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The distribution, excretion, and hepatic metabolism of [^{3}H]microcystin-LR ([^{3}H]MCYST-LR, sublethal, i.v.) were measured in mice. Plasma elimination was biexponential with α- and β-phase half-lives of 0.8 and 6.9 min, respectively. The apparent volume of distribution of the β-phase was 10 ml and the area under the curve was 1036 pmol min/ml. At 60 min, liver contained 67 ± 4% of dose. Through the 6 day study the amount of hepatic radioactivity did not change while 23.7 ± 1.7% of the dose was excreted; 9.2 ± 1 and 14.5 ± 1.1% in urine and feces, respectively. Approximately 60% of the urine and fecal radiolabel 6 and 12 hr post injection was the parent toxin. Hepatic cytosol, which contained 70 ± 2% of the hepatic radiolabel (1 hr through 6 days), was prepared for HPLC analysis by heat denaturation, pronase digestion, and C-18 Sep Pak extraction. One hr post exposure, 35 ± 2% of the radiolabel was insoluble or C-18 Sep Pak-bound; 43 ± 3% was associated with a peak of retention time (rt) 6.6 min, and 16 ± 3% with parent toxin. After 6 days, 8 ± 1% was C-18 Sep Pak-bound or insoluble; 5 ± 0% occurred at rt 6.6 min, 17 ± 1% with (continued on back)

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parent and 60 ± 2% was associated with rt 9.1 min. Two other radioactive peaks, rt 4.9 and 5.6 min, appeared transiently. Analysis of hepatic cytosol by desalting chromatography under native and denaturing conditions revealed that all of the radiolabel was associated with cytosolic components, and 83 ± 5% was covalently bound decreased to through 1 day. By day 6 the amount covalently bound decreased to 42 ± 11%. This study is the first to describe the long-term hepatic retention of MCYST toxin and documents putative detoxication products.

Tissue Distribution, Excretion, and Hepatic Biotransformation of Microcystin-LR in Mice[1,2]

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Abbreviations: [^{3}H]MCYST-LR, [^{3}H]microcystin-LR; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BUN, blood urea nitrogen; C_o, maximum plasma concentration; Cl, clearance; C_{ss}, steady state concentration; C_t, concentration at time t; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; R_f, partition coefficient; rt, retention time; SDH, sorbitol dehydrogenase; SDS, sodium dodecyl sulfate; $t_{\frac{1}{2}}$, half-life; Tris, tris(hydroxymethyl)aminomethane; V_c, apparent volume of the central compartment; V_β, apparent volume of distribution during the terminal exponential phase.

ABSTRACT

The distribution, excretion, and hepatic metabolism of [^{3}H]microcystin-LR ([^{3}H]MCYST-LR, subletnal, i.v.) were measured in mice. Plasma elimination was biexponential with α- and β-phase half-lives of 0.8 and 6.9 min, respectively. The apparent volume of distribution of the β-phase was 10 ml and the area under the curve was 1036 pmol min/ml. At 60 min, liver contained 67 ± 4% of dose. Through the 6 day study the amount of hepatic radioactivity did not change while 23.7 ± 1.7% of the dose was excreted; 9.2 ± 1 and 14.5 ± 1.1% in urine and feces, respectively. Approximately 60% of the urine and fecal radiolabel 6 and 12 hr post injection was the parent toxin. Hepatic cytosol, which contained 70 ± 2% of the hepatic radiolabel (1 hr through 6 days), was prepared for HPLC analysis by heat denaturation, pronase digestion, and C-18 Sep Pak extraction. One hr post exposure, 35 ± 2% of the radiolabel was insoluble or C-18 Sep Pak-bound; 43 ± 3% was associated with a peak of retention time (rt) 6.6 min, and 16 ± 3% with parent toxin. After 6 days, 8 ± 1% was C-18 Sep Pak-bound or insoluble; 5 ± 0% occurred at rt 6.6 min, 17 ± 1% with parent, and 60 ± 2% was associated with rt 8.1 min. Two other radioactive peaks, rt 4.9 and 5.6 min, appeared transiently. Analysis of hepatic cytosol by desalting chromatography under native and denaturing conditions revealed that all of the radiolabel was associated with cytosolic components, and 83 ± 5% was covalently bound through 1 day. By day 6 the amount covalently bound decreased to

42 ± 11%. This study is the first to describe the long-term hepatic retention of MCYST toxin and documents putative detoxication products.

