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SIMULTANEOUS DETERMINATION OF DANTROLENE AND ITS METABOLITES, 5-HYDROXYDANTROLENE AND NITRO-REDUCED ACETYLATED DANTROLENE (F 490), IN PLASMA AND URINE OF MAN AND DOG BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of the skeletal muscle relaxant dantrolene and its metabolites, 5-hydroxydantrolene and nitro-reduced acetylated dantrolene (F 490), in plasma and urine of man and dog. The substances are detected spectrophotometrically at 375 nm. The detection limits are 0.02 mg/l. A preliminary extraction step into a chloroform—butanol mixture is required for the plasma samples. The method is suitable for pharmacokinetic studies of dantrolene.

INTRODUCTION

Dantrolene sodium, 1-{[5-(*p*-nitrophenyl)-furfurylidene]-amino}-imidazolidine-2,4-dione sodium salt hydrate, first reported by Snyder et al. [1], is used as a skeletal muscle relaxant which appears to act by blocking muscle contraction beyond the neuromuscular junction [2—5]. It is used for the symptomatic relief of clinical spasticity resulting from serious disorders such as cerebral palsy, stroke, spinal cord injury, and multiple sclerosis [6—13]. More recently, it has also been recommended for the prevention and treatment of malignant hyperthermia, a syndrome recognized as one of the causes of anaesthesia-related deaths [14—16].

Metabolism of dantrolene (see Fig. 1) has been shown to proceed through both reductive and non-reductive pathways [17]. The nitro group of dantrolene is reduced to the corresponding amine, and in man and some animals subsequently acetylated to yield nitro-reduced acetylated dantrolene (F 490).

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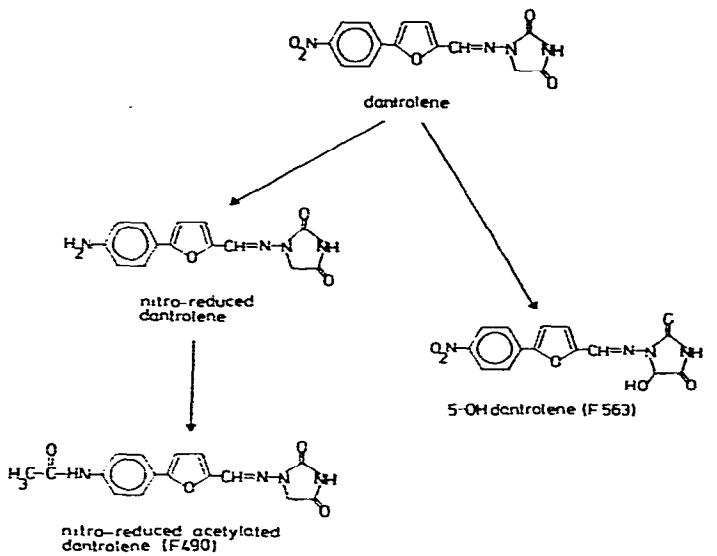

Fig. 1. Proposed metabolic scheme of dantrolene [18].

Of these reduced metabolites, which do not possess muscle relaxant properties [18], only F 490 has been detected in human blood [17,19] and urine [17,20]. Oxidation of dantrolene results in 5-hydroxydantrolene [21], a metabolite with muscle relaxant effects; the activity is less than dantrolene on an equimolar basis [18]. This 5-hydroxy metabolite has been identified in human blood [17,19,20,22,23] and urine [17,20].

Several analytical methods have been developed for the determination of dantrolene in blood and urine, including spectrophotofluorimetry [24,25], differential pulse polarography [17], high-performance liquid chromatography (HPLC) [26,27], and a qualitative colorimetric procedure [28]. The fluorimetric and polarographic methods require complicated differential analytical techniques to measure the drug in the presence of its metabolites. Meyler et al. [22], who used the fluorimetric procedure for plasma concentration measurements in volunteers and patients, encountered a discrepancy in the method which was not further investigated. They found that the total fluorescence of an extract from plasma containing dantrolene and 5-hydroxydantrolene (direct method) was considerably less than the fluorescence measured after the extract had passed through a Sephadex column and the two fractions containing 5-hydroxydantrolene and dantrolene, respectively, had been combined (indirect method).

