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Metabolism of 2,3,5,6-tetrachloronitrobenzene (tecnazene) in rat

G. J. LAPPIN*, D. PRITCHARD, R. B. MOORE and W. J. D. LAIRD

Zeneca Central Toxicology Laboratory, Alderley Park SK10 4TJ, UK

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1. The metabolic fate of [U-^{14}C]-2,3,5,6-tetrachloronitrobenzene (tecnazene) has been determined in the male and female rat following a single dose of 1 mg/kg and in surgically prepared, bile-duct-cannulated rats following a single oral dose of 135 mg/kg.

2. Radioactivity in the female rat was excreted mainly in urine (82 %). The male rat, however, excreted approximately equal amounts of radioactivity in urine and faeces (the latter *via* bile).

3. The principal metabolic pathway was conjugation with glutathione (GSH) and concomitant nitro-displacement. The GSH-conjugate and related metabolites were excreted in the bile and ultimately in the urine as the mercapturic acid conjugate. The cysteine conjugate underwent β-lyase-mediated metabolism to yield a thiol that underwent subsequent methylation to the thioanisole followed by *S*-oxidation.

4. A novel tetrachloromethyldisulphide metabolite was also formed.

Introduction

2,3,5,6-Tetrachloronitrobenzene (tecnazene) is used as a sprout inhibitor on stored potatoes and as a fungicide in smoke generators in greenhouses. The metabolism of tecnazene has been reported for rabbit (Bray *et al.* 1951, 1953, 1957, Betts *et al.* 1955) and pigeon (Wit and Leeuwangh 1968). There is also some limited information on its metabolism in guinea pig and rat (Bray *et al.* 1958). The principal metabolic pathway in all species studied, except apparently the guinea pig, was displacement of the nitro moiety by glutathione (GSH) conjugation evidenced by the presence of the mercapturic acid conjugate in urine. This pathway is consistent with the metabolism of several other chlorinated nitrobenzenes in several species (e.g. rat (Renner and Nguyen 1984), goat and sheep (Aschbacher and Feil 1983), and fish (Bahig *et al.* 1981)). Tecnazene also formed the aniline derivative in the rabbit, along with its *para* phenol and the corresponding glucuronide and sulphate. Apart from the mercapturic acid conjugate, no other metabolites of tecnazene have been reported in rat.

Here we describe the biotransformation of [U-^{14}C]-tecnazene in the male and female rat following oral administration. The major metabolic pathway and several minor pathways were elucidated.

Materials and methods

[U-^{14}C]-tecnazene (specific activity 3 GBq/mM and radiochemical purity > 95 %) and unlabelled tecnazene, with a chemical purity of 98·7 %, were obtained from ICI Agrochemicals (Jealott's Hill Research Station, Bracknell, UK). 2,3,5,6-Tetrachlorothioanisole and 2,3,5,6-tetrachloroaniline (purities > 90 %) were obtained from Lancaster Synthesis (Morcambe, UK). 1,2,4,5-Tetrachlorobenzene

* Author for correspondence at: Corning Hazleton, Otley Road, Harrogate HG3 1PY, UK.

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(purity > 90 %) was obtained from Aldrich Chemical Co. (Gillingham, UK). 2,3,5,6-Tetrachlorophenol, 2,3,5,6-tetrachlorothioanisole sulphoxide and 2,3,5,6-tetrachlorothioanisole sulphone (purities unknown) were synthesized by Dr D. W. Brown (University of Bath, School of Chemistry, Claverton Down, UK).

2,3,5,6-Tetrachlorobenzenethiol was prepared essentially by the method of Renner (1978).

2,3,5,6-Tetrachloroaniline was diazotized with a mixture of sodium nitrite in hydrochloric acid and acetic acid and allowed to react with potassium ethyl xanthate to give *O*-ethyl-*S*-2,3,5,6-tetrachlorophenylxanthate. This compound was hydrolyzed with sodium hydroxide to give 2,3,5,6-tetrachlorobenzenethiol, m. pt. 106 °C. ^{1}H-nmr d ($CDCl_3$) 7·77 (s, 1H). EI-MS (*m/z*) 248 (M, 100), 213 (57), 176 (27), 140 (9), 105 (15).

Octachlorodibenzenedisulphide was prepared using the general method for oxidation of thiols to disulphides developed by Aida *et al.* (1976). 2,3,5,6-Tetrachlorobenzenethiol was dissolved in a mixture of toluene and dimethyl sulphoxide and treated with iodine at 20 °C for 20 h. The reaction mixture was washed with dilute sodium hydroxide solution and evaporation of the organic solvent gave pure octachlorodibenzenedisulphide m. pt. 134 °C. ^{1}H-nmr d (DMSO) 8·23 (s, 1H). EI-MS (*m/z*) 494 (M^+, 32), 245 (52), 212 (100).

Tetrachlorobenzenemethyldisulphide was prepared by using the method of Mukaiyama and Takahashi (1968) for mixed disulphides.