Microcystin-LR (MCYST-LR), isolated from the cyanobacterium *Microcystis aeruginosa* strain PCC-7820, is one of several related cyclic peptide hepatotoxins (Botes *et al.*, 1985; Rinehart *et al.*, 1988). This family of toxins has been implicated in the death of both wild and domestic animals that consumed water containing dense blooms of blue-green alga (Soll and Williams, 1985; Beasley *et al.*, 1989; Galey *et al.*, 1987). A consistent pathological finding after lethal exposure to toxin is blood-engorged livers with diffuse hepatic centrilobular necrosis (Konst *et al.*, 1965; Adams *et al.*, 1988; Jackson *et al.*, 1984; Theiss *et al.*, 1988). The proposed cause of death is massive intrahepatic hemorrhage (Hooser *et al.*, 1989; Theiss *et al.*, 1988). Human fatality has not been associated with the MCYST toxins; however, a heavy bloom of *M. aeruginosa* in a reservoir at Malpus Dam, Australia, was correlated with a rise in plasma gamma-glutamyl transpepidase in humans, suggestive of hepatic injury (Falconer *et al.*, 1983).

While information has been published concerning the tissue distribution of MCYST toxins in rat (Falconer *et al.*, 1986; Pace *et al.*, 1990b) and in mouse (Robinson *et al.*, 1989; Brooks and Codd, 1987; Runnegar *et al.*, 1986), the pharmacokinetics have not been completely described in either species. The reports agree that liver, the target organ, accumulates toxin; however, the time required for maximal accumulation varies from 1 min (Brooks and Codd, 1987) to 60 min (Robinson *et al.*, 1989) after i.p. administration in mice. Additionally, metabolism and elimination of the toxin have not been measured comprehensively.

The only information concerning metabolism of MCYST toxins is a report by Pace *et al.* (1990c) using an isolated perfused liver model. After a one hr perfusion with a toxic dose of [^{3}H]MCYST-LR, hepatic cytosol (90% of hepatic radiolabel) was isolated, heat denatured, pronase digested, C-18 Sep Pak extracted and analyzed by C-18 reverse phase high performance liquid chromatography (HPLC). Radiolabel was localized in three fractions; a) insoluble plus Sep Pak resin-bound (35%), b) parent toxin (rt 5.5 min, 10%), and c) a radioactive peak of rt 5.0 min (55%). It is not known which species is responsible for toxic action. It is reasonable to propose that metabolism of MCYST-LR be necessary for its toxic effects to be expressed, based upon the following considerations: hepatic damage occurs in the centrilobular (perivenous) region, which is the location of the highest concentration of many metabolic enzymes (Jungermann and Katz, 1989); liver specificity could be explained by prerequisite hepatic metabolism for toxicity; and neonatal rats and mice are not as susceptible to the MCYST toxins (Foxall and Sasner, 1981; Stoner *et al.*, 1989), possibly because many metabolic enzymes are regulated developmentally (Pelkonen, 1979).

We described the pharmacokinetics of a sublethal, i.v. bolus dose of [^{3}H]MCYST-LR in the mouse model. Reported here are the kinetics of elimination from plasma, the time course of distribution to tissues over a 60-min interval, excretion in urine and feces through 6 days post injection, and hepatic, urinary and fecal metabolic fate of [^{3}H]MCYST-LR.

Materials and Methods

Reagents. MCYST-LR (>95% purity) was supplied by W. W. Carmichael (Wright State University, Dayton OH). [^{3}H]MCYST-LR (Amersham Corp, Arlington Heights, IL, 40% radiochemical purity) was produced by chemical tritiation of MCYST-LR (Matsuo and Narita, 1975) and purified by reverse-phase HPLC (99% radiochemical purity, 194 mCi/mmol, Robinson *et al.*, 1989).

Animals. Male, VAF/plus CD-1 mice [crl:CD-1(1CR)BR Charles River, Wilmington, MA], weighing 20-27 g, were maintained on a 12-hr light/dark cycle. Mice were fasted 17-20 hr before studies but allowed water *ad libitum*.

Time course of radiolabel distribution. Fasted mice were injected i.v. via the tail vein with 35 μg/kg [^{3}H]MCYST-LR. Two min prior to obtaining blood samples, animals were anesthetized with 50 mg/kg each xylazine and ketamine by i.m. injection (hind limb). After the brachial artery was severed, blood was collected from a skin pouch formed medial to the forelimb. Heparin (1000 U/ml, 50 μl) was added to the blood and plasma was separated by centrifugation at 700 x *g* for 10 min. At 1, 3, 6, 10, 15, 20, 30, 40, and 60 min post injection, tissues (heart, lung, liver, gut, kidney, and spleen) were removed, weighed, minced, and digested in 2N KOH for 48 hr at 65°C. The remainder of the carcass was digested for 72 hr at 65°C. After digestion,

100 µl of each tissue sample was added to 200 µl 2N HCl and 10 ml Hydrofluor (National Diagnostics, Manville, NJ). Radioactivity was measured in a Beckman 5800 liquid scintillation counter (Berkley, CA).