The first reported HPLC procedure for dantrolene has the disadvantage of using a non-aqueous mobile phase [19,26]. In a short communication Hackett and Dusci [27] reported a reversed-phase HPLC procedure with, however, limited sensitivity and selectivity. Only dantrolene itself was measured, in concentrations down to 0.25 mg/l. To investigate the pharmacokinetics of dantrolene in volunteers and patients a new reversed-phase HPLC procedure was developed which allows the measurement of the drug and its metabolites at levels as low as 0.02 mg/l.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals were of analytical grade. Three separate standard solutions were prepared in N,N-dimethylformamide containing 0.5, 0.5 and 0.3 mg/ml dantrolene sodium, 5-hydroxydantrolene, and nitro-reduced acetylated dantrolene (F 490), respectively, (a gift from Eaton Laboratories, Norwich, NY, U.S.A.). Ultrasonic treatment was used to increase the rate of dissolution of F 490. The extraction fluid was chloroform—1-butanol (95:5, v/v). Phosphate buffer (pH 6.8) was 50% (v/v) of dibasic sodium phosphate·$2H_2O$ solution (11.88 g/l) and 50% (v/v) of monobasic potassium phosphate solution (9.08 g/l). The mobile phase was acetonitrile—phosphate buffer (pH 6.8) (33.3:66.6, v/v).

Apparatus and chromatographic conditions

A liquid chromatograph (Kipp Analytica No. 9208) with a variable-wavelength detector (Schoeffel Instruments; SF 770 Spectroflow, GM 770 Monochromator), and a column packed with CP-Spher C_8 (Chrompack, Middelburg, The Netherlands; Cat. No. 28502, particle size 8 μm, 25 cm × 4.6 mm I.D.) was used. The flow-rate was 1.5 ml/min (pressure approx. 5 MPa). Samples were introduced by means of a 50-μl loop. The substances were detected at 375 nm, and the peak heights were measured.

Plasma

Calibration curve. A mixture of the standard solutions was diluted with blank plasma (concentrations 0.02—4 mg/l). To 1.0 ml of each solution were added 0.5 g of ammonium sulfate and 4.0 ml of the extraction fluid. The solutions were shaken for 10 min in a rotary mixer (Cenco Instruments, Cat. No. 23426). After centrifugation for 10 min at 2000 *g* (Heraeus Christ, type UJ1S), an aliquot of the lower layer was collected and evaporated to dryness in a hot (50°C) water bath under nitrogen. The residue was mixed with 1.0 ml of the mobile phase on a vortex mixer and injected onto the column. A control with N,N-dimethylformamide in blank plasma was treated in the same manner.

Samples. To 1.0 ml of plasma were added 0.5 g of ammonium sulfate and 4.0 ml of the extraction fluid; this mixture was further treated as described for the calibration curve.

Urine

Calibration curve. A mixture of the standard solutions was diluted with blank urine and diluted further 1 in 10 with the mobile phase to final concentrations of 0.02—4 mg/l. These solutions were directly injected onto the column. A control with N,N-dimethylformamide in blank urine was treated in the same manner.

Samples. Urine samples of 100 μl were diluted 1 in 10 with the mobile phase and directly injected.

Dog experiment

A male beagle dog of 12 kg body weight was anaesthetized with pento-

barbitone and subsequently given 12 mg of dantrolene sodium (dantrium) intravenously. Blood samples were collected at scheduled intervals. Urine samples were collected by means of a catheter for the first 7 h, thereafter spontaneously voided urine was used.

Recovery

Solutions in urine, prepared as described under the calibration curve for urine, and extracts from water and plasma, using the procedure as described under *Calibration curve* for plasma, were compared to a direct assay of standards in the mobile phase. The recoveries were determined for three different concentrations.

Stability

The standard solutions, which were kept protected from light at 4°C, were periodically measured spectrophotometrically (Beckman spectrophotometer, Model 3600), and by the described HPLC procedure.

RESULTS

A chromatogram of a plasma sample obtained from a patient treated with a daily oral dose of 5 mg/kg body weight dantrolene sodium is given in Fig. 2.

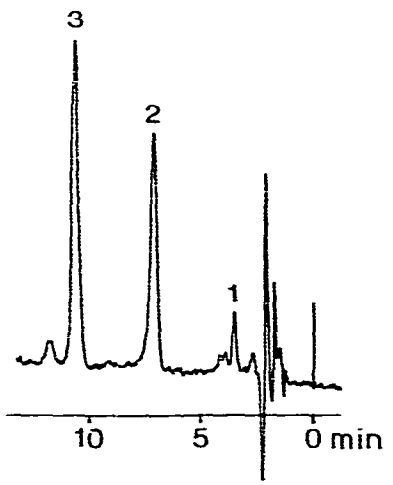

Fig. 2. Liquid chromatogram of patient plasma containing 0.705 mg/l dantrolene (3), 0.624 mg/l 5-hydroxydantrolene (2), and 0.055 mg/l F 490 (1).