2,3,5,6-Tetrachlorobenzenethiol was treated with diethyl azodicarboxylate to give diethyl *N*-(2,3,5,6-tetrachlorophenylsulphenyl) hydrazodicarboxylate. This compound was heated with sodium thiomethoxide to give *S*-methyl-*S'*-2,3,5,6-tetrachlorophenyl disulphide, which was purified by silica column chromatography eluting with hexane. ^{1}H-nmr d ($CDCl_3$) 2·55 (s, 3H), 7·63 (s, 1H). EI-MS (*m/z*) 294 (M^+, 100), 259 (22), 222 (37), 212 (56).

2,3,5,6-Tetrachlorobenzene mercapturate was prepared as follows: a sample of tecnazene (1·00 g, 3·83 mmol) was dissolved in dioxan (10 ml) and added to a solution of *N*-acetyl cysteine (0·625 g, 3·83 mmol) in saturated aqueous sodium bicarbonate solution (10 ml). The mixture was stirred at 20 °C for 16 h, filtered, and the filtrate extracted with ethyl acetate after adjusting to pH 2 with concentrated hydrochloric acid. After evaporation of the solvent, the residue was triturated with diethyl ether to give pure 2,3,5,6-tetrachlorobenzene mercapturate. ^{1}H-nmr d (DMSO) 1·77 (s, 3H), 3·3 (m, 2H), 4·3 (m, 1H). EI-MS (*m/z*) 377 (M^+, 12), 318 (63). 248 (38), 212 (26), 87 (100).

Animals

Wistar-derived male and female rats (Alpk:ApfSD strain, weight range 180–280 g) were obtained from the Barriered Animal Breeding Unit (Zeneca Pharmaceuticals). The animals were maintained under controlled environmental conditions (19–23 °C, 40–72 % relative humidity, 12 h of light cycled with 12 h of dark). To determine metabolic profiles, five male and five female rats were each given 1 mg [U-^{14}C]-tecnazene/kg as a single oral dose by gavage, using corn oil as the dosing vehicle. The dose was administered at 4 ml/kg and the amount of radioactivity was 2 MBq/kg. To obtain sufficient mass of ^{14}C-metabolites for characterization, five male and five female rats were administered four consecutive daily doses of 135 mg [U-^{14}C]-tecnazene/kg as described above. In addition, the bile ducts of one male and one female rat were cannulated under Fluothane (Zeneca Pharmaceuticals) anaesthesia. The cannulae were exteriorized and the animals allowed to recover. On the morning following surgery each rat was given a single oral dose of 135 mg [U-^{14}C]-tecnazene/kg. Urine and faeces were collected into containers cooled by solid CO_2. For the 1-mg/kg dosing experiment, urine was collected during 0–6 h and urine and faeces during 6–12, 12–24, 24–36 and 36–48 h and then at 24-h intervals until 144 h. For the bile-duct cannulation experiment, bile was collected at room temperature during 0–2, 2–4, 4–6, 6–12, 12–24, 24–28, 28–36 and 36–48 h after dosing. Urine was collected from bile duct cannulated rats 0–12, 12–24, 24–36 and 36–48 h after dosing. Faeces were collected during 0–24 and 24–48 h after dosing. Samples were stored at −20 °C prior to analysis.

Determination of radioactivity

Duplicate 1-ml samples of urine and bile were mixed with Optiphase-MP scintillant (10 ml) and analyzed for radioactivity (1 % of the SD or 10 min) using a Packard Tricarb 2000 CA liquid scintillation counter. Values were corrected for counting efficiency using a ^{133}Ba external standard. Faeces were ground until homogeneous. Samples (*c.* 200 mg) were oxidized (Packard Tricarb B306) and $^{14}CO_2$ absorbed in Optisorb '1' and mixed with Optisorb 'S' scintillant (total volume 10 ml). Radioactivity was analysed by liquid scintillation counting as described above.

High-performance liquid chromatography

For urine samples, approximately 10 % of each sample weight was combined forming separate pools for males and females.

Samples of bile were similarly combined over the 0–48-h collection times. Untreated urine or bile was injected (100 µl) onto an APEX ODS, 5-µm particle size, 15 cm × 4·5 mm column (Jones Chro-

matography, Hengoed, UK). The column was eluted with an acetonitrile–water gradient (solvents modified with 1 % (v/v) acetic acid). The gradient started at 1:40 and changed to 100 % acetonitrile over 0–90 min, which was held for a further 30 min. The flow rate was 1·5 ml/min. Detection was by UV absorption at 254 nm. One-minute fractions were also collected, mixed with Optiphase MP (10 ml) and analysed by liquid scintillation counting.

The amount of radioactivity in each hplc peak was used to calculate the quantities of metabolites present as the percentage of administered dose. The prolonged hplc analysis times were necessary to separate the numerous metabolites, with varying polarities, in the presence of constitutive compounds which interfered with the chromatographic separation.

Hplc-mass spectroscopy (hplc-ms)

Metabolites in urine and bile were separated by a similar method to that described above. The column was eluted with an acetonitrile–water gradient (solvents modified with 1 % (v/v) acetic acid). The gradient started at 1:40 and changed to 100 % acetonitrile over 0–15 min and the flow rate was 0·5 ml/min. Ammonium acetate (0·2 M) was continuously added post-column at 0·5 ml/min to the eluate, which was passed into a VG 70-70E mass spectrometer fitted with a thermospray/plasmaspray interface at 230 °C. Negative ion spectra were acquired.

