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Note

Determination of a dantrolene metabolite, 5-(*p*-nitrophenyl)-2-furoic acid, in plasma and urine by high-performance liquid chromatography

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The direct-acting muscle relaxant dantrolene (Fig. 1) is used in patients with spasticity and also in humans and animals, including dogs at risk of malignant hyperthermia [1,2]. Although its efficacy is well established, knowledge concerning the fate of this drug in the body is limited owing to the complicated and extensive metabolism, which is also the case for the structurally related nitrofurans [3]. Known metabolic pathways for dantrolene are hydroxylation of the hydantoin ring and reduction of the nitro group, which may then be acetylated [1]. Many methods have been developed for the quantification of dantrolene and its oxidized and reduced metabolites in plasma and urine, the latest by Lalande et al. [4], but when these are applied to pharmacokinetic studies, the major part of the mass balance is missing. A pilot study in the dog showed that less than 1% of the intravenously administered dose was excreted in the urine [5] and renal recovery in humans amounted to only 15% [6]. Other pathways of elimination must exist to explain this incomplete recovery.

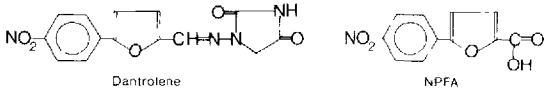

Fig. 1. Structures of dantrolene and its metabolite 5-(*p*-nitrophenyl)-2-furoic acid (NPFA).

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Cleavage of the azomethine (–CH=N–) linkage is one pathway in need of further investigation. Analogously to nitrofurans [3,7], in vitro acidic hydrolysis at body temperature has resulted in aldehyde formation [8], and the corresponding acid, 5-(*p*-nitrophenyl)-2-furoic acid (NPFA) (Fig. 1), has been identified in vivo (Norwich Eaton Pharmaceuticals, product information). This paper describes a modification of our previously developed assay for dantrolene [5], which allows quantification of NPFA in plasma and urine of humans and dogs.

EXPERIMENTAL

Reagents and chemicals

All chemicals were of analytical grade. Norwich Eaton Pharmaceuticals (Norwich, NY, U.S.A.) kindly supplied NPFA. A stock solution (0.5 mg/ml) was prepared in N,N-dimethylformamide and kept in the dark at 4°C. Phosphate buffer (pH 6.8) was 50% (v/v) of dibasic sodium phosphate dihydrate solution (11.88 g/l) and 50% (v/v) of monobasic potassium phosphate solution (9.08 g/l).

Apparatus and chromatographic conditions

The HPLC system was similar to that previously described [5]. The mobile phase, acetonitrile–phosphate buffer (25:75, v/v for plasma, 20:80, v/v for urine), was used at room temperature at a flow-rate of 1.5 ml/min with a CP-SpherC_8 column (25 cm × 4.6 mm I.D., particle size 8 μm) (Chrompack, Middelburg, The Netherlands). NPFA was detected at 354 nm.

Procedure

Plasma. To standard or unknown plasma (200 μl) in a small glass centrifuge tube, 50 μl of 2 *M* hydrochloric acid and 1.4 ml of the extraction mixture, chloroform–1-butanol (95:5, v/v) were added. After vortex-mixing for 60 s, the tubes were centrifuged at 2000 *g* for 5 min. An aliquot of the lower layer (1.0 ml) was then collected and evaporated to dryness in a water-bath (37°C) under nitrogen. The residue was reconstituted with 250 μl of the mobile phase and subsequently injected into the liquid chromatograph (50-μl loop).

Urine. Standard or unknown urine (200 μl) was mixed with 200 μl of the mobile phase and directly injected (50 μl).

Calibration curves were constructed by plotting the concentrations against the peak heights. The calibration range was based on the expected concentrations and established in the range 0.1–10 mg/l.

Recovery experiments

Samples containing four concentrations of the standard compound in plasma in the range 0.1–10 mg/l were prepared in duplicate and treated as described

above. The percentage recovery was determined by comparing the heights of the peaks obtained with the heights obtained from standards that were diluted with mobile phase to identical concentrations and directly injected.

Samples

Dantrolene was administered to dogs and human volunteers in different dosages. Dogs also received 5-hydroxydantrolene. Plasma and urine were collected and stored in the dark at −20 °C until analysis. More details will be published elsewhere.

