**In Vitro Interference with Hepatocellular Transport of Taurocholate by 1,1,2-Trichloro-1,2,2-Trifluoroethane***

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**Abstract**—In recent years workers in our laboratory have shown that several industrial chlorinated aliphatic hydrocarbon solvents interfere with the transport of bile acids by hepatocytes and this interference may account for the raised concentration of serum bile acids that has been observed after occupational exposure to solvents. There has been concern about the effects on workers of a selective solvent used in degreasing electrical equipment, 1,1,2-trichloro-1,2,2-trifluoroethane (FC 113). However, this compound has not been investigated for effects on bile acid transport. Therefore we undertook the present study to examine the direct *in vitro* effects of FC 113 on uptake and efflux of bile acids by isolated rat hepatocytes. FC 113, at non-cytotoxic doses after a 20-min equilibration time, showed significant (*P* < 0.05) inhibitory effects on the initial rate of uptake of taurocholate (TC), whereas accumulation of TC over an extended incubation time was not affected. Kinetic analysis revealed a non-competitive inhibition of TC uptake as evidenced by a decline in *V*ₘₐₓ and an unaltered *K*ₘₐₙ. The initial rate of efflux of TC and the continuous efflux of this model substrate from preloaded cells incubated with different doses of solvent were not significantly different from those of controls. However, the highest dose of solvent inhibited the process of efflux at the early time points. The data suggest that FC 113 interferes with bile acid transport in a reversible manner similar to that of the chlorinated aliphatic hydrocarbons. It would be expected, therefore, that this solvent would cause an increase in serum bile acids in exposed workers.

**INTRODUCTION**

Organic solvents are widely used in industry and although their neurotoxicity and dermal toxicity are well defined, their potential for hepatotoxicity is less clear (Whitby, 1986). However, only a few of the thousands of industrial chemicals have been studied for hepatotoxicity and the number of suspected and established hepatotoxicants is continually increasing (Dossing and Skinhoj, 1985). Furthermore, only a low frequency of abnormal results is obtained using conventional liver tests for screening for occupational liver injury (Edling and Tagesson, 1984). The uncertainties regarding the evaluation of liver function and concern among exposed workers have led to a demand for a reliable and more sensitive test of liver function which can be used for early diagnosis and prevention of occupational liver disease (Edling and Tagesson, 1984; Franco *et al.*, 1989). Bile acids (BAs) have been suggested as useful indicators of hepatobiliary dysfunction, because almost all aspects of hepatobiliary function are involved in BA metabolism (Palmer, 1982). The higher sensitivity, specificity and positive predictive value of serum bile acids (SBAs) in comparison with traditional liver function tests have been reported in several studies (Driscoll *et al.*, 1992; Edling and Tagesson, 1984; Franco, 1991; Wang and Stacey, 1990). In some studies it has been found that serum bile acids (SBAs) are elevated in workers exposed to solvents, although conventional parameters of liver dysfunction are often unaltered (Driscoll *et al.*, 1992, Edling and Tagesson, 1984; Franco *et al.*, 1986). The observation that exposed workers had increases in SBA, without evidence of liver injury, has been supported by our recent animal studies (Bai *et al.*, 1992a, b; Hamdan and Stacey, 1993; Wang and Stacey, 1990). There is also evidence to show that elevated SBAs after exposure to chlorinated solvents is likely to be due to the ability of these substances to interfere with BA transport by hepatocytes (Bai and Stacey, 1993a, b; Kukongviriyapan *et al.*, 1990).

