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Note

High-performance liquid chromatographic determination of bromofenofos and its metabolite in rat plasma

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Bromofenofos, 3,3′,5,5′-tetrabromo-2,2′-biphenyldiol mono(dihydrogenphosphate) (BF), is used extensively in the treatment of fascioliasis in ruminants; the recommended therapeutic dose is 12–16 mg/kg [1]. This compound is known to be excreted in cow's milk, in which its residue has been assayed by thin-layer chromatography (TLC), gas chromatography (GC) and GC–mass spectrometry (MS) [2,3]. De Boer and Kleinepier [4], using TLC, found that only the metabolite dephosphate-bromofenofos, 3,3′,5,5′-tetrabromo-2,2′-biphenyldiol (DBF), was detectable in cow's milk, and the parent compound BF not at all. Their observation was later confirmed by Fukuhara et al. [5] using GC–MS. Recently BF was found to be embryolethal and teratogenic in rats [6,7]. Similar effects were also observed with DBF [8]. A more rapid and selective method applicable to the quantification of these two compounds in biological materials is thus required for toxicological studies. The purpose of the present work was to develop a high-performance liquid chromatographic (HPLC) method capable of separating and quantifying BF and DBF distributed in plasma samples. As an application of this method, BF and DBF could be quantified in plasma from rats that had received BF by the intraperitoneal route.

EXPERIMENTAL

Chemicals and standards

BF, DBF and the internal standard (I.S.), 3,3′-dibromo-5,5′-dichloro-2,2′-biphenyldiol (DDB), were supplied by ACF Chemiefarma (Amsterdam, The Netherlands) through Denka Pharmaceutical (Tokyo, Japan). The structures

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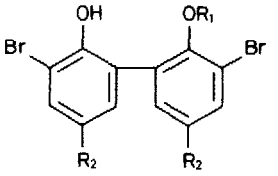

	R_1	R_2
Bromofenofos (BF)	$PO(OH)_2$	Br
Metabolite (DBF)	H	Br
Internal Standard (DDB)	H	Cl

Fig. 1. Structures of bromofenofos (BF), metabolite (DBF) and the internal standard (DDB).

of these compounds are presented in Fig. 1. Analytical-grade acetonitrile, ethyl acetate, methanol, phosphoric acid, and hydrochloric acid were obtained from Wako (Osaka, Japan).

Stock solutions of BF, DBF, and DDB (100 μg/ml) were prepared in methanol and stored in the dark at 4°C.

Instrumentation and HPLC conditions

The HPLC system comprised a Model 635A pump (Hitachi, Tokyo, Japan), a Model SIL-6A autoinjector (Shimadzu, Kyoto, Japan), a Model Uvidec-100-V detector (Japan Spectroscopic Co., Tokyo, Japan) set at 210 nm and a Model C-R3A integrator (Shimadzu). The column was a 25 cm×4.6 mm I.D. stainless-steel column packed with Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.). The mobile phase consisted of acetonitrile–methanol–water–phosphoric acid (35:35:30:0.1). The injection volume was 10 μl, and the flow-rate was 1.0 ml/min. The column temperature was 40°C. The chromatograms were recorded with a chart speed of 5 mm/min.

Extraction procedure

To a rat plasma sample (1 ml) in a 50-ml glass centrifu tube were added 9 ml water. The mixture was extracted twice with 20 ml of ethyl acetate by shaking for 2 min. The organic phase obtained by centrifugation at 1200 *g* for 10 min was evaporated to dryness at 40°C (fraction 1). The inorganic phase was then made acidic by the addition of 10 ml of 0.1 *M* hydrochloric acid. This solution was also extracted twice with 20 ml of ethyl acetate by shaking for 2 min, and the organic phase obtained by centrifugation at 1200 *g* for 10 min was evaporated to dryness at 40°C (fraction 2). The fractions were separately dissolved in 1 ml of mobile phase containing 10 μg/ml DDB, filtered through Ekicrodisc 13CR (Gelman Sciences Japan, Tokyo, Japan) if necessary, and injected into the HPLC system.

