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Note

Simultaneous determination of nitrofuran derivatives in various animal substrates by high-performance liquid chromatography

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Nitrofuran derivatives have been used for more than 30 years in veterinary medicine, alone or in combination with other drugs. Nitrofurans are metabolized extensively[1], but the metabolic pathways have still not been elucidated[2]. Since the demonstration that nitrofurans are mutagenic and (pro)carcinogenic, their use has been strictly regulated in many countries. Recently, the EEC has recommended a tolerance level of 1–2 ppb[a] for the parent nitrofurans[3,4] in edible tissues of slaughtered animals. Hitherto, drug instability and the lack of sensitive detection methods has made the detection of nitrofurans at the 1 ppb level complicated.

A previous study[1] revealed that degradation of furaltadone and nitrofurazone dissolved in calves' urine could be prevented by dilution of the urine with 1 *M* phosphate buffer (pH 5.0) containing 0.2% sodium azide (enzyme blocker) with immediate storage at −20°C and taking strict precautions to protect them from light.

This study describes an *in vitro* tested stabilization procedure that prevents degradation of nitrofurans in organic tissue and a rapid, sensitive and selective high-performance liquid chromatographic (HPLC) method for the simultaneous determination of four nitrofuran derivatives (nitrofurantoin, nitrofurazone, furazolidone and furaltadone) in plasma, urine and body tissues at the 1 ppb level.

The application of the procedure to the routine determination of nitrofuran derivatives is demonstrated.

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC equipment consisted of an SF 400 pump (Kratos, Rotterdam, The Netherlands), a Rheodyne 7125 injector with a 100-μl sample loop, a Spectroflow 783 detector (Kratos) and a Shimadzu CR-3a integrator. Separations were carried out with a Zorbax CN (5 μm) column (250 × 4.6 mm I.D.) (DuPont, Wilmington, DE, U.S.A.). A guard column (50 × 2.1 mm I.D.) tap-packed with a pellicular reversed phase (Chrompack, Middelburg, The Netherlands) was fitted in front of the analytical column.

The eluent was prepared by mixing 600 ml of 0.01 *M* sodium acetate buffer (pH

[a] Throughout this paper, the American billion (10^9) is meant.

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5.0) with 400 ml of methanol. The eluent was degassed and filtered before use. The flow-rate was 1.50 ml/min, the analyses were performed at 20°C and the detection of the nitrofuran derivatives was performed at 365 nm with a detector setting of 0.001 a.u.f.s.

Chemicals and reagents

Furaltadone hydrochloride (Aesculaap, Boxtel, The Netherlands), nitrofurazone (Furazin; Stricker, Berne, Switzerland), furazolidone (Sigma, St. Louis, MO, U.S.A.) and nitrofurantoin (Sigma) were used. Their structures are shown in Fig. 1.

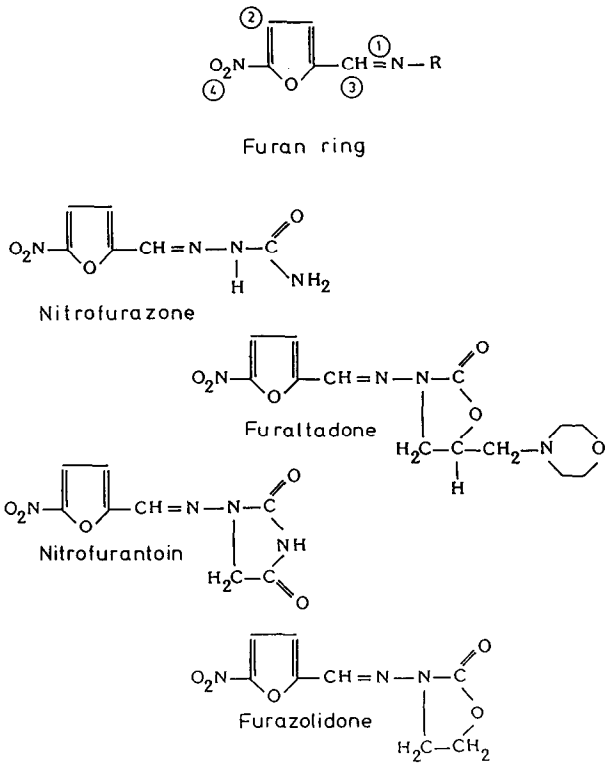

Fig. 1. Structure of nitrofurans and of furaltadone, furazolidone, nitrofurantin and nitrofurazone.