To date, our investigations have mainly focused on the chlorinated solvents. Recently, concern about workers exposed to FC 113 was raised in an
Australasian workplace, prompting an investigation of effects on the liver (study in progress). As SBAs are being investigated we considered it was necessary to investigate the effects of FC 113 on BA transport. FC 113, (1,1,2-trichloro-1,2,2-trifluoroethane) the only fluorocarbon product that liquefied at room temperature (Rasmussen et al., 1988) was first used as a refrigerant (McGee et al., 1990). Because of its good wetting action and rapid evaporation (May and Blotzer, 1984), it is used as a selective solvent in degrading electrical equipment and precision instruments and as a dry-cleaning solvent (Mackison et al., 1981). Although FC 113 is generally considered to have low toxicity (Trochimowicz et al., 1988) because of its stability, short biological half-life and rapid elimination (Savolainen and Pfaffli, 1980). acute inhalation exposure to high concentrations of the solvent vapour may be fatal. Some studies in experimental animals, occupation-ally exposed individuals and volunteers have failed to find any evidence of hepatotoxicity of FC 113 (US EPA/OTS, 1989a,b; Imbus and Adkins, 1972; Rinhardt et al., 1971; Trochimowicz et al., 1988). However in other studies there have been reports of mild liver effects (lipid droplets) in rats and rabbits exposed to FC 113 (Edling and Soderkvist, 1982; International Program on Chemical Safety, 1990; Mahurin and Bernstein, 1988), synergistic hepatocarcinogenicity (Epstein et al., 1967a,b) in mice after combined treatment with FC 113 and piperonyl butoxide (a relatively non-toxic synergist that is widely used with pesticides and freon propellants), and a FC 113 dose-related hepatotoxicity in rats and mice which was marked by ultrastructural changes in hepatocytes, reduced activity of hepatic drug metabolizing enzyme, NADPH cytochrome c reductase, enhancement of membrane-bound UDP-glucuronosyltransferase activity and binding to cytochrome P-450 (McKnight and McGraw, 1983; Vainio et al., 1980).

Since the hepatotoxic potential of FC 113 remains uncertain, and liver effects in workers are currently under investigation, our studies were undertaken to investigate any possible role of inhibition of BA transport that may be related to changes in parameters of liver function following exposure to this chemical.

Isolated hepatocyte suspensions were chosen for these experiments for a number of reasons. Cellular functions, such as membrane transport and metabolizing capacity, in isolated hepatocytes are generally similar to those found in the intact organ (Berry and Friend, 1969; Berry et al., 1992; Eaton and Klaassen, 1978; Grein, 1980; Klaassen and Stacey, 1982; Sandker et al., 1994; Tyson, 1987; Tyson and Stacey, 1989; Zhu and Liu, 1994). Furthermore, a specific comparison of freshly isolated hepatocytes in suspension with cells in primary culture showed that hepatocytes lose their ability to take up taurocholate with increasing time in culture (Kukongviriyapan and Stacey, 1989). In addition, the cytochrome P-450-related metabolic capacity of freshly isolated hepatocytes is greater than that of cells in primary culture (Klaassen and Stacey, 1982; Tyson and Stacey, 1989).

The general metabolic competence of cells prepared by this method has been previously documented (Klaassen and Stacey, 1982; Tyson and Stacey, 1989).

**MATERIALS AND METHODS**

**Chemicals**

[9-1]H] Taurocholic acid (TC) (2 Ci/mmol) was purchased from Du Pont (Sydney, Australia). FC 113 was purchased from Sigma Chemical Co. (St Louis, MO, USA). Percoll was obtained from Pharmacia Biotech AB (Uppsala, Sweden). Collagenase (144 U/mg solid, Type CLS2) was supplied by Worthington Biochemical Corporation (Freehold, NJ, USA). HEPES was purchased from Calbiochem-Behring (San Diego, CA, USA). All other chemicals were of the highest purity commercially available and were purchased from local agents and distributors.

**Animals**

Male Sprague-Dawley rats weighing 250-350 g (9-12 wk of age) were obtained from the University of Sydney Animal House and served as liver donors. They were fed with normal laboratory diet (Rodent Cubes, Allied Stock Feeds, Sydney) and water ad lib. Animals were housed in plastic cages with a stainless-steel cover in an environmentally controlled room which was automatically maintained at a temperature of about 25°C and a relative humidity of 50 ± 10% and a daily cycle of 12-hr light/12-hr dark. Food was removed from the animals 2 hr before hepatocyte isolation. Surgery under halothane anaesthesia was performed at about 11.00 hr for each experiment.