Quantitation of radiolabel in urine, feces, and liver. Mice were housed three per Nalgene metabolic cage, (Harvard Apparatus, South Natick, MA) after i.v. injection of 35 µg/kg [^{3}H]MCYST-LR. Urine and feces were collected at 6 and 12 hr and 1, 2, 3, 4, 5, and 6 days post exposure. Sodium fluoride (25 mg) was added to the urine samples and 100-500 µl was analyzed for radioactivity. The calculated limit of detection in urine was 1 pmol/ml. Feces were ground in liquid nitrogen with a mortar and pestle. Weighed portions (20-30 mg) of the ground fecal material were digested in 0.2 ml of 1 N KOH for 48 hr at 65°C. The radioactivity of the entire sample was measured as described above for tissues. The calculated limit of detection in feces was 16 pmol/g. At 1, 6, and 12 hr and 1, 2, 4, and 6 days, three animals were anesthetized and their livers were removed, freeze-clamped with liquid nitrogen cooled-aluminum tongs, and ground in liquid nitrogen with a mortar and pestle. Radioactivity of weighed portions (20-30 mg) of liver was measured after digestion in 2N KOH. The remainder of the carcass was digested and radioactivity measured as described above.

HPLC analysis of urine, feces and liver metabolites. HPLC

was performed using a Waters system (Milford, MA) equipped with a Waters 490 multiwavelength detector. The C-18 column (Adsorbosphere HS, 4.6 x 250 mm, 5 μm, Alltech, Deerfield, IL) was run at a flow rate of 1 ml/min of 73% 10 mM ammonium acetate, pH 6.0, and 27% acetonitrile for 15 min. Elution of radioactivity was monitored with a Flow I β-radiodetector (Radiomatic Instruments and Chemical Co., Tampa, FL), through a 5-ml cell with Flo-Scint III scintillation fluid (Radiomatic) pumped at 2 ml/min. Variation in the retention time of standard MCYST (probably due to column age) was assessed by spiking each radioactive sample with 5 μg of unlabeled MCYST and monitoring the elution of this internal standard by UV absorption at 238 nm.

Urine (100-250 μl) was analyzed directly by C-18 HPLC. Feces (0.1-0.5 g) were homogenized with a Polytron homogenizer (Brinkman Instrument Co, Westbury, NY) in two 4-ml portions of 50 mM ammonium acetate, pH 6.0. The supernatant fraction was obtained after centrifugation at 15,000 x *g* for 15 min. The fecal extract was subjected to solid-phase extraction on C-18 Sep Pak cartridges (Waters, Milford, MA). Radioactivity was eluted with 2 ml of 100% methanol. The methanol fraction was dried under N_2 at room temperature and the residue dissolved in 200 μl of 27% acetonitrile and 73% 10 mM ammonium acetate, pH 6.0.

Ground liver samples (1.5-2 g) were homogenized with a motor-driven, teflon pestle in 5 ml of 100 mM tris(hydroxymethyl)aminomethane (Tris-Cl), pH 7.2, and centrifuged at 10,000 x *g* for 10 min. The pellet was

rehomogenized (3 ml buffer) and recentrifuged. The combined supernatants were centrifuged at 100,000 x *g* to obtain the cytosolic fraction. The hepatic cytosol was prepared for HPLC analysis as described by Pace *et al.* (1990c). The cytosol (1 ml, 18.3 ± 1 mg/ml) was heat-denatured for 30 min at 90°C after addition of an equal volume of 100 mM potassium phosphate, pH 7.5. Pronase (1 mg in 1 ml buffer, Boehringer Mannhiem Biochemicals, Indianapolis, IN) was added to the heat-denatured slurry and the sample incubated for 1 hr at 37°C. The resulting solution was centrifuged to remove any particulate material and the supernatant chromatographed on C-18 Sep Pak cartridges. Radioactivity was eluted with methanol, and the sample concentrated and prepared for HPLC analysis as described for fecal samples. The sample preparation procedure did not cause degradation of parent toxin when [^{3}H]MCYST-LR in buffer was analyzed or when heat-denatured hepatic cytosol was spiked with [^{3}H]MCYST-LR prior to addition of pronase.

Analysis of hepatic cytosol by desalting chromatography. Econo-Pac 10DG disposable columns (Bio-Rad, Rockville Center, NY), supplied prepacked with Bio-Gel P-6 desalting gel, were used to analyze samples of hepatic cytosol isolated from livers 1 hr to 6 days post exposure to [^{3}H]MCYST-LR. Columns were equilibrated with 100 mM Tris-Cl, pH 7.2, (native conditions) and 0.8 ml of hepatic cytosol was applied to the columns. Fractions (1 ml) were collected and analyzed for radioactivity by liquid

scintillation.