Ethyl acetate (Uvasol), acetonitrile (Uvasol), dichloromethane (analytical-reagent grade) and *n*-hexane (analytical-reagent grade) were obtained from Merck (Darmstadt, F.R.G.). Phosphate buffer (0.67 *M*, pH 5.0) was prepared by dissolving 22.69 g of KH_2PO_4 and 0.64 g of $Na_2HPO_4 \cdot 2H_2O$ in 250 ml of distilled water.

Stability studies

The *in vitro* stability of the nitrofuran derivatives was studied in meat and liver of cows, swine and poultry. These tissues were refrigerated at 4°C for 1 day. Meat and

liver were homogenized with (i) physiological saline, (ii) 1 *M* phosphate buffer (pH 5.0) and (iii) 1.5 *M* KH_2PO_4 solution (pH 4.5) containing 0.2% sodium azide. Pools were spiked to a concentration of 50 ppb, kept in complete darkness and analysed at different times.

Preliminary studies revealed that degradation of the nitrofurans could be prevented by the last procedure. Therefore, the following preparation procedures were performed for the validation of the method.

Preparation of the samples

For urine, the sample was diluted 1:1 (v/v) with 1.5 *M* KH_2PO_4 containing 0.2% sodium azide. For plasma, 5 ml of 1.5 *M* KH_2PO_4 with 0.2% of sodium azide were added to a 50-ml sample. For edible tissues (bovine muscle and liver), the samples were homogenized (Waring blender) with 1.5 *M* KH_2PO_4 containing 0.2% sodium azide (1:2, w/v).

All samples were wrapped in tin-foil and immediately stored at −20°C until analysis.

Extraction procedures

During the extraction procedures the samples were protected from light.

Plasma and urine. To 10 ml of buffered plasma or urine, 10 ml of dichloromethane–ethyl acetate (1:1, v/v) were added, mixed by rotation (Heidolph rotator) at 20 rpm for 20 min and centrifuged at 4500 *g* for 10 min. An 8-ml volume of the organic layer was removed and evaporated to dryness at 50°C under a gentle stream of nitrogen, the residue was dissolved in 500 μl of phosphate buffer (0.67 *M*, pH 5.0) and 1 ml of *n*-hexane was added. After mixing and centrifugation, 100 μl of the aqueous phase were injected into the HPLC system.

Organic tissues. To 30 g of the homogenate, 20 ml of acetonitrile were added, mixed by rotation for 15 min and then centrifuged at 4500 *g* for 10 min. A 35-ml volume of the supernatant was transferred into another 50-ml tube and 5 ml of dichloromethane–ethyl acetate (1:1, v/v) were added, mixed by rotation for 15 min and centrifuged at 4500 *g* for 10 min. A 12-ml volume of the upper organic layer was removed, evaporated to dryness at 50°C under a gentle stream of nitrogen and 2 ml of *n*-hexane and 500 μl of phosphate buffer (0.67 *M*, pH 5.0) were added to the residue. After mixing and centrifugation, 100 μl of the aqueous phase were subjected to HPLC.

Quantification and confirmation

Quantification was carried out using calibration graphs obtained from spiked samples in the concentration range 1–100 ppb. The concentration of the drug was determined by comparison of peak areas or peak heights.

Interferences

To study interference, 1000 ppb of the antimicrobial agents chloramphenicol, sulphadimidine, sulphamethoxazole, sulphanilamide, sulphatroxazole, sulphathiazole, dapsone, oxytetracycline and carbadox were injected into the HPLC system.

RESULTS AND DISCUSSION

Homogenization of meat and liver with physiological saline or 1 *M* phosphate buffer (pH 5.0) showed rapid degradation of the nitrofurans. The degradation half-lives of nitrofurantoin, furazolidone, furaltadone and nitrofurazone bovine liver homogenates prepared in physiological saline were approximately 12, 12, 31 and 44 h, respectively (see Fig. 2). Different degradation half-lives for nitrofurans in the organs from different species should be emphasized. For sample in fresh goat and cattle livers the degradation half-life was about 13 min[5]. Decreasing the pH to 4.5 using 1.5 *M* KH_2PO_4 containing 0.2% sodium azide instead of saline or pH 5.0 buffer revealed an *in vitro* tested stabilization of the various nitrofuran derivatives for 24 h in edible tissues of swine, cows and poultry.