**Isolation of hepatocytes**

Hepatocytes were isolated from untreated rats by the collagenase digestion method as originally described by Berry and Friend (1969) with a few modifications (Stacey et al., 1980) plus an additional Percoll centrifugation step (Kreamer et al., 1986). After isolation, hepatocytes were resuspended in Hanks-HEPES buffer (NaCl, 136.9 mM; KCl, 5.37 mM; MgSO4, 7H2O, 0.81 mM; d-glucose, 5.55 mM; HEPES, 20 mM; Na2HPO4, 0.33 mM; KH2PO4, 0.44 mM; NaHCO3, 4.17 mM; CaCl2; 2H2O, 1.26 mM; pH 7.45-7.50, 37°C) at a concentration of 1.4 x 106 cells/ml for uptake and 1 x 105 cells/ml for efflux. Viability, as determined by the trypan blue exclusion test, was in the range of 90 to 95%.

**Determination of intracellular potassium ion (K+) and hepatocyte enzyme activities**

30 min after incubation of the cells with FC 113 at 37°C, intracellular K+ content and leakage of cytosolic enzymes were determined as indices of cell viability. Aliquots of 200 µl cell suspension were...
centrifuged for 5 sec in a Beckman microfuge (Fullerton, CA, USA) for the separation of the cells from the incubation medium using the silicone oil layer method (Stacey and Klaassen, 1980). Assays for ALT, AST and LDH were performed on the supernatants with a Roche Centrifichem (Model 400) and appropriate kits [Roche Diagnostic System for ALT and AST and Trace Scientific Pty. Ltd (Melbourne, Australia) for LDH]. The bottom portion of the centrifuged cell suspension below the silicone oil layer, which contained the cell pellet, was used for the determination of intracellular K⁺, using a Corning Flame-Photometer (Model 405, Essex, England), as previously described (Stacey et al., 1980).

In vitro uptake of TC

TC was selected as the model substrate because it is known to enter hepatocytes by active transport across the sinusoidal membrane and it is secreted by hepatocytes across the canalicular membrane in the process of efflux (Carey and Calahalane, 1988; Eaton and Richards, 1986; Schwarz et al., 1976), and because it has been shown to be one of the bile acids that is most sensitive to solvent exposures (Bai et al., 1992a; Driscoll et al., 1992; Hamdan and Stacey, 1993; Wang and Stacey, 1990). To study the in vitro uptake of this substrate, 1.99 ml of a suspension of freshly isolated hepatocytes (1.4 × 10⁶ cells/ml) were pre-incubated (in 25-ml Erlenmeyer flasks that were specially designed with a small internal middle cup to allow solvent to come into contact with the cell suspension in the vapour phase) at 37°C with shaking at 80 oscillations/min for 10 min. FC 113 (1–20 µl) was then added to the middle cup with a micro-syringe, and the flasks were then quickly sealed to prevent any loss due to volatilization. Preliminary experiments showed that these amounts of chemical had no cytotoxic effects. 20 min after the addition of FC 113 (a period that was considered to be sufficient for the solvent to equilibrate between the aqueous and vapour phase), 10 µl radiolabelled substrate in physiological saline was added to each flask (final concentration 10 µM for dose-response experiments and 2–100 µM for kinetic studies) and quickly mixed.

