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Note

Determination of dantrolene and its reduced and oxidized metabolites in plasma by high-performance liquid chromatography

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Dantrolene sodium, 1-[5-(*p*-nitrophenyl)furfurylideneamino]hydantoin sodium salt hydrate, is a muscle relaxant which acts directly on skeletal muscle [1,2]. More recently, it has been used for the treatment of malignant hyperthermia occurring during anaesthesia [3,4]. The metabolites of dantrolene have been identified as the 5-hydroxydantrolene resulting from the oxidation of the hydantoin ring [5] and aminodantrolene obtained by reduction of the nitro group on the phenyl ring. This latter compound is further metabolized by acetylation [6] giving acetamidodantrolene. Of these metabolites, only 5-hydroxydantrolene has been shown to have some pharmacological activity [7]. Numerous assays have been developed for the determination of dantrolene and its metabolites [6,8–13]. Previous assays used spectrofluorometry [8], pulse polarography [6] and colorimetry [9]. These assays measure dantrolene and perhaps one, if any, of its metabolites. High-performance liquid chromatography (HPLC) has also been used by different groups [10–13] but each assay has drawbacks such as the lack of an internal standard, the requirement for a large amount of biological material, the lack of identification or quantitation of the three metabolites. In order to determine the pharmacokinetics of dantrolene and its metabolites in patients with malignant hyperthermia and in animals, we developed a simple assay that measures all three metabolites as well as dantrolene. This HPLC method uses a small volume of plasma (50 μl) and has a sensitivity of 1.0 μg/ml.

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EXPERIMENTAL

Reagents and chemicals

Dantrolene sodium and its three metabolites were kindly supplied by Norwich-Eaton Pharmaceuticals (Norwich, NY, U.S.A.). Stock solutions (1 mg/ml) of each compound were prepared in N,N-dimethylformamide and were kept at −20°C in the dark. Other materials were obtained from known commercial sources and were of HPLC grade.

Synthesis of methyldantrolene

Dantrolene sodium (0.05 mmol) was dissolved in 0.1 *M* sodium hydroxide containing 0.35 mmol of tetrabutylammonium hydroxide. Extractive alkylation was performed with methyl iodide (0.5 mmol) in methylene chloride with vigorous shaking for 60 min at room temperature. Afterwards, the organic phase was washed twice with 5 ml of water, dried over sodium sulfate and evaporated to dryness. Upon analysis of the residue, under the same HPLC conditions described below, only one peak was obtained showing that the reaction was complete. A stock solution (1 mg/ml) of methyldantrolene was also prepared in N,N-dimethylformamide and diluted to 10 μg/ml in acetonitrile as the internal standard.

Apparatus

The HPLC system (Waters Scientific Assoc.) consisted of a solvent pump (Model 45), a U6K injector, a Model 480 spectrophotometer set at 375 nm (0.02 a.u.f.s.) and a Houston Instruments strip-chart recorder. The filtered (0.2 μm) and degassed mobile phase, acetonitrile–20 m*M* glycine (35:45, v/v), final pH 3.6 with phosphoric acid, was run at room temperature at a flow-rate of 2 ml/min through a LiChrosorb RP-18 10 μm, 250 cm×4.6 mm I.D. column (Merck).

Sample preparation and calibration

Blank human or rat plasma was spiked with dantrolene and its metabolites over the range 1.0–10 μg/ml. To standard or unknown plasma (50 μl) in a small polypropylene centrifuge tube were added 100 μl of acetonitrile containing the internal standard (10 μg/ml). After vortexing for 30 s, the tubes were centrifuged at 1500 *g* at room temperature for 5 min. An aliquot of the supernatant (25 μl) was then injected into the liquid chromatograph.

From the chromatogram of the standard plasmas, we calculated the peak-height ratio of each compound as compared to methyldantrolene. A calibration curve was then constructed by plotting the concentration of each compound against its respective peak-height ratio. Unknown samples were quantified by reference to these standard curves.

Recovery experiments

Samples containing all the standard compounds at two concentrations, 1.5 and 10 μg/ml, as well as methyldantrolene at 10 μg/ml were prepared in plasma or acetonitrile and treated as described above. The percentage recovery was deter-

mined by comparing the heights of the peaks obtained from the acetonitrile samples with the heights obtained from the standards.

RESULTS AND DISCUSSION

Fig. 1 presents a chromatogram obtained from the above procedure with (A) drug-free plasma, (B) from plasma spiked with 5 μg/ml of each of the compounds and (C) from a patient sample which was taken 24 h post-operation. Under these conditions, the retention times of the compounds of interest are: acetamidodantrolene, 2.6 min; aminodantrolene, 4.8 min; 5-hydroxydantrolene, 5.6 min; dantrolene, 6.4 min; and methyldantrolene, 11.2 min.

The intra-day variations at three different concentrations are shown in Table I. Five independently prepared samples were used for each set of concentrations. The results obtained were within satisfactory limits and the highest variation was obtained (8%) with dantrolene at a concentration of 5 μg/ml. In Table II, we have listed the variations of the same samples run on different days over a period of seven days. Again, the back-calculated concentrations as well as the coefficients of variation were within acceptable limits.

