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Determination of GX-071 and Its Major Metabolite in Rat Blood by Cold On-Column Injection Capillary GC/ECD

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GX-071 (*N*-ethylperfluorooctanesulfonamide) is currently undergoing general toxicologic studies to support its registration for use as a pesticide. To aid in these investigations, methodology was developed for the determination of GX-071 and its major metabolite as their pentafluorobenzyl derivatives from 20–40 μL of rat blood at ppm to ppb levels using cold on-column injection capillary GC/ECD. The compounds were extracted from an alkaline aqueous phase to a methylene chloride phase as ion pairs and derivatized with pentafluorobenzyl bromide. The major metabolite of GX-071, perfluorooctanesulfonamide (GX-071M), was identified by cold on-column injection capillary GC/MS from the blood of both rats and dogs that had been given oral doses of GX-071. The percent RSD of the procedure was 4.7 and 2.8 at 100 ppm and 17 and 21 at 400 ppb for GX-071 and GX-071M, respectively.

The establishment of critical toxicokinetic parameters of a chemical or a drug such as absorption, distribution, biotransformation, and elimination requires many repeated analyses of blood and other biological fluids from laboratory animals dosed with the compound of interest. The laboratory animal of choice for these investigations is often the rat. However, the size of laboratory rats places constraints on the volume of blood that can be taken at any one time without seriously compromising the results of later analyses. Therefore, methods for the determination of a compound in rat blood must be able to do so from small sample sizes if they are to be routinely useful in toxicokinetic/pharmacokinetic investigations.

Identification of major metabolites and the determination of the parent compound and major metabolites in biological fluids are essential parts of pharmacological studies of a chemical in regard to its toxicity from in vivo exposure. This work was done as part of a study of the pharmacokinetics and toxicokinetics of GX-071 (*N*-ethylperfluorooctanesulfonamide) to support its registration as a pesticide. GX-071 is being evaluated as a pesticide for the red imported fire ant. Such investigations are crucial to the evaluation of a chemical in regard to its intended uses and anticipated human exposures (Yakatan and Iglesia, 1984; Garrett, 1974). GX-071 and its major metabolite, perfluorooctanesulfonamide (GX-071M), were identified in the blood of rats and dogs that had been given oral doses of GX-071. This methodology included the separation of an ethyl acetate extract of whole blood by cold on-column injection capillary gas chromatography (GC) (Arrendale and Chortyk, 1985). Capillary gas chromtography/mass spectrometry (GC/MS) was used to confirm the identification of GX-071 and GX-071M.

Fluorocarbons, such as GX-071, are known for their chemical and thermal stabilities (Metcalf, 1970; Smith, 1970). Among its physical properties, GX-071 possesses thermal stability and resistance of hydrolyses. The hydrogen attached to the sulfonamide nitrogen is acidic, and the compound forms salts with strong bases. However, the compound is lipophilic and has very low solubility in water, and solutions exhibit a neutral pH. Although it is a sulfonamide, GX-071 does not fit the general description often applied to derivatives of sulfanilamide (Anand, 1979). Thus, its pharmacokinetic/toxicokinetic properties including metabolism were not expected to parallel those normally associated with the classical sulfonamides (Hekster and Vree, 1982).

Both GX-071 and GX-071M are lipophilic in nature and can be extracted from blood with ethyl acetate. Furthermore, their sulfonamide hydrogens are acidic, and their anionic species form ion pairs with tetrabutylammonium as the counterion. Methodology was developed for the extraction of GX-071 and GX-071M as ion pairs with the tetrabutylammonium counter ion from 20–40 μL of rat blood in an alkaline aqueous phase to a methylene chloride phase. This was followed by the addition of pentafluorobenzyl bromide to the methylene chloride with subsequent formation of their PFB derivatives, which were analyzed by cold on-column injection capillary GC with electron capture detector (ECD) using an internal standard spiking technique at ppm to ppb levels.

A recent study (Schnellmann, 1989) indicated that GX-071 and GX-071 M were potent uncouplers of oxidative phosphorylation. Therefore, both compounds were han-

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dled in accordance with standard operating procedures for handling toxic substances. More definitive information on toxicity will be included in future publications as it becomes known.

EXPERIMENTAL SECTION

Materials. GX-071 ($C_8F_{17}SO_2NHCH_2CH_3$) and GX-071M ($C_8F_{17}SO_2NH_2$) for use as standards were provided by Griffin Corp. (Valdosta, GA). The internal standard (ISTD), decanoic acid, was obtained from Analabs, Inc. (New Haven, CT); the methylene chloride and 2,2,4-trimethylpentane (isooctane, resianalyzed) were from J. T. Baker Chemical Co. (Phillipsburg, NJ); the tetrabutylammonium hydrogen sulfate was from Aldrich Chemical Co. (Milwaukee, WI); the pentafluorobenzyl bromide was from Pierce Chemical Co. (Rockford, IL); the 13 × 100-mm Pyrex screw-cap culture tubes were from Corning Glass Works (Corning, NY); and sodium hydroxide was from Mallinckrodt, Inc. (Paris, KY).