Recovery

BF and DBF recoveries were determined by comparing peak-area ratios of each compound (BF/I.S., DBF/I.S.) extracted from spiked plasma (range 1–100 μg/ml) with peak-area ratios obtained by direct injection of pure compounds.

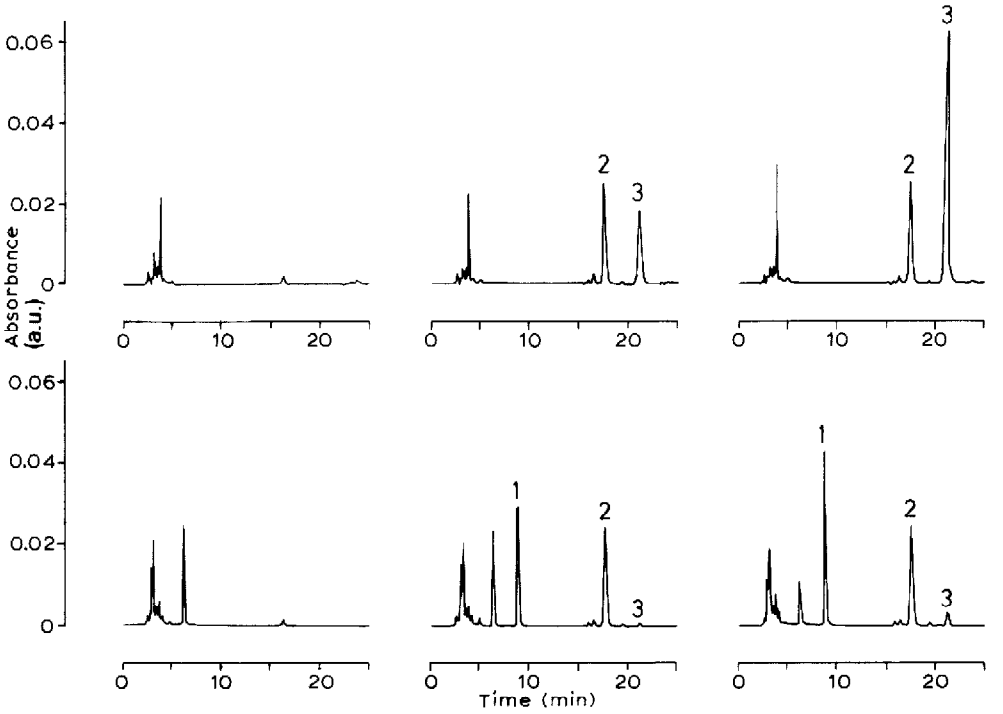

Fig. 2. Chromatograms of rat plasma extracts. Each panel shows the following chromatograms (from left to right): blank plasma; plasma spiked with 10 μg/ml BF and DBF; and plasma from a rat 1 h after a single 12 mg/kg intraperitoneal dose of BF. Peaks in fraction 1 are shown in the upper row and those in fraction 2 in the lower row. Peaks: 1 = BF; 2 = internal standard (DDB); 3 = DBF.

Application

Female Sprague-Dawley rats (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) weighing ca. 200 g received an intraperitoneal injection of BF (12 mg/kg) suspended in olive oil. Rats were sacrificed 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 or 48 h later. Blood samples were heparinized, and plasma was obtained by centrifugation for 10 min at 1200 *g*. The plasma was kept frozen at −20 °C until used.

RESULTS AND DISCUSSION

Chromatograms of fractions 1 and 2 extracted from plasma are shown in Fig. 2. Retention times (t_R) for BF and DBF were 8.8 and 21.1 min, respectively. Although DBF was extracted in fraction 1, a small amount of it remained in fraction 2. BF was shown to be extracted in fraction 2, but not at all in fraction 1. DDB gave one major (t_R = 17.5 min) and two minor (t_R = 15.8 and 19.2 min) peaks, clearly separated. The areas of the minor peaks were 3–4% of that of the major peak. We used the major peak for calculation. The assay was not interfered with by the minor peaks, which are presumably due to impurities. No interfering

TABLE I

RECOVERIES OF BF AND DBF FROM SPIKED RAT PLASMA

Values are the mean ± S.D. of three samples.