Different sets of samples were run for the estimation of the linearity of this assay over the range 1–10 μg/ml. For each compound, the intercepts obtained were not significantly different from zero ranging from −0.04 to 0.03. The slopes were within a 10% difference from day to day and the coefficients of linear correlation (r) were always higher than 0.997 ($n=10$). The mean recoveries for dantrolene, aminodantrolene, acetamidodantrolene, 5-hydroxydantrolene and methyldantrolene were 115, 118, 111, 121 and 106%, respectively. The limit of

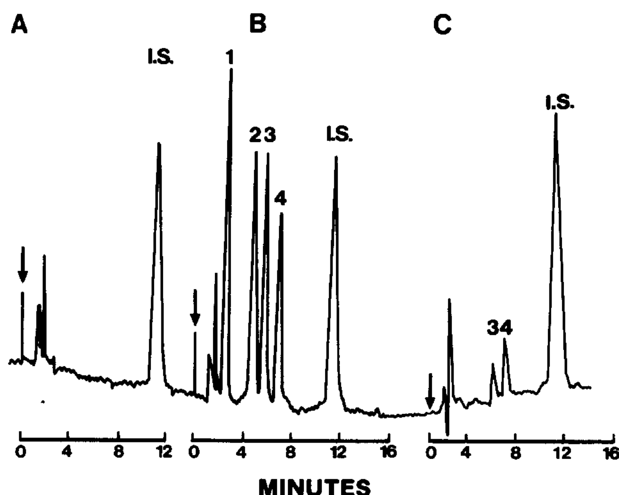

Fig. 1. (A) Drug-free plasma containing 10 μg/ml methyldantrolene (I.S.). (B) Drug-free plasma spiked with 5 μg/ml of each of acetamidodantrolene (1), aminodantrolene (2), 5-hydroxydantrolene (3), dantrolene (4) and I.S. (10 μg/ml). (C) Patient plasma sample which was taken 24 h post-operation. The concentrations of 5-hydroxydantrolene and dantrolene are 0.8 and 1.3 μg/ml, respectively. The arrows indicate the injections.

TABLE I

INTRA-DAY VARIATIONS FOR DANTROLENE AND ITS METABOLITES FROM INDEPENDENTLY PREPARED PLASMA SAMPLES ANALYZED ON THE SAME DAY

Values in parentheses are within-day precisions (percentage of variation).

Nominal concentration (μg/ml)	Mean determined concentration ($n=5$) (μg/ml)			
	Dantrolene	Amino-dantrolene	Acetamido-dantrolene	5-Hydroxy-dantrolene
1.0	1.1 (5.6)	1.2 (6.7)	1.1 (5.9)	1.1 (7.0)
5.0	4.7 (8.0)	4.9 (5.5)	4.8 (4.0)	4.9 (5.6)
10.0	10.0 (0.7)	10.0 (0.8)	10.0 (1.1)	10.1 (3.5)

TABLE II

INTER-DAY VARIATIONS FOR DANTROLENE AND ITS METABOLITES AS DETERMINED BY THE ANALYSES OF STANDARDS ON DIFFERENT DAYS

Values in parentheses are inter-day precision (percentage of variation).

Nominal concentration (μg/ml)	Mean determined concentrations ($n=5$) (μg/ml)			
	Dantrolene	Amino-dantrolene	Acetamido-dantrolene	5-Hydroxy-dantrolene
1.0	1.1 (5.0)	1.2 (3.3)	1.1 (4.5)	1.2 (8.0)
5.0	4.8 (2.1)	4.7 (2.2)	4.7 (2.4)	4.5 (2.2)
10.0	10.1 (0.6)	10.1 (0.5)	10.1 (0.6)	10.1 (0.6)

reliable concentration was conservatively set at 1.0 μg/ml. The stability of the plasma standards was checked over a one-week period. If protected from light, and kept at −20°C, the plasma standards are stable for this period of time as has been previously reported [12]. Due to the use of a visible wavelength, we did not encounter interference from other drugs or plasma constituents despite the protein precipitation technique we employed. Even drugs with absorption around 375 nm such as tetracycline and certain benzodiazepines do not interfere with the analysis. Furthermore, the application of this procedure is demonstrated (Fig. 2) with the plasma dantrolene concentration–time profiles obtained from two patients. Each patient received a total dose of 5 mg/kg of oral dantrolene in four doses, every 6 h with the last dose 4 h before their operation. Plasma samples were taken at induction of anesthesia and every 6 h thereafter. In order to increase the sensitivity, we tried a solvent extraction with diethyl ether. However, due to the instability of dantrolene at low pH and to the poor extraction of aminodantrolene, it was impossible to obtain reliable recoveries even if, at pH 4.0, the human plasma was clean. Other solvents such as ethyl acetate and chloroform–butanol which has been used [6,12] gave interfering peaks with acetamidodantrolene.

In conclusion, this HPLC assay is rapid, requires a small amount of plasma

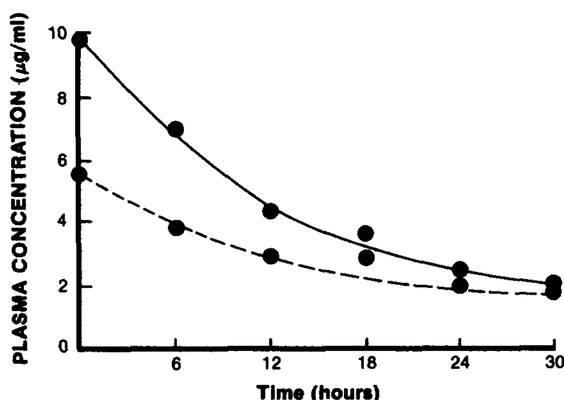

Fig. 2. Plasma dantrolene concentration-time curves following oral dantrolene (5 mg/kg) given in four divided doses prior to induction of anesthesia (time 0) in two patients.

and has a related internal standard. Although less sensitive than two previous methods [12,13], it has enough sensitivity for steady-state pharmacokinetics in patients with malignant hyperthermia as well as metabolite pharmacokinetic studies in animals.

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