Collection of Blood Samples. Blood samples from female Sprague–Dawley rats and male purebred beagle dogs (*Canis familiaris*) were utilized in the initial studies to determine the chemical structure of the metabolite of GX-071 in the blood. Dogs were administered 100 mg/kg per day GX-071 for 5 days each week for 23 days, allowed to recover for 34 days, and then administered 50 mg/kg per day for 5 days each week for 18 days. The dose was given orally in loosely packed gelatin capsules. Blood samples were taken 77 days after the dosing regime had begun with dogs actually receiving GX-071 on 31 days. Samples were collected from the jugular vein, placed in heparinized tubes, and frozen until analyzed. Female rats were given GX-071 in the diet in concentrations of 30 or 100 ppm (for 9 days) and 500 pm (for 13 days). Animals were anesthetized at the end of each trial, and a blood sample was taken via cardiac puncture. Blood was placed into heparinized tubes and frozen until analyzed.

Blood samples from male and female Sprague–Dawley rats were used in studies for the development of the gas chromatographic assay. Upon arrival and prior to use, animals were housed in solid stainless steel cages (two per cage) with glass front plates and wire grid floors in a limited-access animal room. The animal room was maintained at 22 °C, 40% relative humidity, and had 12 air exchanges/h, with a 12-h light/dark cycle (full-spectrum fluorescent lights). Animals were supplied with a commercial rat ration and tap water ad libitum. Twenty-four hours prior to oral or iv administration of GX-071, indwelling cannulas were surgically implanted in the carotid artery and/or jugular vein of rats. Both arterial and venous cannulas were implanted in rats used for iv studies, but only an arterial cannula was implanted in rats used for oral studies. The cannulas were held to place with a harness apparatus, allowing the rat to move about freely in the cage. GX-071 was administered as a solution in poly(ethylene glycol) 400 in both the oral and iv experiments. Rats were dosed at 9:00 a.m. (2 h into the light cycle). Blood samples were taken immediately prior to dosing, to serve as controls, and sequentially after the doses were administered. Samples, 20–40 μL, were taken from the arterial cannula, placed in chilled new Pyrex screw-cap culture tubes (13 × 100 mm) with Teflon-lined caps, and frozen until analyzed.

Ion-Pair Extraction. One milliliter of double-distilled water (0.02 M, NaOH, 0.01 M TBAHS), 2 mL of methylene chloride, and 1 μg of decanoic acid (ISTD) in 200 μL of methylene chloride were added to the rat blood (20–40 μL) in a 30 × 100 mm Pyrex screw-cap culture tube cooled in an ice bath. The phases were thoroughly mixed with a Lab-Line Super-Mixer and allowed to stand for 15–20 min in the ice bath. Next, the mixture was centrifuged for 2 min, and the aqueous phase was discarded. The organic phase (CH_2Cl_2) was partitioned three times with 1-mL portions of double-distilled water that contained 0.02 M NaOH and 0.01 M TBAHS. In each of the three partitioning steps, the phases were thoroughly mixed, allowed to stand for 5 min in an ice bath, and centrifuged, and the aqueous phases were discarded to yield a GX-071/GX-071M ion pair isolate (organic phase).

Formation of PFB Derivatives. One milliliter of double-distilled water (0.02 M NaOH, 0.01 M TBAHS) and 10 μL of PFBB were added to the GX-071/GX-071M ion-pair isolate in the 30 × 100 mm screw-cap culture tube. The capped culture tube was heated for 2 h at 43 °C in a heating block with occasional mixing, or in a thermostated mechanical shaker. Next, 4 mL of 2,2,4-trimethylpentane was added, the phases were thoroughly mixed, and the tube was centrifuged for 2 min. The organic phase (upper layer) was transferred to a 30 × 100 mm screw-cap culture tube that had been tapered in a flame and blown to dryness in a stream of dry N_2 under mild heat (43 °C). Finally, 100 μL of 2,2,4-trimethylpentane was added, and the PFB derivatives were stored in a freezer (0 °C) until analyses.