Concentration (μg/ml)	Recovery (%)			
	Fraction 1		Fraction 2	
	BF	DBF	BF	DBF
1	0±0	99.8±1.0	82.8±2.2	0 ±0
3	0±0	100.0±1.0	100.7±3.3	0 ±0
5	0±0	98.8±1.9	92.7±0.6	0 ±0
10	0±0	95.2±0.5	95.3±1.4	1.2±1.0
30	0±0	94.9±1.6	95.8±1.4	4.5±0.1
100	0±0	97.5±0.6	92.0±1.3	2.9±0.4
Mean		97.7±2.3	93.3±5.8	

peaks appeared in fractions 1 and 2 of blank plasma at the retention times corresponding to these three compounds.

The Nucleosil 5 C_{18} column gave a better separation of BF and DBF from plasma components than did a Nucleosil 5 C_8 column. Maximum absorptions in the mobile phase were observed at 209 and 295 nm for BF, and 208 and 299 nm for DBF, so 210 nm was selected as the detection wavelength for the compounds in HPLC analysis, because both compounds have too weak an absorption at higher wavelengths for the sensitivity to be satisfactory. The optimum HPLC operating conditions were derived from experiments based on parameters such as column temperature, flow-rate and mobile phase composition. Optimum results for detecting BF and DBF were obtained using acetonitrile–methanol–water–phosphoric acid as the mobile phase. Phosphoric acid added to mobile phase improved the peak shape of BF and prevented background interference by plasma components to BF peak. Acetic acid was not effective.

Calibration curves were linear and reproducible over the range 0–50 μg/ml for both BF and DBF ($r=0.999$, $n=6$). Table I shows the recovery data for BF and DBF. Overall mean recoveries were 97.7±2.3% for DBF in fraction 1 and 93.3±5.8% for BF in fraction 2. BF was not recovered at all from fraction 1. DBF was recovered in small amounts from fraction 2 at higher concentrations, which may explain a corresponding small peak on the chromatograms in Fig. 2.

When plasma was first made acidic and extracted, the recovery of BF was enough, but that of DBF varied from 30 to 91% over the range 5–30 μg/ml (data not shown). This is why DBF was first extracted in fraction 1 and BF next in fraction 2.

Purification using Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) and ion-pair extraction for BF and DBF with a tetra-*n*-butylammonium salt as the ion-pairing agent did not prevent background interference or result in high recoveries of BF and DBF (data not shown).

The detection limit of the method presented was as low as 0.1 μg/ml for both

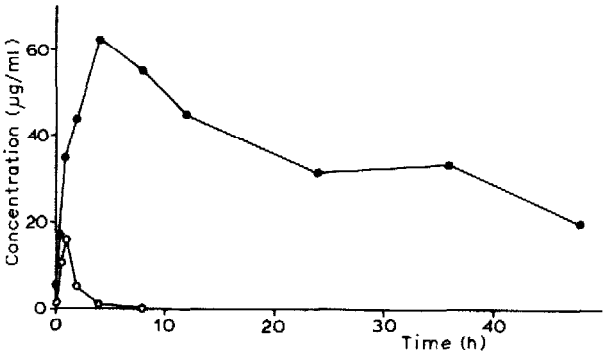

Fig. 3. Plasma concentrations of BF (○) and DBF (●) versus time in rats that had received a single intraperitoneal dose of BF (12 mg/kg).

compounds, at a signal-to-noise ratio of 2. The recoveries and the detection limit were satisfactory for use in pharmacokinetic studies of BF.

The plasma concentration–time curves for BF and DBF obtained in rats are shown in Fig. 3, indicating that the present method is applicable in vivo. The advantages of the present HPLC method over the reported GC and GC–MS methods [2,3,5] are that sample treatment is rapid and simple, no derivatization is needed, and it is capable of the simultaneous quantification of BF and DBF in plasma treated with the clinically relevant dose of BF. However, it is less sensitive than the GC method [2,3], which had a limit of detection of 0.01 μg/ml in cow's milk. Application of the present method is also indicative of the utility in studying the fate of BF in the body.

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