Gas chromatography-mass spectroscopy (gc-ms)

A VG 70-70E mass spectrometer, with an EI source, interfaced to a Hewlett Packard 5790 GC was used. The chromatograph was fitted with a SGE BP1, 25 m, 1-μm film thickness, 0·53-mm i.d. column, preceded with *c.* 50-cm deactivated silica retention gap. Helium was the carrier gas. The temperature program was 75–150 °C at 35 °C/min, then, 150–300 °C at 15 °C/min, which was held for 10 min. Samples were extracted from the urine using ethyl acetate and injected on-column. For the identification of metabolite **2**, the ethyl acetate extract was first methylated with ethereal diazomethane.

Results

Excretion of radioactivity

The female rat administered 1 mg [U-^{14}C]-tecnazene/kg excreted approximately 82 % of the administered radioactivity in urine and approximately 13 % in faeces over the duration of the experiment. In contrast, the male rat excreted approximately 43 % of the dose in the urine and 49 % in the faeces over the same time (table 1, figure 1). The rate of urinary excretion for the female rat was slightly higher than for the male. Bile-duct-cannulated rats administered 135-mg tecnazene/kg excreted approximately 81 and 31 % of the dose in bile for the male and female respectively (figure 2). The male bile-duct-cannulated rat excreted approximately 5·5 % of the dose in the urine and 6·4 % in faeces. For the female, approximately 33 % was excreted in the urine and 15 % in the faeces. The rate of biliary excretion was higher in the male rat than in the female.

Identification of metabolites

Metabolites were identified, using gc-ms or hplc-ms, in urine from the male and female rat administered four consecutive daily doses of [U-^{14}C]-tecnazene at 135 mg/kg/day. Their presence was confirmed in the urine from rats administered 1-mg tecnazene/kg by co-chromatography. Metabolites in bile were identified by hplc-ms. Following a provisional identification based on the mass spectra, the majority of the metabolites were obtained as authentic standards, either commercially or by synthesis (see Materials and methods). Identification was confirmed by comparison of the chromatographic retention time and mass spectra obtained from the metabolite and reference standard. The principal ions in the EI + and negative ion hplc-ms spectra along with retention time data for identified metabolites are given

Table 1. Percentage recovery of radioactivity following a single oral dose of 1 mg/kg tecnazene in the rat (mean of five rats).

	Male (% of dose)		Female (% of dose)	
Sample	Mean	SD	Mean	SD
Urine	43·2	4·84	81·8	1·31
Faeces	49·1	5·43	13·2	1·70
Cage wash	0·1	0·03	0·2	0·11
Total	92·4		95·2	

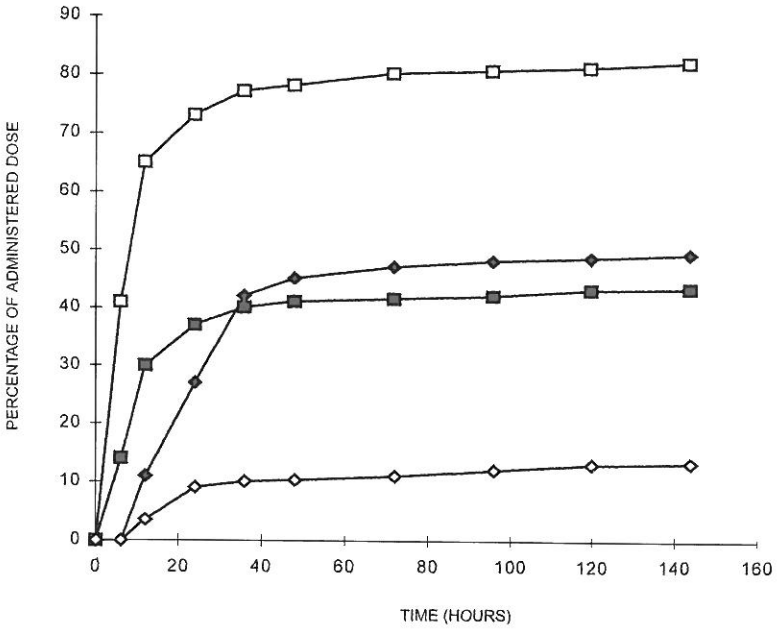


Figure 1. Excretion of radioactivity in male urine (■), female urine (□), male faeces (◆), and female faeces (◇) ($n = 5$ rats of each sex) following the administration of a single oral dose of ^{14}C-tecnazene (1 mg/kg).

in table 2, which quotes the most abundant ion in a given chlorine isotopic cluster (i.e. for four chlorines, $^{35}Cl_3{}^{37}Cl$ and for eight chlorines, $^{35}Cl_6{}^{37}Cl_2$).

Metabolite **1** was presumed to be unmetabolized tecnazene, present in urine and bile, based on its hplc retention time (88·5 min) when compared with that of the standard. Metabolite **1** was present in very small quantities (< 0·1 % of dose) and spectroscopic identification was not possible.