Cold On-Column Capillary GC/ECD Analyses. Capillary GC analyses of the PFB derivatives of GX-071 and GX-071M were done with a Hewlett-Packard 5880 gas chromatograph equipped with a laboratory-constructed cold on-column inlet (Arrendale and Chrotyk, 1985), an electron capture detector (ECD), and a FS immobilized stationary phase SE-54 capillary column (Arrendale and Martin, 1988). GC conditions were as follows: column dimensions, 30 m × 0.3 mm (i.d.); temperature program, 90 °C for 1 min, 90–280 °C at 8 °C/min; column flow rate, 40 cm/s He; injection volume, 1 μL of 2,2,4-trimethylpentane; injection mode, cold on-column; makeup gas for the ECD, 40 mL/min N_2.

Identification of GX-071M. The GC separations of the ethyl acetate extracted from dog and rat blood were performed with a Hewlett-Packard 5840 gas chromatographed equipped with a laboratory-constructed cold on-column inlet system (Arrendale and Chortyk, 1985). The fused silica (FS) immobilized stationary phase SE-54 capillary columns were prepared by the method of Arrendale and Martin (1988). Gas chromatographic conditions for the analyses of the ethyl acetate extracts of rat and dog blood were as follows: column dimension, 30 m × 0.3 mm (i.d.); liquid phase film thickness, 0.1 μm; temperature program, 60 °C for 1 min, 60–280 °C at 8 °C/min; column flow rate, 40 cm/s H_2; injection volume, 1 μL of ethyl acetate; injection mode, cold on-column; flame ionization detector (FID). The capillary GC/MS analyses of the ethyl acetate extracts of rat and dog blood were performed with a Hewlett-Packard 5985B GC/MS system equipped with an open split interface (Arrendale et al., 1984). Mass spectrometer conditions were as follows: source temperature, 200 °C; interface zone temperature, 280 °C; electron energy, 70 eV; electron multiplier voltage, 2200 V; scan range, 40–600 amu; scan rate, 277 amu/s.

RESULTS AND DISCUSSION

Determination of GX-071 from rat blood was a relatively easy analytical problem as its gas chromatographic characteristics and its electron capture detector response were very good. However, the metabolite, perfluorooctanesulfonamide, lacked the *N*-ethyl group, which resulted in an increase in reactivity, poor gas chromatographic peak shape, and inconsistent detector response. Studies have shown that acids, phenols, and sulfonamides can be converted to stable pentafluorobenzyl derivatives with high electron capture response (Ehrsson, 1971; Gyllenhaal and Ehrsson, 1975). In this instance, derivatization served primarily to improve the GC characteristics and to a lesser extent the detector response of GX-071M. The derivatization procedure involved two steps: The anionic form of an acidic species was extracted from an alkaline aqueous phase as an ion pair with a lipophilic counterion such as tetrabutylammonium to a poorly solvating organic phase such as methylene chloride. The anionic form of an ion pair possesses high reactivity in nucleophilic displacement reactions (Brandstrom and Junggren, 1969) with pentafluorobenzyl bromide to form pentafluorobenzyl derivatives (PFB).

Capillary GC/FID analyses of the ethyl acetate extracts of whole blood from both rats and dogs that had been given oral doses of GX-071 had two peaks not present in the profiles of normal blood (Figure 1). Both peaks exhibited good response with the electron capture detector (ECD), but the chromatographic characteristics of the first eluting peak were good while those of the second were poor. The capillary GC retention and GC/MS data clearly showed that the first peak was GX-071 (Figures 1 and 2). The base peak in the mass spectrum of GX-071 occurs at m/z 108 and results from cleavage α to the sulfonamide group,

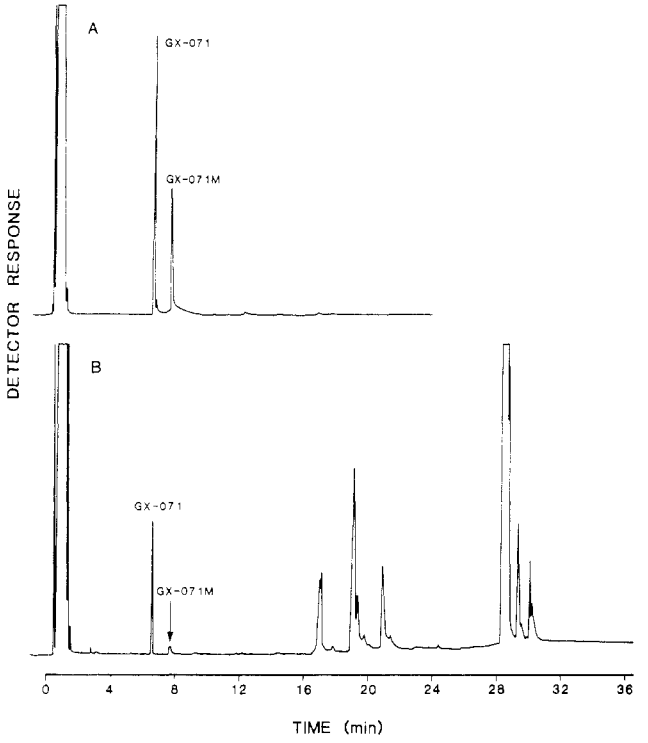

Figure 1. Chromatograms showing the cold on-column injection capillary GC/FID analyses of (A) a standard mixture of GX-071 and GX-071M and (B) ethyl acetate extract of blood from a dog given oral doses of GX-071.