The amount of radioactivity was approximately 80 nCi/ml of cell suspension. Sampling for uptake determination was carried out by the silicone oil centrifugation method (Stacey and Klaassen, 1980) at appropriate times. After the pellet had been dissolved in 3 M potassium hydroxide overnight, the samples containing 3H were placed in scintillation vials containing 30 µl 3 M hydrochloric acid to prevent chemiluminescence being produced by potassium hydroxide. After addition of 5 ml scintillation fluid to the vials and vortexing for 10 sec, the amounts of radioactivity in the supernatant and pellet were quantified using a Tri-Carb 4430 liquid scintillation counter (Packard Instrument Co., Downers Grove, IL, USA). Measurements of the amount of radiolabel and the protein concentration (Lowry et al., 1951) allowed the calculation of uptake per mg of protein. The initial rate of uptake (V₀) was determined from the slope of the lines in the linear range (20–80 sec). Apparent kinetic parameters were calculated using initial rate of uptake of TC (final concentration 2–100 µM) and the Eadie–Hofstee plot.

Table 1. Concentrations of C₂Cl₂F₃ (FC 113) in the cells and medium and effects on activities of hepatocyte enzymes and intracellular potassium content

<table>
<thead>
<tr>
<th>FC 113 exposure (µl/flask)</th>
<th>FC 113 conds (µM)*</th>
<th>Activity (% relative to control)</th>
<th>Potassium (µmol/g cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Medium</td>
<td>ALT (%)</td>
</tr>
<tr>
<td>0</td>
<td>32 ± 11</td>
<td>5 ± 1</td>
<td>1.5</td>
</tr>
<tr>
<td>1</td>
<td>71 ± 24</td>
<td>12.0 ± 1.7</td>
<td>10.3 ± 0.7</td>
</tr>
<tr>
<td>2.5</td>
<td>150 ± 37</td>
<td>25.0 ± 5.6</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>270 ± 40</td>
<td>35.0 ± 14.3</td>
<td>9.8 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>522 ± 71</td>
<td>159.0 ± 20.3</td>
<td>10.6 ± 1.0</td>
</tr>
</tbody>
</table>

*Cell suspensions were incubated with different amounts (1–20 µl) of FC 113 by vapour phase in 25-ml flasks for 20 min at 37°C. FC 113 concentrations were measured by gas chromatography.

ALT, LDH, and AST were assayed with enzymatic kits and compared with the homogenates of control hepatocytes.

Each value represents the mean ± SEM of three to five separate experiments. The percentage of cytosolic enzyme leakage and level of intracellular potassium content in control cells were not significantly different from those in treated groups (Duncan’s multiple comparison test; P < 0.05).
In vitro efflux of TC

For these studies, freshly isolated hepatocytes were preloaded with radiolabelled TC by incubation of $2 \times 10^7$ cells/2 ml for 20 min at 37°C with shaking at 80 oscillations/min. The final concentration of TC was 25 $\mu$M and the amount of radioactivity was 160 nCi/ml of cell suspension. After incubation, an aliquot (100 $\mu$l) of this concentrated cell suspension was added to 1.9 ml of fresh incubation medium that had been pre-incubated with various amounts (1–20 $\mu$l) of FC 113. Samples were then taken at
Effects of FC 113 on bile acid uptake and efflux

Table 2. In vitro effects of C₃ClF₃ (FC 113) on kinetic parameters of taurocholate uptake by isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.26 ± 0.35</td>
<td>25.5 ± 3.5</td>
</tr>
<tr>
<td>FC 113 (20 μl/flask)</td>
<td>1.31 ± 0.12*</td>
<td>21.7 ± 2.9</td>
</tr>
</tbody>
</table>

*Values for $K_m$ and $V_{max}$ were calculated from Eadie-Hofstee plot. Each value represents the mean ± SEM (n = 5 or 6), and the value marked with an asterisk is significantly different from the corresponding control value (Student’s t-test; $P < 0.05$).

Appropriate intervals for determination of efflux of TC as described and referenced for the uptake studies. Initial rate of efflux ($V_0$) was determined from the slope of the lines in the linear portion (1–5 min).