Metabolite **2** was detected by gc-ms in the ethyl acetate extract of urine following methylation with diazomethane. Following methylation, two products were obtained, the methyl ester and *N*-methylated methyl ester of the mercapturic acid. The mass spectra and retention times for both derivatives were the same as those for a reference standard methylated in the same way. The EI+ spectrum of the *N*-methylated methyl ester showed a M^+ at 405 and a loss of 59 ($COOCH_3$) at 246. The methyl ester of **2** had a M^+ at 391 and showed a loss of 59 at m/z 332. Metabolite **2** was also identified in unprocessed urine by lc-ms. The thermospray spectrum had a M^- at 377 and an ion at 248, which was attributed to $[HSH\text{-}Ar\text{-}Cl_4]^-$.

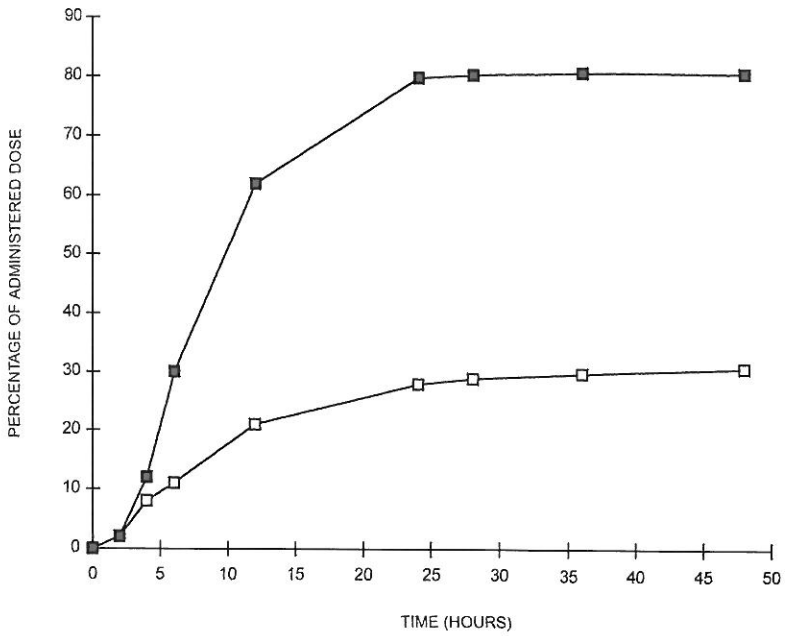


Figure 2. Excretion of radioactivity in bile from one male (■) and one female (□) rat following the administration of a single oral dose of tecnazene (135 mg/kg).

Metabolite **3** was detected in urine by hplc-ms and was assigned as the sulphoxide of tetrachlorobenzene mercapturate. The ESP-spectrum showed a $[M-1]^-$ ion at 393.

Metabolite **4** was detected in urine by hplc-ms and was assigned as the sulphone of tetrachlorobenzene mercapturate although the ESP spectrum was very weak. An ion at 409 was present which was attributed to the molecular ion. A major ion at 393 was assigned to [M-oxygen]$^-$.

Metabolites **5**, **7**, **8**, **9**, **11**, **12** and **13** were all detected in ethyl acetate extracts of urine by gc-ms and comparison with the authentic standard. These metabolites were identified as follows:

Metabolite **5**—tetrachloroaniline. The EI+ spectrum showed a M^+ ion at 231. The other major ion (135) was attributed to background interference.

Metabolite **6** was identified in ethyl acetate extracts of urine by gc-ms and was assigned as 4-hydroxytetrachloroaniline. The EI+ spectrum showed a M^+ ion at 247.

Metabolite **7**—tetrachlorobenzene. The EI+ spectrum showed a M^+ ion at 214 and an ion at 179 attributed to $[M-Cl]^+$. An ion at 143 was assigned to the $[M-HCl-Cl]^+$ or $[M-Cl-HCl]^+$ fragments.

Metabolite **8**—tetrachlorophenol. The EI+ spectrum showed a M^+ ion at 232. An ion at 196 was assigned to $[M-HCl]^+$. The fragment at 166 was consistent with ring fragmentation, forming $[Cl-C=C(Cl)-C(H)=C-CCl]^+$.

Metabolite **9**—tetrachlorobenzenethiol. The EI+ spectrum showed a M^+ ion at 248. An ion at 213 was assigned the $[M-Cl]^+$ fragment and the ion at 178 was the $[M-Cl_2]^+$ fragment.

Metabolite **10** was identified by direct analysis of urine by hplc-ms and

Table 2. Tabulated mass spectra and retention times for metabolites of tecnazene.