Dog experiment with the metabolite

A female Beagle dog with a body weight of 14 kg was anaesthetized with pentobarbitone and subsequently given 13 mg of NPFA by constant-rate intravenous infusion for 45 min. Serial 2-ml blood samples were collected in heparinized tubes at scheduled intervals. Urine samples were collected by means of a catheter for the first 7 h, and subsequently spontaneously voided specimens were used. Urinary pH was measured in each sample.

RESULTS AND DISCUSSION

Typical chromatograms of NPFA in dog plasma and urine are shown in Figs. 2 and 3. Neither the chromatograms of the drug-free specimens for the dog (A) nor those for the human volunteers contained any interfering substances. The concentration of NPFA in the spiked samples (B) was 0.49 mg/l (plasma) and 0.57 mg/l (urine). After intravenous administration of 5-hydroxydantrolene (3.9 mg/kg) the concentrations of NPFA in the samples shown (C) were 0.86 mg/l (plasma) and 0.84 mg/l (urine). The detection limit of NPFA was ca. 1 ng, defined as three times the noise level. The retention times (t_R) were 3.4 and 7.4 min for plasma and urine, respectively. Calibration graphs showed good linearity in the ranges mentioned (r^2 always greater than 0.99). The precision

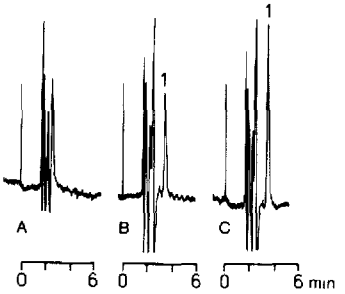

Fig. 2. Chromatograms of (A) drug-free dog plasma, (B) dog plasma spiked with 0.49 mg/l NPFA, (C) dog plasma sample taken after intravenous administration of 5-hydroxydantrolene (3.9 mg/kg) containing 0.86 mg/l NPFA Peak 1 = NPFA.

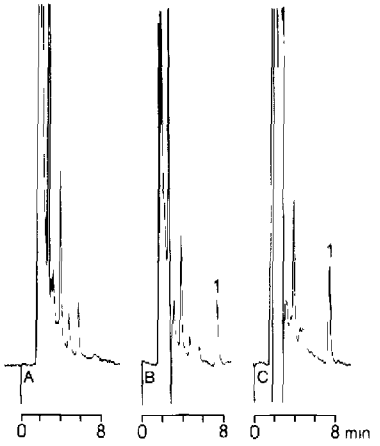

Fig. 3. Chromatograms of (A) drug-free dog urine, (B) dog urine spiked with 0 57 mg/l NPFA, (C) dog urine sample taken after intravenous administration of 5-hydroxydantrolene (3 9 mg/kg) containing 0.84 mg/l NPFA Peak 1 = NPFA

of the determinations in plasma and urine, measured within the same ranges at three concentrations in quadruplicate, was within acceptable limits (coefficients of variation always less than 7.6%). The mean ($\pm$S.D.) recovery for plasma was $83 \pm 5\%$.

When dantrolene and its reduced and oxidized metabolites were introduced into this HPLC system, all compounds could be separated within ca. 30 min. Because of band broadening, however, low concentrations escaped detection. This implies that for pharmacokinetic purposes the method as such is inadequate to determine the five compounds simultaneously. Other refinements, such as gradient elution, might favour the sensitivity.

In a preliminary survey of canine and human urine collected after administration of dantrolene and 5-hydroxydantrolene, NPFA was found in low concentrations. Total urinary recovery of this metabolite accounted for a few per cent of the dose, contributing accordingly to the mass balance of dantrolene in humans and dogs. Since in vitro hydrolysis of the azomethine bond results in aldehyde formation, urine samples were also screened for the presence of 5-(*p*-nitrophenyl)furfural. By increasing the concentration of acetonitrile in the mobile phase to 50%, the otherwise unchanged HPLC system could measure this compound (kindly supplied by Norwich Eaton Pharmaceuticals) in the spiked urine ($t_R = 6.3$ min). However, in neither human nor dog samples was the aldehyde detected.