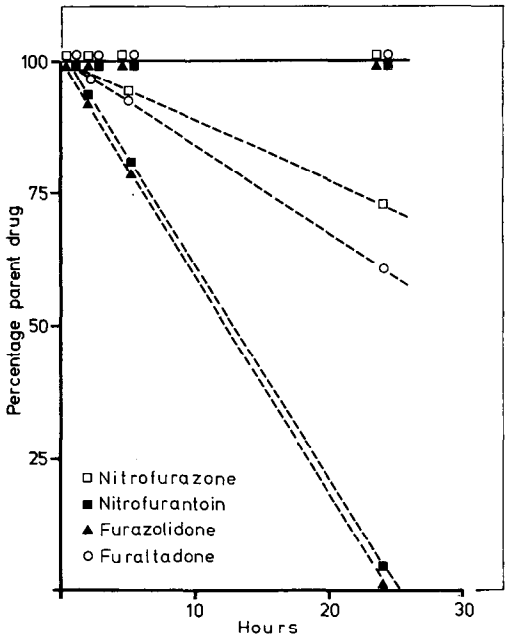

Fig. 2. Stabilization of nitrofurans in liver homogenates made with (broken lines) physiological saline and (solid line) 1.5 *M* KH_2PO_4 solution (pH 4.5) and 0.2% sodium azide.

Fig. 3 shows typical chromatograms of bovine plasma and meat spiked with nitrofurazone, nitrofurantoin, furazolidone and furaltadone. The four nitrofuran derivatives eluted as distinct peaks with retention times of 4.2, 4.6, 5.5 and 7.4 min, respectively. Calibration graphs for the four nitrofuran derivatives in plasma, urine, meat and liver (range 1–100 ppb) were linear ($r = 0.999$). The limit of detection was 1 ppb, yielding a detector response approximately equal to five times the detector noise.

Table I shows the recovery and reproducibility of the procedure for the nitrofuran derivatives in the various substrates. The method showed good precision

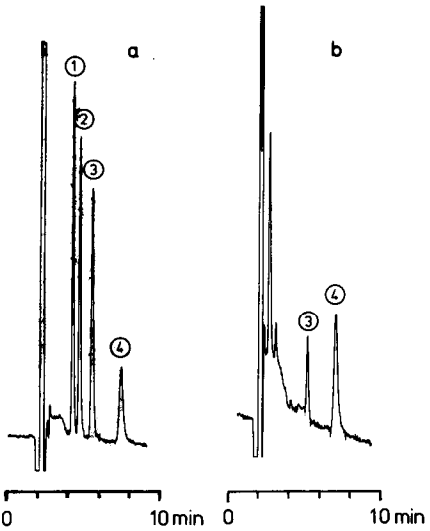

Fig. 3. Representative chromatograms (0.002 a.u.f.s.) of (a) plasma spiked with 10 ppb each of nitrofurantoin (1), nitrofurazone (2), furazolidone (3) and furaltadone (4) and (b) a meat sample with 1.13 ppb of furazolidone (3) and 4.78 ppb of furaltadone (4).

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE PROCEDURES

Drug concentration range 1–100 ppb.

Sample	*Nitrofurazone*		*Nitrofurantoin*		*Furazolidone*		*Furaltadone*	
	Av.[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*
Water								
Mean	91.5	2.6	90.2	3.0	97.7	2.6	87.2	3.4
S.D.	1.5	0.8	1.8	1.5	0.4	1.2	1.0	0.7
Linearity	0.9999		0.9999		0.9999		0.9999	
Bovine plasma								
Mean	69.7	3.0	72.8	3.9	89.7	2.7	88.1	4.0
S.D.	1.6	1.9	1.1	1.0	1.6	1.5	1.0	1.5
Linearity	0.9999		0.9999		0.9998		0.9998	
Bovine meat								
Mean	60.7	2.2	52.5	1.1	100.3	2.8	99.1	2.3
S.D.	0.4	1.5	0.5	0.4	2.2	2.2	1.9	1.5
Linearity	0.9998		0.9999		0.9998		0.9999	
Bovine liver								
Mean	60.7	2.0	52.3	1.8	99.9	1.9	99.1	1.5
S.D.	0.3	1.0	0.4	0.4	1.3	1.7	1.3	1.2
Linearity	0.9998		0.9999		0.9998		0.9998	

[a] Average of 40 determinations.

[b] Coefficient of variation.

and accuracy (coefficient of variation 1.1–4.0%). The combination of the low pH and sodium azide as enzyme blocker gave good stabilization and prevented *in vitro* degradation.

No interference in the chromatograms of nitrofurans was observed from chloramphenicol, sulphadimidine, sulphametoxazole, sulphanilamide, sulphatroxazole, sulphathiazole, dapsone and oxytetracycline; only carbadox eluted just before nitrofurazone. A diode-array detector can give good results for the identification of these two components.

Recent monitoring of 250 slaughtered diseased animals for nitrofuran residues with the method described revealed that the method was easy to perform and reliable for the simultaneous determination of nitrofuran derivatives in both body fluids and tissues.

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