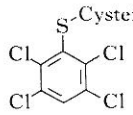
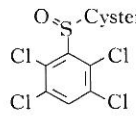
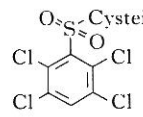
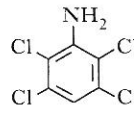
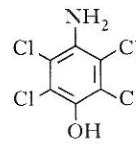
Metabolite	El+ or −ve thermospray (TSP−)	Hplc retention time (min)
2 (methyl ester) 	GC-MS (El+)	Not applicable GC used for identification
M^+ 391 (2)[c], 332 (70)[a], 290 (10)[c], 261 (2)[c], 238 (3)[c], 212 (3)[c], 144 (5)[c], 117 (20)[d], 88 (40)[d], 59 (10)[d], 43 (100)[d]		
2 (*N*-methyl, methyl ester)	GC-MS (El+)	GC
M^+ 405 (2)[a], 346 (100)[a], 290 (42)[a], 274 (5)[c], 248 (10)[c], 212 (17)[a], 178 (3)[c], 158 (11)[d], 131 (24)[d], 102 (55)[d], 74 (18)[d], 43 (71)[d]		
2 Underivatized	TSP−	44.5
437 (12)[a], $[M-1]^-$ 377 (100)[a], 248 (12)[a]		
3 	TSP−	42.5
M^- 393 (100)[a], 324 (10)[c], 294 (20)[a], 248 (25)[c], 225 (5)[d], 189 (25)[d], 129 (28)[d]		
4 (weak spectrum) 	TSP−	48.2
M^- 409 (40)[c], 393 (90)[a], 188 (100)[c]		
5 	El+	80
M^+ 231 (45)[a], 158 (8)[c], {135 (100)[d], background}, 107 (23)[d]		
6 	El+	22
M^+ 247 (100)[a], 183 (45)[c], 146 (60)[c]		

Table 2. (*cont.*)

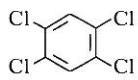
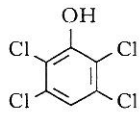
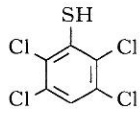
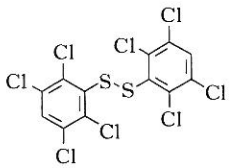
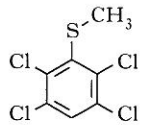
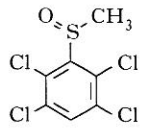
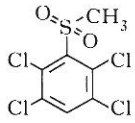
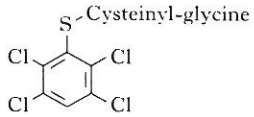
Metabolite	El+ or −ve thermospray (TSP−)	Hplc retention time (min)
7 	El+	96
M^+ 214 (100)[a], 179 (23)[b], 143 (22)[c], 108 (21)[e], 84 (14)[d], 74 (26)[d]		
8 	El+	65.5
M^+ 232 (100)[a], 196 (18)[b], 166 (20)[c], 131 (34)[d], 107 (8)[e], 96 (15)[e]		
9 	El+	99
M^+ 248 (100)[a], 213 (58)[b], 178 (25)[c], 140 (20)[d], 105 (24)[e],		
10 	TSP−	120
M^- 494 (15)[f], 245 (47)[a], {100 (100)[d], background}		
11 	El+	101.5
M^+ 262 (100)[a], 247 (8)[a], 229 (37)[e], 212 (34)[b]		
12 	El+	35
M^+ 278 (49)[a], 263 (100)[a], 108 (31)[e]		

Table 2. (*cont.*)

Metabolite	El+ or −ve thermospray (TSP−)	Hplc retention time (min)
13 weak spectrum 	El+	50
M^+ 294 (94)[a], 230 (88)[e], 215 (100)[a], 180 (40)[e], 143 (61)[e], 108 (63)[e], 79 (96)[d]		
14 (see figure 3)	El+ and accurate mass	106
15 	TSP−	25.2
M^+ 392 (20)[a], 248 (100)[a], 212 (50)[b], 146 (90)[d], 96 (65)[d]		

Isotopic cluster consistent with: [a]4Cl, [b]3Cl, [c]2Cl, [d]no chlorine atoms, [e]not determinable, and [f]8Cl. Molecular ions and relevant fragments are based on the most abundant ion in the isotopic cluster (i.e. four chlorines, $^{35}Cl_3{}^{37}Cl$ and eight chlorines, $^{35}Cl_6{}^{37}Cl^6$).

comparison with the authentic standard. This metabolite was identified as the octachlorodibenzenedisulphide. The molecular ion at 494 had a isotopic cluster consistent with 8Cl. An ion at 248 was the $[HS\text{-}Ar\text{-}Cl_4]^-$ fragment.

Metabolite **11**—tetrachlorothioanisole. The EI+ spectrum showed a M^+ ion at 262. An ion at 247 was attributed to the $[M\text{-}CH_3]^+$ fragment. A cluster of ions at 229 was not directly interpretable to a single structure but was thought to represent a mixture of $[M\text{-}Cl]^+$ and $[M\text{-}SH]^+$ ions. An ion at 212 was attributed to the $[S\text{-}Ar\text{-}Cl_3]^+$ fragment.

Metabolite **12**—the sulphoxide of tetrachlorothioanisole. The EI+ spectrum showed a M^+ ion at 278 and a fragment at 263 attributed to $[M\text{-}CH_3]^+$.