giving rise to an ion with the molecular formula SO_2NH-CH_2CH_3 (Figure 2). Examination of the GC/MS data for the second eluting peak showed that the m/z 108 ion was absent, and the base peak now at m/z 80 (SO_2NH_2) indicated the absence of the ethyl group as this ion also appears to have resulted from loss of the perfluorooctane portion of the molecule by cleavage α to the sulfonamide group (Figure 3). Comparison of GC retention and GC/MS data with authentic standards confirmed that the second eluting peak (Figure 1) was perfluorooctanesulfonamide (Figure 3). The absence of the ethyl group in perfluorooctanesulfonamide also explains its poor chromatographic peak shape in comparison to the *N*-ethyl isomer as reactivity of the primary amine would result in greater interaction with active sites in the chromatographic system.

Determination of GX-071 and its major metabolite, perfluorooctanesulfonamide, from rat blood by capillary GC was complicated by two factors: First, the small size of laboratory rats limited the amount of blood that could be taken at one time without compromising future samples. The sensitivity required here was supplied by the use of cold on-column injection capillary GC with an electron capture detector (Arrendale and Chortyk, 1985). Second, the chemical characteristics of GX-071M often resulted in GC peak tailing, peak broadening, and generally poor chromatographic response. Even using cold on-column injection, the method of choice for labile components (Grob and Grob, 1978), and new, low surface activity FS capillary columns, results were unacceptable as only a few injections of relatively clean samples produced the characteristic peak distortions for GX-071M. Therefore, derivatization was essential for accurate determination of GX-071M. However, GX-071M formed both mono and bis derivatives. For example, silylation yielded both mono- and bis(trimethylsilyl) (TMS) derivatives of GX-071M. Steric hinderance may play a significant role here since GX-071 readily forms stable TMS derivatives, but the addition of two TMS groups on the sulfonamide nitrogen of GX-071M appears to occur at a very slow and unpredictable rate.

A relatively common procedure for the formation of stable pentafluorobenzyl derivatives of compounds with acidic hydrogens, i.e., carboxylic acids, phenols, and sulfonamides, involves the extraction of the compounds as ion pairs from an alkaline aqueous phase to a methylene chloride phase containing the derivatization reagent pentafluorobenzyl bromide (Ehrsson, 1971; Gyllenhaal and Ehrsson, 1975). Under these conditions, ion pairs composed of the anionic forms of acids, phenols, and/or sulfonamides and an alkylammonium cation are extracted from the aqueous phase to the methylene chloride phase according to the formula ($K_{ex(CA)} = [CA_{org}]/[C_{aq}] + [A_{aq}]$ where C_{aq} is the anionic form of the acid in the aqueous phase, A_{aq} is the alkylammonium counterion, CA is the ion pair, and $K_{ex(CA)}$ is the extraction constant (Smith and Stewart, 1981). Thus, the distribution ratio (Dc) can be written as follows: $Dc = [CA_{org}]/[C_{aq}] = K_{ex(CA)}[A_{aq}]$. According to these relationships, a lipophilic compound

