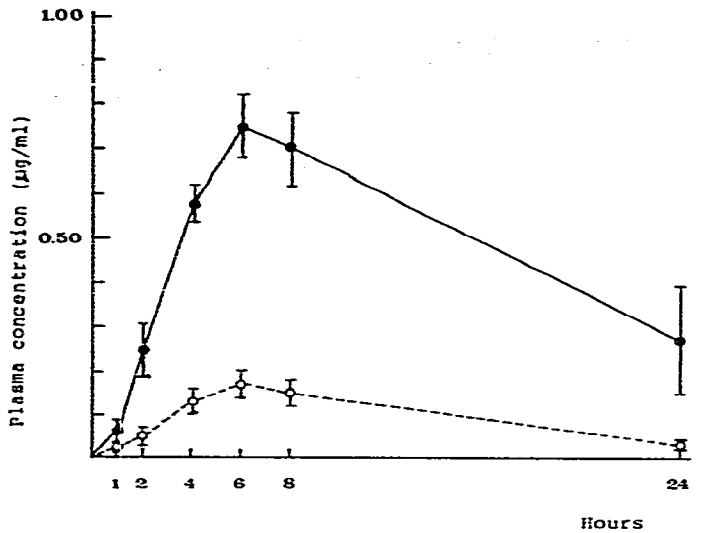


Fig. 2. Plasma concentrations of dantrolene (●——●) and 5-hydroxydantrolene (○— — —○) in plasma after a single 50-mg oral dose of dantrolene sodium given to a human. Each point represents the mean ± S.E. from six normal humans.

the metabolite following oral administration of a clinical dose of dantrolene sodium.

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