Metabolite **13**—the sulphone of tetrachlorothioanisole. The EI+ spectrum was weak and therefore contained a number of ions not interpretable directly to the structure. The spectrum was nevertheless consistent with that of the standard and had the same retention time on gc and hplc. The spectrum showed a M^+ ion at 294 and a fragment at 215 attributed to $[Ar\text{-}Cl_4]^+$.

Metabolite **14** was detected in ethyl acetate extracts of urine by gc-ms, comparison with the authentic reference standard and accurate mass MS at a resolution of 5000 measured against perfluorokerosene. This metabolite was identified as the tetrachlorobenzenemethyldisulphide. The EI+ spectrum is shown in figure 3. The accurate mass was 293·8479 (consistent with $C_7{}^{35}Cl_3{}^{37}ClS_2H_4$).

Metabolites **15**, **16** and **17** were all detected in bile by hplc-ms.

Metabolite **15** was assigned as the tetrachlorobenzene cysteinyl–glycine conjugate. The ESP spectrum showed a $[M\text{-}1]^-$ ion at 392. The fragment at 248 was attributed to $[HS\text{-}Ar\text{-}Cl_4]^-$ and the fragment at 212 was attributed to $[HS\text{-}Ar\text{-}Cl_3]^-$.

Metabolite **16** produced a spectrum similar to that of the mercapturate (**2**) showing a molecular ion at 377, but its retention time (21·5 min) was different to that

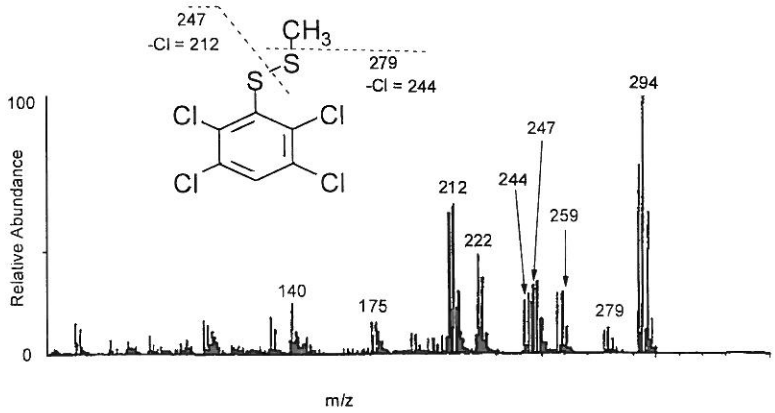


Figure 3. EI + mass spectrum of metabolite **14**, identified as tetrachlorobenzenemethyldisulphide. The mass spectrum of an authentic standard was identical to the one shown here for the metabolite.

of the mercapturate standard (22·5 min). Since ammonium acetate was added post-column, **16** was therefore assigned as the cysteine conjugate and it was thought that the metabolite was acetylated in the ion source.

Metabolite **17** failed to produce a spectrum interpretable to the structure. Since the mercapturate (**2**), cysteinyl–glycine (**15**) and cysteine (**16**) were present it was possible that **17** was the GSH conjugate. Metabolite **17** was more polar on hplc than **2**, **15** and **16**, which is consistent with it being the GSH. It is the experience of this laboratory that GSH conjugates do not yield molecular ions under thermospray conditions.

Quantitation of metabolites

The most abundant metabolite in female rat urine was the mercapturic acid (**2**) (*c.* 56 % of the dose and 74 % of the urinary radioactivity). In male rat urine this metabolite accounted for 19·3 % of the dose and 35 % of the urinary radioactivity. In male rat bile the most abundant metabolite was **17**, which was thought to be the GSH conjugate, representing 48 % of the dose. In female rat bile this metabolite accounted for 24 % of the dose. The cysteine conjugate accounted for 22 % of the dose in male bile and 2·6 % in female. Metabolite **15**, assigned as the cysteinyl–glycine conjugate, was not sufficiently well resolved to be reliably quantified. All other identified metabolites did not individually exceed 1·3 % of the dose.

Discussion

From the amounts of radioactivity recovered in the faeces of the bile-duct-cannulated rat it was established that at least 85 % of a single oral dose of 135-mg tecnazene/kg was absorbed. The difference in the amounts of radioactivity in the faeces of the male (6·4 %) and female (15 %) bile-duct-cannulated rat was not considered significant given that the data were derived from one animal of each sex and they had been surgically prepared. Moreover, given the lipophilicity of tecnazene, absorption was likely to be reduced by the absence of bile salts in the gastrointestinal tract following cannulation.