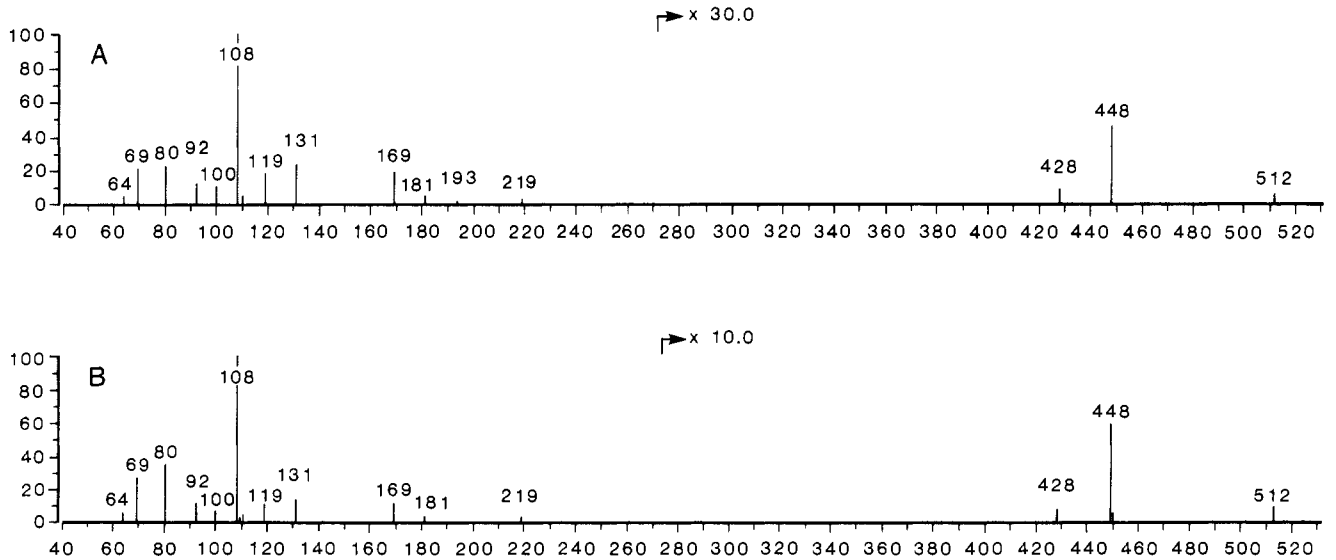

Figure 2. Mass spectra of (A) standard GX-071 and (B) GX-071 from the ethyl acetate extract of blood from a dog given oral doses of GX-071.

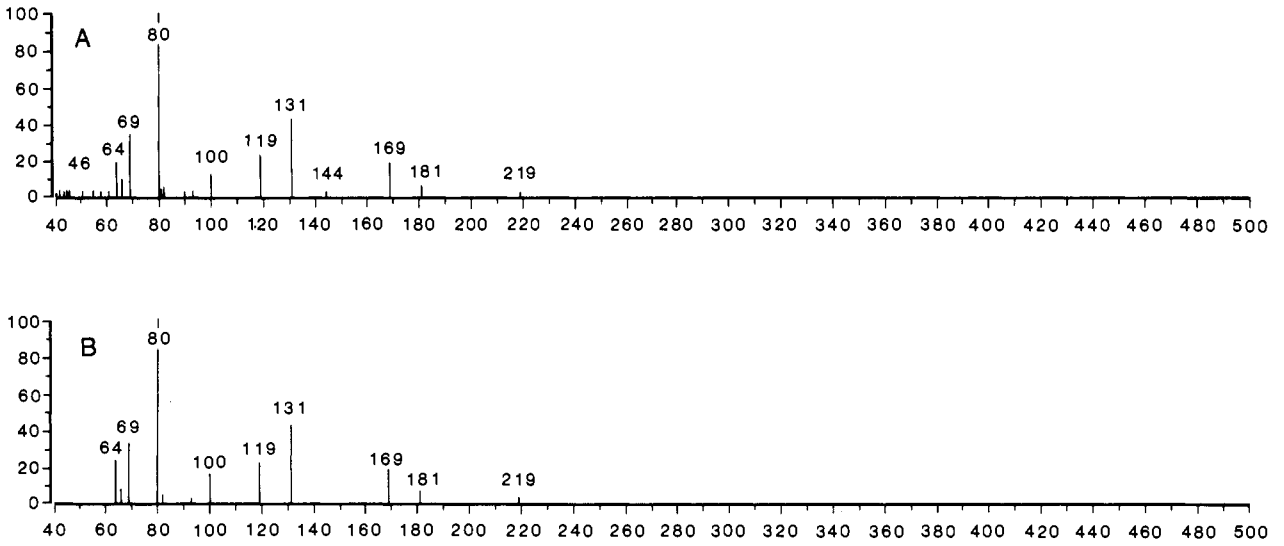

Figure 3. Mass spectra of (A) standard GX-071M and (B) GX-071M from the ethyl acetate extract of blood from a dog given oral doses of GX-071.

Table I. Efficiency of Ion-Pair Extraction[a]

no. of extractns	% recovery[b,c]		
	GX-071	GX-071M	ISTD
1	97.6	97.2	85.7
2	2.4	2.8	11.4
3	0	0	2.9

[a] Determined by repeated extraction of 1 mL of aqueous phase (0.02 M NaOH, 0.01 M TBAHS) containing 40 μL of rat blood spiked with 50 μg of ISTD (decanoic acid), 100 μg of GX-071, and 100 μg of GX-071M with 2-mL portion of CH_2Cl_2. [b] Analyses of the PFB derivative were performed by cold on-column injection capillary GC/FID. [c] Average of three repetitions, percent RSD values for recovery of GX-071, GX-071M, and ISTD in the first 2-mL extract were 0.8, 0.6, and 4.2, respectively.

will require less counterion and a hydrophilic compound will require more counterion to achieve the same distribution ratio.