Calibration curves in plasma and urine showed good linearity between peak heights and concentrations from 0.02 to 4.0 mg/l (r^2 = 0.999 for all substances). Chromatograms of blanks did not show any interfering substances at the detection wavelength of 375 nm. Retention times and capacity ratios are given in Table I.

The detection limit of dantrolene and its metabolites was 1 ng, defined as three times the noise level. The precision of the determination was measured for two different concentrations; the coefficients of variation are given in

TABLE I

RETENTION TIMES OF DANTROLENE AND ITS METABOLITES

For chromatographic conditions, see text.

Substance	Retention time (min)	Capacity ratio (k')
Dantrolene	10.9	3.5
5-Hydroxydantrolene	7.1	2.0
F 490	4.1	0.7

TABLE II

COEFFICIENTS OF VARIATION

For description of analytical procedure, see text.

Substance	Plasma			Urine		
	Concentration (mg/l)	Coefficient of variation (%)	n*	Concentration (mg/l)	Coefficient of variation (%)	n*
Dantrolene	0.051	3.5	6	0.043	7.0	6
	1.63	3.9	5	1.30	5.7	6
5-Hydroxydantrolene	0.066	3.1	6	0.055	6.0	6
	2.13	4.3	5	1.68	6.3	6
F 490	0.039	6.1	4	0.031	5.8	6
	1.24	3.9	5	1.07	6.3	6

*n = number of determinations.

TABLE III

RECOVERIES OF DANTROLENE AND ITS METABOLITES

For description of analytical procedure, see text.

Substance	Recovery* (%)		
	Water (extracted)	Plasma (extracted)	Urine
Dantrolene	97 ± 4	99 ± 8	103 ± 7
5-Hydroxydantrolene	100 ± 3	86 ± 2	99 ± 1
F 490	101 ± 4	89 ± 4	101 ± 4

*Means and standard deviations of three different concentrations (n = 3): dantrolene 3.25, 0.406, and 0.051 mg/l; 5-hydroxydantrolene 4.20, 0.525, and 0.066 mg/l; F 490 2.68, 0.335, and 0.042 mg/l.

Table II. The results of the recovery experiments are mentioned in Table III.

The standard solutions of all three substances did not show any deterioration for at least three months when kept at 4°C and when protected from light. Upon standing in light, however, F 490 deteriorates extremely quickly. On the chromatogram two peaks develop (Fig. 3). After 4 h at room temperature only about 50—60% of the peak heights relative to a freshly prepared solution could be measured (Fig. 4).

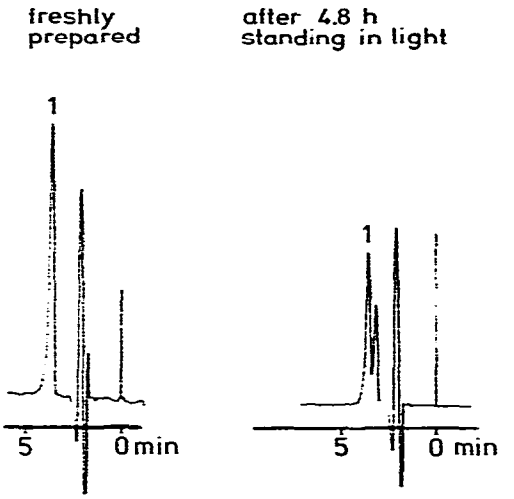

Fig. 3. Liquid chromatograms of a solution containing F 490 (1), showing instability upon standing in light.

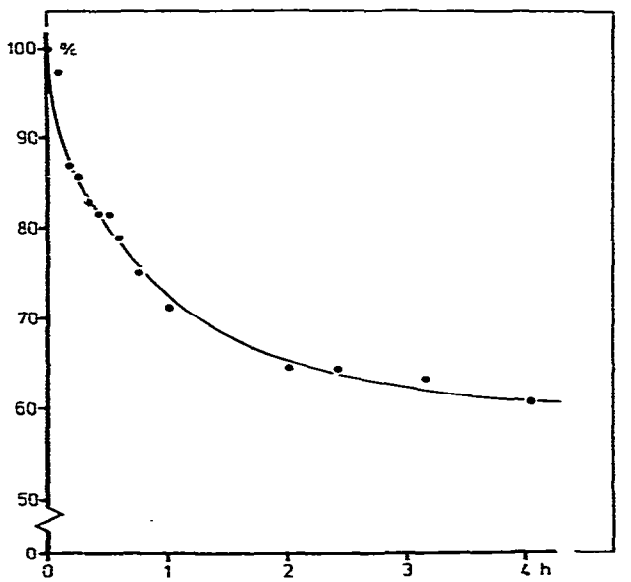

Fig. 4. Degradation of a solution containing F 490 upon standing in light.