In the bile-duct-cannulated female rat, 33 % of the dose was excreted directly in the urine and an approximately equal amount in the bile. In contrast, the male bile-duct-cannulated rat excreted 5·5 % of the dose in urine and 81 % in bile. Given that the male non-cannulated rats (given a dose of 1 mg/kg) excreted approximately equal amounts in urine and faeces, a proportion of the radioactivity must be reabsorbed in the gastrointestinal tract following biliary excretion. Consequently, there was the possibility that tecnazene metabolites undergo enterohepatic recirculation. Such an effect was also seen in the female although higher levels of radioactivity were excreted directly in the urine. The enterohepatic recirculation of pentachlorothioanisole in the rat is known to occur (Bakke *et al.* 1990) and biliary excretion has been reported to be more pronounced for the male (Bakke *et al.* 1993). The rate of biliary excretion of tecnazene was also higher for the male rat. No such sex difference in the excretion of tecnazene has been previously reported in the rat or any other species, although a sex difference in the metabolism of hexachlorobenzene in the rat has been reported, with the female rat producing more sulphur-containing metabolites (Richter *et al.* 1981). A sex difference in the excretion of sulphur-containing metabolites has also been reported in rats receiving oral doses of pentachloronitrobenzene at 50 mmol/kg/day for 7 days. There was no difference in the amounts of pentachlorobenzethiol and tetrachloro-1,4-benzenedithiol (derived from the mercapturic acid conjugates of pentachlorobenzene) excreted in the bile or faeces, but the urinary excretion of both thiols was > 10-fold greater in the males. A similar result was obtained following a single intraperitoneal dose of 20 mmol/kg pentachlorophenyl-*N*-acetyl-L-cyseine. There was also evidence that the sulphur-containing metabolites were excreted by an active renal secretion, particularly in the female rat (Smith and Francis 1983).

Thermospray analysis did not result in a spectrum interpretable to the GSH conjugate (**17**). It is the experience of this laboratory that GSH conjugates do not readily form molecular ions using thermospray. The cysteinyl–glycine, cysteine and mercapturic acid conjugates were all identified as metabolites, which leads to the conclusion that GSH conjugation was an important metabolic pathway. The electrophilic carbon adjoined to the nitro group was conjugated with GSH, with the concomitant displacement of the nitro moiety. GSH conjugation of tecnazene was previously assumed to be the main metabolic pathway in the rabbit and possibly the rat, although no bile-duct cannulation experiments have been previously reported (Bray *et al.* 1951, 1953, 1957, Betts *et al.* 1955). GSH conjugation is also an important pathway in the metabolism of similar compounds (e.g. pentachloronitrobenzene and hexachlorobenzene in the rat (Renner and Nguyen 1984); tetrachlorobenzene in the monkey (Schwartz *et al.* 1985)).

There is evidence to indicate that the GSH conjugate underwent a series of metabolic transformations resulting in several other metabolites. There was evidence for the presence of the cysteinyl–glycine metabolite (**15**) in bile along with the cysteine conjugate metabolite (**16**). *N*-acetylation of the cysteine conjugate, probably in the kidney following reabsorption, results in the excretion of the mercapturic acid conjugate (**2**) in urine. Both the sulphoxide and sulphone of the mercapturic acid conjugate were detected in the rat urine (**3** and **4** respectively). The *S*-oxidation of mercapturates are known to occur in mammals (Davison *et al.* 1990). The presence of tetrachlorothioanisole (**11**) in rat urine, along with the free thiol (**9**), is evidence of β-lyase-mediated metabolism of the cysteine conjugate followed by *S*-methylation (Davison *et al.* 1990). A similar process has been seen in the metabolism