Fortunately, GX-071 and GX-071M are both lipophilic and require relatively low concentrations (0.01 M) of the tetrabutylammonium counterion to achieve quantitative recovery from the aqueous phase to the methylene chloride phase. High concentrations of NaOH and TBAHS (0.2 and 0.1 M, respectively) often resulted in significant breakdown of naturally occurring components in the blood, which interferred with the GC/ECD determination of GX-071 and GX-071M. Regardless of the composition of the aqueous phase, it was essential that the mixture be cooled in an ice bath until the aqueous phase containing the rat blood was discarded to avoid excessive contamination of the organic phase with blood breakdown products. Data on the efficiency of extraction of GX-071, GX-071M, and the ISTD from the aqueous phase (1 mL, 0.02 M NaOH, 0.01 M TBAHS) containing 40 μL of rat blood with 2-mL portions of methylene chloride are given in Table I. Both GX-071 and GX-071M were quantitatively recovered in the first 2-mL extract as was 86% of the ISTD. We have successfully used a single 2-mL methylene chloride extract for analytical determinations since the reproductibility of recovery of the ISTD from rat blood (% RSD 4.2) was reasonable; however, a second 2-mL extract may also be done if desired, followed by reduction in volume of the methylene chloride to about 2 mL and continuation of the analytical procedure described in the Experimental Section. Recovery of the ISTD was higher (96%) in the first 2-mL extract when higher concentrations of NaOH and TBAHS (0.1 and 0.2 M, respectively) were used. However, as discussed above, contamination from blood breakdown products negated any advantage gained in higher recovery of the ISTD. Alkalinity of the aqueous phase was essential to ion-pair formation and efficient recovery of the components. The pH of the aqueous phase containing the blood may be adjusted with slightly higher concentrations of acid and base or an increased volume of aqueous phase to maintain alkalinity. Partitioning of the organic phase with 3 × 1 mL portions of the aqueous phase served two purposes: (1) the removal of any extraneous aqueous phase containing rat blood, and (2) the breakup of the emulsion at the interface of the phases.

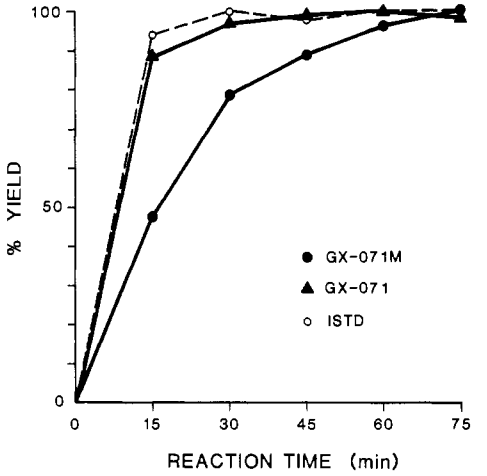

Figure 4. Reaction rates for formation of PFB derivatives.

The anionic species of acids in the form of ion pairs with alkylammonium counterions in a poorly solvating solvent such as methylene chloride are highly susceptible to nucleophilic displacement reactions (Brandstrom and Junggren, 1969; Ehrsson, 1971). Under these conditions, GX-071 and the ISTD readily form PFB derivatives in about 30 min as illustrated in Figure 4. However, complete derivatization of GX-071M requires a minimum of 75 min. The rate of reaction of GX-071M was probably slower because it forms a $(PFB)_2$ derivative and formation of the PFB derivative probably introduced steric hinderance resulting in a slower rate for attachment of the second PFB group. The exact nature of the GX-071M/TBA ion pair is unknown, but analysis of the reaction media by GC/MS failed to demonstrate the presence of a PFB derivative.