Quantitation of FC 113 by gas chromatography

The concentration of FC 113 in cell suspensions and hepatocytes was determined as follows. Aliquots of 200 μl cell suspension or cell free media were extracted with 1.6 ml of heptane (Chromanorm, Prolabo, Paris, France) in teflon-capped glass tubes without head-space. Samples were kept at 4°C until analysis, which was carried out using a Hewlett Packard 5890 gas chromatograph equipped with a 0.25 mm × 30 m column (cyanopropylphenyl-substituted methylpolysiloxane) and an electron capture detector. The column temperature was 70°C, the injector temperature was 220°C and the detector temperature was 280°C. The carrier gas was nitrogen at a flow rate of 2 ml/min. The difference between the solvent concentration in cell suspension and cell-free media was used to estimate solvent concentration in the cells.

Data analysis and statistical procedures

The data were statistically evaluated using Student’s t-test, analysis of variance and Duncan’s test with a preset probability of $P < 0.05$. Each value is a mean that has been obtained from three to six experiments using hepatocytes from individual rats.

RESULTS

FC 113 and cytotoxicity

The concentration of FC 113 in cells, and cell viability (as indicated by leakage of cytosolic enzymes and intracellular potassium ion content) were determined 20 and 30 min after application of the solvent, respectively. There was no indication of cytotoxicity at any of the doses of FC 113, as shown by the lack of any significant increase in ALT, LDH or AST in the medium, and no significant decrease in intracellular

![Graph](image-url)

Fig. 4. In vitro effect of FC 113 on the initial rate of efflux ($V_0$) of TC (25 μM). Columns represent the mean and bars the SEM (n = 4). None of the values was significantly different from any other (Duncan’s multiple comparison test; $P < 0.05$).
Fig. 5. *In vitro* effect of FC 113 on the initial efflux of TC (25 μM). Hepatocytes were exposed to 0 (control, ■), 1 (▲), 2.5 (▼), 5 (◇), 10 (▽) or 20 (□) μl FC 113. Symbols represent the mean and bars the SEM (n = 4). Values marked with an asterisk are significantly different from the corresponding control value (Duncan’s multiple comparison test; P < 0.05).

potassium ion content (Table 1). Furthermore, there was no significant difference between cell viability (as determined by trypan blue exclusion test) before exposure to solvent and that at the end of the experiments.

**Uptake of TC**

Incubation of hepatocytes with FC 113 for 20 min resulted in a dose-dependent inhibition of the initial rate of uptake ($V_0$) of TC by hepatocytes (Fig. 1).

Fig. 6. *In vitro* effect of FC 113 on continuous efflux of TC (25 μM) from hepatocytes over time. Hepatocytes were exposed to 0 (control, □), 1 (▲), 2.5 (▼), 5 (◇), 10 (●) or 20 (■) μl FC 113. Symbols represent the mean and bars the SEM (n = 4). There was no statistically significant difference between any of the means (Duncan’s multiple comparison test; P < 0.05).
The lowest dose of solvent that resulted in a significant decrease in \( V_0 \) was 2.5 \( \mu l / flask \) (71 \( \mu M \)). The accumulation of TC during 30 min of incubation was not significantly inhibited by FC 113 (Fig. 2).

**Kinetic parameters of TC uptake**

The effects of FC 113 on the handling of TC by hepatocytes were investigated in more detail by examining uptake of concentrations of substrate ranging from 2 to 100 \( \mu M \) in the presence and absence of the highest dose of solvent. As shown in Fig. 3, for uptake of all concentrations of substrate by hepatocytes, the \( V_0 \) in the presence of solvent was significantly inhibited. These data were used to calculate \( K_m \) and \( V_{max} \). The unchanged \( K_m \) and significantly decreased \( V_{max} \) (Table 2) demonstrated that the inhibition was non-competitive.