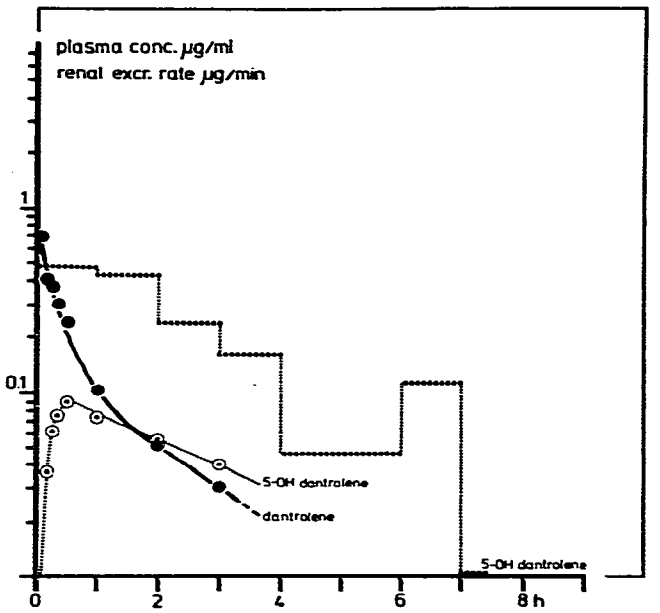

Fig. 5. Plasma concentration and renal excretion rate profiles of dantrolene and 5-hydroxydantrolene in a dog after intravenous administration of 1 mg/kg dantrolene sodium.

Fig. 5 shows the plasma concentration and renal excretion rate profiles of dantrolene and 5-hydroxydantrolene in a dog after intravenous administration of 1 mg/kg body weight dantrolene sodium. No dantrolene is excreted unchanged in dog urine.

DISCUSSION

The very low solubility of dantrolene and its metabolites in many solvents, including water, and the degradation by light, are complicating factors in the analysis. Dantrolene sodium is slightly soluble in water (15 mg/l), but it hydrolyzes quickly. The extremely insoluble (less than 1 mg/l) free acid dantrolene precipitates. The water solubilities of 5-hydroxydantrolene and F 490 are less than 10 mg/l (Data Sheet, Eaton Laboratories). Protection from light during the whole procedure appears to be important, especially for F 490, which is very unstable upon standing in light (see Figs. 3 and 4). N,N-dimethylformamide appeared to be a suitable solvent in preparing standard solutions. The solutions were stable for at least three months when kept protected from light at 4°C. No interference from this solvent was observed in the chromatographic assay.

Extraction of 1 ml of plasma with 4 ml of the chloroform—1 butanol (95:5) mixture yielded good recoveries. For the recovery of dantrolene from plasma (see Table III) a higher value than reported by Saxena et al. [26] was found; they used 80 ml of a chloroform—butanol (70:30) mixture.

On the chromatogram, the peaks of parent drug and metabolites were well resolved (see Fig. 2). With the reversed-phase method of Hackett and Dusci

[27] a relatively poor resolution between dantrolene and 5-hydroxydantrolene was obtained.

Slightly different chromatographic conditions may be needed for measurements in urine samples because of interference by endogenous substances with low retention times.

At present only a few pharmacokinetic studies of dantrolene have been published. Varying values for plasma concentrations and drug metabolite ratios have been reported. Vallner et al. [19] found dantrolene plasma concentrations between 0.03 and 0.2 mg/l, with slightly higher values for the metabolites in patients on chronic oral therapy, with daily dosages ranging from 50 to 200 mg. However, Lietman et al. [20] and Meyler [23] found plasma dantrolene concentrations of between 1 and 2 mg/l in patients on daily dosages of 4—12 mg/kg and 200—400 mg, respectively; the concentration of the 5-hydroxymetabolite appeared to be only 30—50% that of the parent compound. In a preliminary survey of patients on low dosages (0.7—5 mg/kg) we found large inter-individual differences in plasma concentrations and drug metabolite ratios, with plasma concentrations of 0.2—2.0 mg/l, 0.1—1.0 mg/l, and about 0.02 mg/l for dantrolene, 5-hydroxydantrolene, and F 490, respectively. In the dog experiment dantrolene and 5-hydroxydantrolene could be detected in plasma up to 3 h after the intravenous dose. The amount of 5-hydroxydantrolene excreted in the urine accounted for less than 1% of the dose. No dantrolene or F 490 could be detected in the urine.

The reported differences in the concentrations of the drug and of the metabolites have stimulated the investigation of the clinical pharmacokinetics of dantrolene. The described method provides adequate sensitivity and selectivity to make it applicable to pharmacokinetic studies.

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