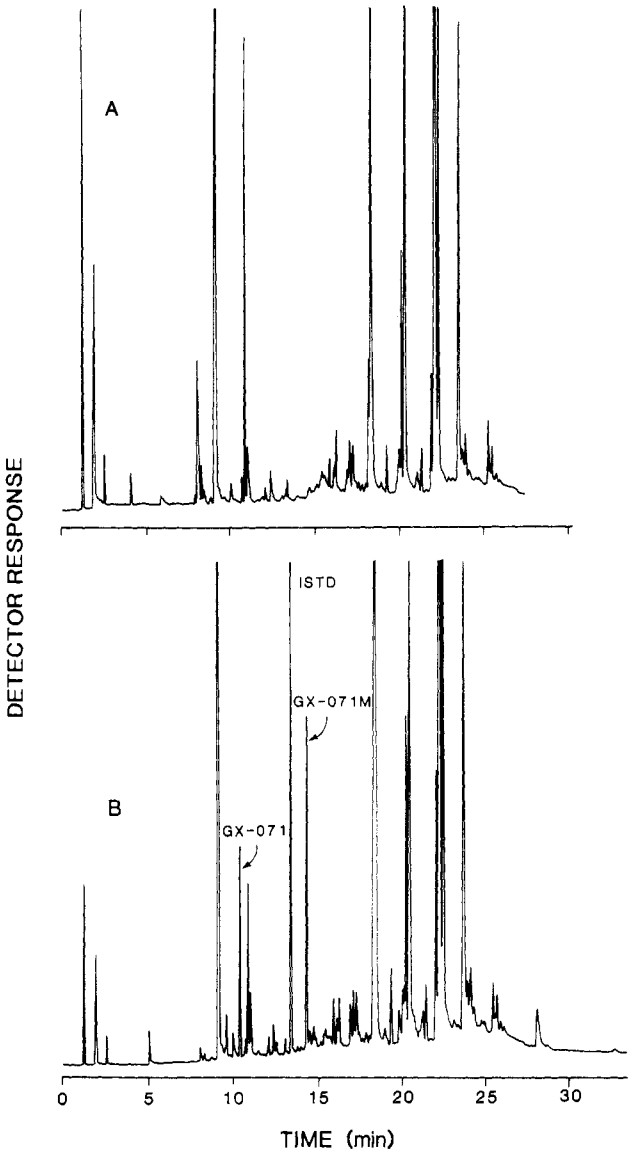

Figure 5. Chromatograms of the capillary GC/ECD analyses of (A) 40 μL of blank rat blood and (B) 40 μL of rat blood spiked with 10 μg/mL GX-071, ISTD, and GX-071M.

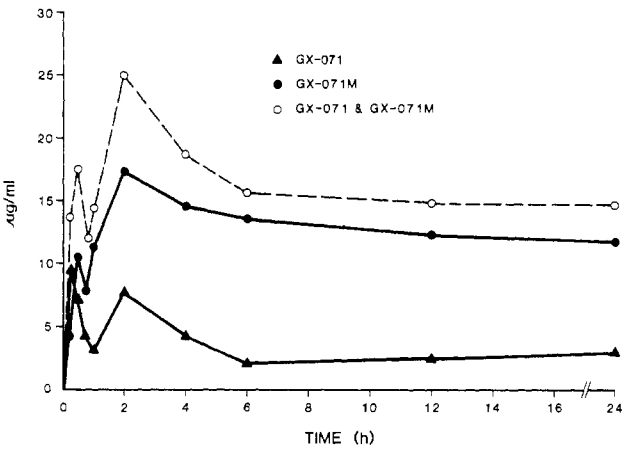

Figure 6. Blood levels of GX-071 and GX-071M from a rat after a 15 mg/kg iv dose of GX-071.

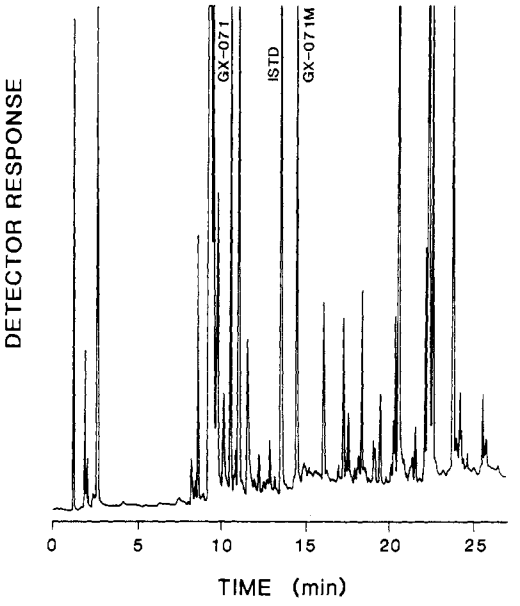

Figure 7. Chromatogram of the capillary GC/ECD analysis of 40 μL of blood from a rat 6 h after administration of a 10 mg/kg iv dose of GX-071.

Higher concentrations of the TBA counterion (0.1 M) had little or no effect on derivatization of GX-071M. Some variation in reaction time was observed; therefore, a reaction period of 2.5 h was used. Occasional mixing of the phases in the reaction mixture or use of a thermostated shaker were used to ensure ion-pair formation as no reaction takes place under these conditions for the three components unless ion pairs are formed first.