When the method described for plasma was followed, NPFA was not detected in the human samples. In pilot studies with dantrolene and 5-hydroxydantrolene administered as the parent drug in dogs, NPFA plasma concentrations in excess of the parent drug were measured, indicating that this metabolic

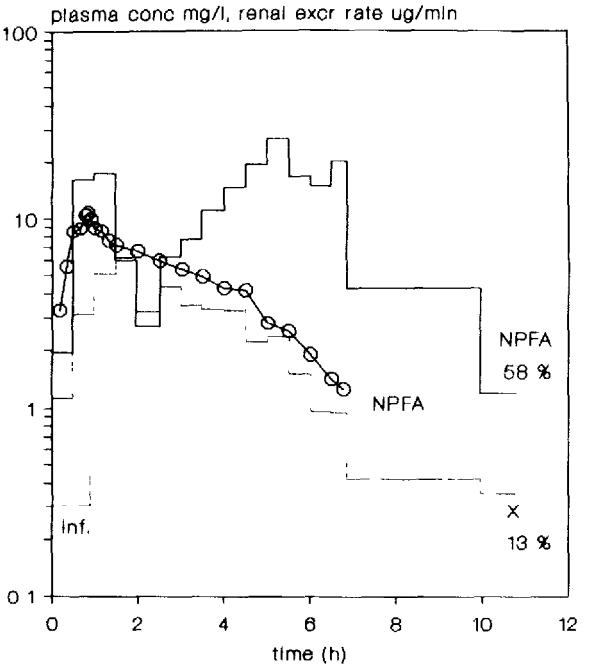

Fig. 4. Plasma concentration–time curve and renal excretion rate–time profile of NPFA following its intravenous administration (0.9 mg/kg) in a dog. The infusion time (inf.) was 45 min. X = unknown metabolite.

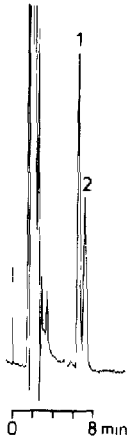

Fig. 5 Chromatogram of dog urine sample taken after intravenous administration of NPFA (0.9 mg/kg) containing 3 7 mg/l NPFA. Peaks: 1 = NPFA; 2 = unknown metabolite.

pathway is important in the dog. The possibility that NPFA was formed in vitro following acidification of the plasma samples was ruled out by the absence of NPFA in standard solutions treated similarly.

The method described was also used in a metabolite kinetic experiment with NPFA administered as the parent drug in the dog. Fig. 4 shows the plasma concentration–time curve and the renal excretion rate–time profile of NPFA after its intravenous administration. The plasma curve shows non-linearity for the dose administered, with a terminal half-life of 1.4 h. When administered as the parent drug, the major excretory product found in the urine was unchanged NPFA (58% of the dose). An unknown metabolite (Fig. 5), not present in plasma, accounted for a further 13% recovery (calculated as NPFA). This unknown metabolite was absent in the samples obtained after dantrolene c.q. 5-hydroxydantrolene administration. Total body clearance (D/AUC) and apparent renal clearance (A_e/AUC) of NPFA were 6.2 and 3.6 ml/min, respectively, where D is the dose administered, AUC is the area under the plasma concentration–time curve, and A_e is the total amount excreted in the urine as NPFA. The apparent renal clearance of NPFA was dependent on the pH of the urine in the 5–7 range. This dependency may explain the unusual renal excretion rate–time course measured during the first 5 h of the experiment.

In conclusion, we have developed an HPLC method that is applicable to pharmacokinetic studies of dantrolene, in particular to metabolite kinetic studies in the dog.

REFERENCES

1 A. Ward, M.O. Chaffman and E.M. Sorkin, Drugs, 32 (1986) 130.
2 A H Kirmayer, A.M. Klide and J.E Purvance, J. Am. Vet Med. Assoc , 185 (1984) 978.
3 S. Swaminathan and G M. Lower, Jr., in G T Bryan (Editor), Carcinogenesis, Vol. 4, Nitrofurans, Raven Press, New York, 1978, Ch. 3, p. 59
4 M. Lalande, P. Mills and R.G. Peterson, J. Chromatogr , 430 (1988) 187.
5 E W Wuis, A.C.L.M. Grutters, T B Vree and E. van der Kleijn, J. Chromatogr., 231 (1982) 401.
6 H Asari, S. Kitamura, S. Higuchi and C. Urano, Hiroshima J. Anesth., 20 (1984) 255.
7 N Inotsume and M. Nakano, Int. J. Pharm , 8 (1981) 111.
8 N. Inotsume and M. Nakano, Int. J. Pharm., 17 (1983) 357.