**Efflux of TC**

The effects of FC 113 on efflux of TC from hepatocytes pre-loaded with 25 \( \mu M \) \([H]^+\)TC were investigated. FC 113 resulted in a slight (but not statistically significant) decrease in \( V_0 \) of efflux of TC (Fig. 4). Furthermore, the highest dose of solvent significantly inhibited the efflux of TC at the early incubation times (up to 4 min) even though \( V_0 \) was not significantly different (Fig. 5). FC 113 did not show any significant inhibitory effect on the continuous efflux of TC (Fig. 6).

**DISCUSSION**

In the present study, different doses of FC 113 significantly inhibited the initial rate of uptake of TC in the absence of cytotoxicity. However, the inhibition seems to be reversible as suggested by a rapid recovery and full restoration of uptake after longer incubation. This observation is consistent with previous studies in which several chlorinated solvents were reported to inhibit the uptake of bile acids and other actively transported substrates in a rapid and reversible manner (Bai and Stacey, 1993a,b; Kukongviriyapan et al., 1990). FC 113 is known to be metabolized to only a very small degree, if at all, and no metabolite(s) of this compound has been identified (Auton and Woollen, 1991; International Program on Chemical Safety, 1990). Furthermore, metabolism of this compound, if it occurs, has only a minor influence on the pharmacokinetics of the parent compound (Auton and Woollen, 1991). Therefore, this inhibitory action may be attributed to the chemical itself. This conclusion again is in agreement with previous studies (Bai and Stacey, 1993b; Hamdan and Stacey, 1993), in which increased SBA after exposure to a chlorinated solvent (trichloroethylene) was due to the direct action of the solvent and not its metabolites. In our studies, hepatocytes pre-exposed to FC 113 restored their uptake function relatively more quickly than after exposure to trichloroethylene (Bai and Stacey, 1993b). This may be explained by rapid loss of the solvent following frequent initial samplings because of its high vapour pressure (334 mm Hg at 25°C, compared with 60 mm Hg at 20°C for trichloroethylene), very high volatility and consequently high rate of evaporation (1.95, compared with 1.0 for ether) and rapid elimination from biological systems (Clark and Tinston, 1982; Marsden and Mann, 1963; May and Blotzer, 1984; Trochimowicz et al., 1988). This was confirmed by direct measurement of the solvent concentration in cell suspensions 30 min after initial sampling which showed 97.7–99.6% loss of solvent in comparison with the initial concentration (0.84–2.55 \( \mu M \) v. 36.7–680.8 \( \mu M \)). The inhibitory action of FC 113 on uptake of TC was non-competitive, as it was with the other chlorinated solvent, trichloroethylene (Rai and Stacey, 1993b).

Dose-dependent depletion of cellular ATP and reduced activities of Na+-, K+- and Mg+-ATPases have been implicated as factors that may be causally linked to the inhibitory action of chlorinated solvents on bile acid transport (Kukongviriyapan et al., 1990). As an analogue of the chlorinated solvents, FC 113 may exert its effect through such mechanisms.

The mild inhibitory effect of the highest dose of FC 113 on the efflux of TC in the absence of any change in \( V_0 \) deserves comment. This may be the result of alteration in the calcium pump in the endoplasmic reticulum of hepatocytes, since halogenated compounds are known to alter cytosolic calcium homoeostasis (Moore et al., 1990) which in turn can affect the intracellular transport process (regulated by the calcium pump in the endoplasmic reticulum) and modify the efflux of bile acid (Franco, 1991). The inhibition at the highest dose in the absence of an effect on \( V_0 \) seems inconsistent. However, a close examination of the data suggests that a decrease in efflux at all the early time points across which \( V_0 \) was calculated resulted in no change in the actual rate of efflux.

In conclusion, the data show that the chlorofluorocarbon FC 113 interferes with the transport of bile acids in a manner similar to that of chlorinated aliphatic hydrocarbons. Consequently, it would be predicted that this solvent might cause increases in SBA of exposed individuals.

**REFERENCES**