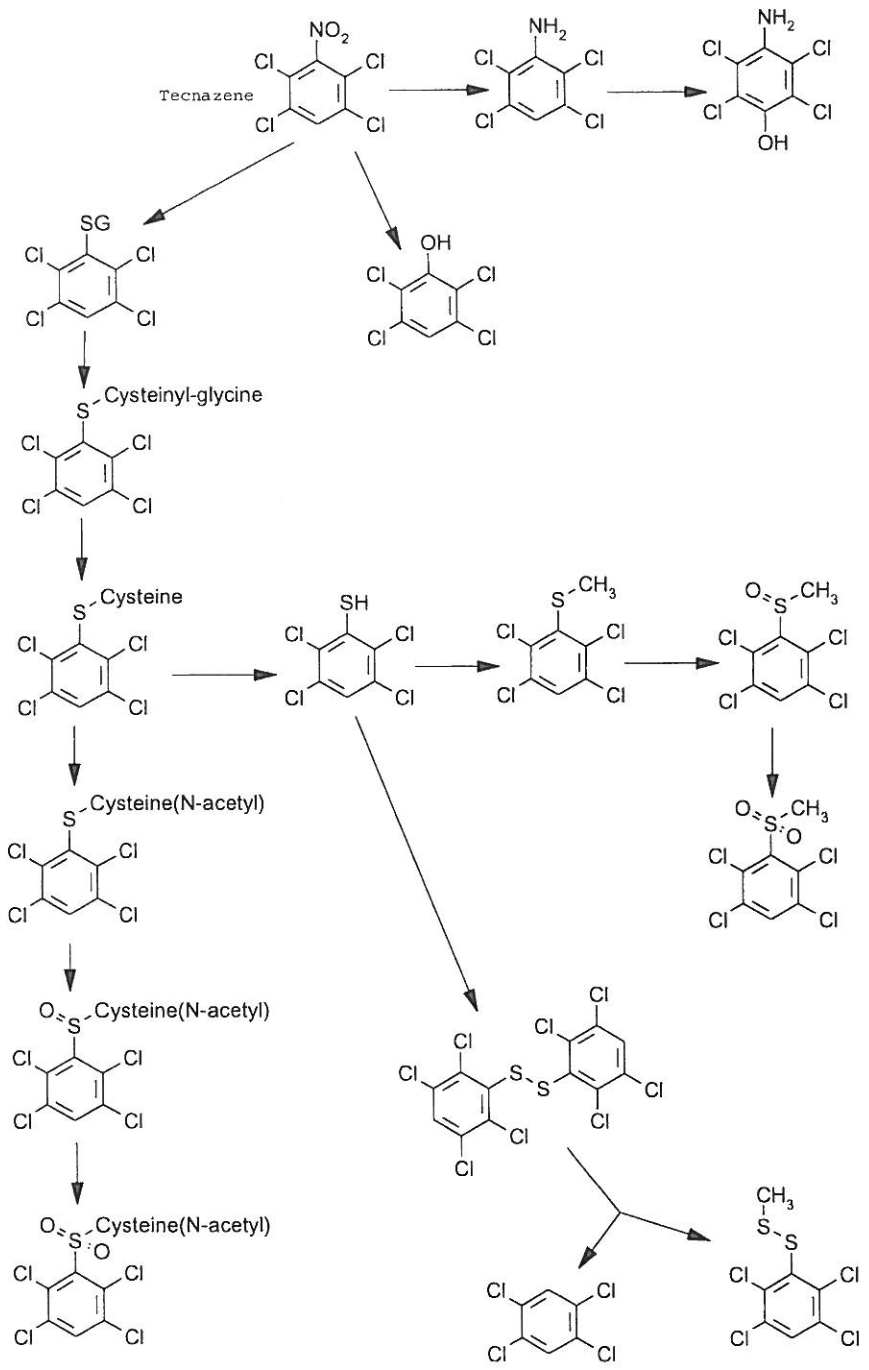


Figure 4. Proposed pathways for the metabolism of tecnazene in the rat. SG, glutathione derivative.

of pentachloronitrobenzene and hexachloronitrobenzene (Renner and Nguyen 1984).

Pentachlorothioanisole is also known to form the GSH conjugate in rat by displacement of the thiomethyl moiety, which in turn forms the cysteine conjugate.

β-lyase activity and methylation regenerates the thioanisole (Huwe *et al.* 1991, Mulford *et al.* 1991). A sex difference has also been reported in the metabolism of pentachlorothioanisole with greater biliary excretion occurring in the male rat (Bakke *et al.* 1993). The sulphoxide and sulphone (**12** and **13** respectively) of these metabolites were also detected in urine in the present study, formed, presumably, by a similar mechanism as to the *S*-oxidation products of the mercapturate. The free thiol compound was found to dimerize when left in solution overnight. The presence of the dimer in urine (**10**) was therefore explained by facile dimerization of the thiol.

The tetrachlorobenzenemethyldisulphide (**14**) was detected in the urine. The metabolic process leading to this very unusual metabolite is difficult to explain but may be formed following a second conjugation of the free thiol with GSH. Such conjugations with sulphur are known, for example *S,S,S*,-tributylphosphorotrithioate forms butyl mercaptan (Bu-SH) in goat (Sahali *et al.* 1994). For an *S*-*S*-methyl moiety to be formed from the *S*-*S*-GSH, it would require sequential cleavage of glutamic acid and glycine forming the cysteine conjugate and then β-lyase-mediated metabolism to the $ArCl_4$-*S*-SH followed by *S*-methylation. Another explanation might be that the dimer (**10**) could be a substrate for a methylase enzyme. Methylation of one of the sulphur atoms in the disulphide moiety of the dimer might result in the methyl disulphide and tetrachlorobenzene (**7**). This latter metabolite was detected in rat urine and has been previously reported as a metabolite of tecnazene in rabbit (Renner 1980). It is known that some thiol methyl transferases favour the sulphur being in close proximity to strong electron-withdrawing groups (Stevens and Bakke 1990). Whether either of the above-proposed mechanisms is true or whether **14** was formed by a more obscure pathway, this metabolite was nevertheless formed by a novel metabolic process.

In addition to GSH-related metabolites, several other minor metabolic pathways were evident. The aromatic nitro moiety was reduced to the aniline (**5**), which was further metabolized to the 2,3,5,6-tetrachloro-4-hydroxyaniline (**6**). This latter metabolite has been reported as present in rabbit urine (Bray *et al.* 1953). Tetrachlorophenol was also detected in rat urine. Although this represents an unusual metabolic process this metabolite has been seen in the urine of rabbit dosed with pentachloronitrobenzene (Renner 1980). The proposed pathways for the metabolism of tecnazene are shown in figure 4.

The early reports on the metabolism of tecnazene in rabbit reported the presence of glucuronide and sulphate conjugates of hydroxylated metabolites (Bray *et al.* 1951, 1953). In the present study no such metabolites of tecnazene were detected in rat. Moreover, the hydroxylated metabolites of tecnazene detected in rat formed only a very small percentage of the administered dose ($< 0{\cdot}1\,\%$). In rabbit, total glucuronides and sulphates were reported present at 11–13 and $< 1\,\%$ respectively. This therefore represents an apparent species difference in metabolism.

Acknowledgements

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