The reproducibility of the entire analytical procedure (extraction, derivatization, GC/ECD analysis) was investigated by repeated analyses of 20–40 μL of rat blood spiked with known concentrations of GX-071 and GX-071M. The method yielded percent relative standard deviations of 4.7 and 2.8 for determination of GX-071 and GX-0.1M, respectively, at the 100 ppm (μg/mL) level from 20 μL at rat blood (n = 4); at the 400 ppb (ng/mL) level from 40-μL samples percent RSD values were 17 and 21 for GX-071 and GX-071M, respectively (n = 4). For our purposes, 400 ppb of GX-071 and/or GX-071M represents the lowest practical detector limits without further sample cleanup steps. Peak areas for the three PFB derivatives were also reproducible from run to run when care was taken to quantitatively transfer phases during extraction and derivatization and manual cold on-column injections were carefully reproduced. The percent RSD at the 100 ppm level was <5% for each of the three components (n = 3). Standard curves generated by spiking blank rat blood with varying quantities of GX-071 and GX-071M showed a high degree of linearity. Linear regression on the standard curves from concentrations ranging from 10 to 100 ppm produced correlation coefficients of 0.998 and 0.999 for GX-071 and GX-071M, respectively, which were significant at the 99.9% level of confidence. Correlation coefficients for standard curves with samples down to the 400 ppb (ng/mL) level were significant at the 99.0–99.9% confidence intervals.

Chromatograms of the cold on-column injection capillary GC/ECD analyses of the PFB derivatives from 40 μL of blank rat blood (Figure 5A) and 40 μL of rat blood spiked with 10 μg/mL of GX-071, ISTD, and GX-071M (Figure 5B) were rather complicated. However, the components of interest were separated sufficiently from contaminants, arising from both the rat blood and the reagents, to allow their determination.

Blood levels of GX-071 and GX-071M were determined for a rat given a 15 mg/kg iv dose of GX-071 (Figure 6). The importance placed on accurate determination of GX-071M was justified since GX-071 was rapidly metabolized to GX-071M in this rat. A combination of factors including

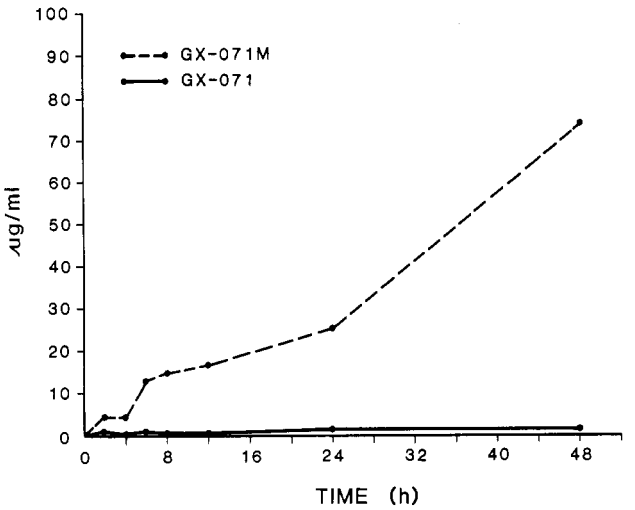

Figure 8. Levels of GX-071 and GX-071M in the blood of a rat after a 150 mg/kg oral dose of GX-071.

poor water solubility, rapid metabolism, and enterohepatic recycling among others resulted in a complex picture for blood levels of these components during the first hour after iv dosage. Analyses of a 40-μL sample of blood taken from a rat 6 h after administration of a 10 mg/kg iv dose of GX-071 are shown in Figure 7. The presence of GX-071 and GX-071M (PFB), in a 40-μL sample of blood taken from a rat 15 min after administration of an iv dose of 25 mg/kg GX-071, was confirmed by cold on-column injection capillary GC/MS by comparison of retention times and mass fragmentation patterns with those of authentic standards. A graph showing the levels of GX-071 and GX-071M in the blood of a rat after a single 150 mg/kg oral dose of GX-071 again indicated that GX-071 was extensively metabolized to GX-071M (Figure 8). These data suggest that GX-071 undergoes a significant hepatic metabolism (first-pass effect) prior to entry into the systematic circulation.

Registry No. GX-071, 4151-50-2; GX-071M, 754-91-6.

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Distribution of Tolclofos-methyl in Potatoes Grown on Soil Treated with Rizolex

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Tolclofos-methyl residue levels in potato tubers are affected both by the applied dosage of the fungicide Rizolex-50 WP and by the type of soil on which the potatoes were raised. All the tolclofos-methyl present in the tuber accumulates in the peels wherein about 70% is located in the fiber fraction and about 20% in the juice fraction. No tolclofos-methyl could be detected in the starch fraction. Applying the recommended amount of Rizolex-50 WP (15 kg/ha), the tolclofos-methyl residue level in the unpeeled potatoes never exceeded 0.05 ppm.

Potato disease caused by infection of *Rhizoctonia solani* (e.g., black scurf and stem canker) are serious agricultural and economical problems in the production of potato crops. It is known that, after desinfection of the seed potatoes, in many cases the potato crop will still be affected by *R. solani* from the soil. Many chemicals have been used in an attempt to control these diseases. In the past several years a new fungicide, with the trade name Rizolex, with

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