Econo-Pac 10 DG columns were also used to analyze hepatic cytosol under denaturing conditions, 6 M urea, 0.1% sodium dodecyl sulfate (SDS) in 10 mM Tris-Cl, pH 7.2 (elution buffer). Hepatic cytosol (0.5 ml) was denatured by the addition of solid urea and SDS, final concentration 6 M and 0.1%, respectively, prior to application (1 ml) to the columns. Fractions (1 ml) were collected and analyzed for radioactivity by liquid scintillation.

Plasma chemistry and histology. Plasma collected from either control mice (1 hr or 6 days post injection of saline) or toxin-treated mice (1, 6, and 12 hr and 1, 2, 4, and 6 days post injection) was analyzed on a COBAS BIO Centrifugal Analyzer (Roche Analytical Instruments, Nutley, NJ). Kits for blood urea nitrogen (BUN), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were obtained from Roche. Sorbitol dehydrogenase (SDH) and Lowry total protein kits were purchased from Sigma Chemical Company (St. Louis, MO). Sections of the left lobe of liver were removed at each time point and fixed for a minimum of 24 hr in 10% neutral buffered formalin. Subsequently, tissue specimens were processed by routine paraffin-embedding techniques and sectioned at -6 μm. All sections were stained with hematoxylin and eosin, and examined by light microscopy.

Data analysis. Tissue distribution data are expressed as toxin molar equivalents. Metabolites were not determined in tissue due to the low amount of radioactivity in all tissues except liver. The plasma concentration data were fit to a biexponential decay equation, $C = Ae^{-\alpha t} + Be^{-\beta t}$, by nonlinear regression using Enzfitter software (Elsevier-BIOSOFT, Cambridge, UK) on a IBM personal computer AT. The half-lives ($t_{½}$) of the α- and β-phases were calculated by the equation

$$t_{½} = 0.693/k$$

where k is the elimination rate constant for the α or β phases. The maximum plasma concentration of toxin, C_o, was calculated by using the defined plasma concentration equation at t = 0. Area under the curve (AUC) was calculated by the trapezoid rule. Clearance (Cl) was calculated by the following equation:

$$Cl = Dose/AUC$$

The apparent volume of the central compartment, V_c, and the apparent volume of distribution during the terminal exponential phase, V_β, were calculated from the following equations:

$$V_c = Dose/C_o$$

$$V_\beta = Dose/(AUC*\beta)$$

Hepatic uptake half-life was calculated from the following equations:

$$C_t/C_{ss} = 1 - e^{-kt}$$

$$t_{½} \text{ uptake} = 0.693/k$$

where C_t is the concentration in liver at time t, and C_{ss} is the steady state concentration. The first order absorption rate

constant k, was estimated from the slope of the logarithmic transform of the equation by the method of least squares. The correlation coefficient for hepatic uptake was 0.86.

Values are expressed as mean ± SE, the number of animals per study are given in Results or the Figure legends. Statistical comparison of values was accomplished with one-way analysis of variance and Duncan's multiple range test. $P < 0.05$ was considered significant.

Results

Biological effects of sublethal [^{3}H]MCYST-LR injection. After an i.v. injection of a sublethal dose (35 μg/kg) of [^{3}H]MCYST-LR, the mice displayed normal behavior. Liver weight did not increase; and histologically, 1-60 min and 6 days post-exposure, livers were indistinguishable from control. The only indication of toxic effect was a transient increase in plasma levels of LDH, SDH, and AST at 6 hr ($P<0.01$, n=3, 10, 25, and 20-fold, respectively over control, n=6), which returned to normal by 12 hr. Levels of BUN and ALT were unaffected. This dose of [^{3}H]MCYST-LR had minimal hepatotoxic effects which could influence the pharmacokinetics of the compound.

Clearance of toxin from plasma. The semilog plot of plasma concentration of toxin equivalents versus time is shown in Fig. 1. Results are presented as molar equivalents of toxin. The curve represents a biexponential decay with a maximum concentration of 428 pmol/ml. The half-lives of the α phase and the β phase were 0.8 and 6.9 min, respectively. The AUC, estimated by the trapezoid rule, was 1036 pmol min/ml, and clearance from plasma was 0.9 ml/min. The apparent volume of the central compartment and V_β were 2 and 10 ml, respectively.

Concentration of toxin in tissues. The concentration of toxin molar equivalents per gram of tissue over time is shown in Fig 2. In liver, there was a rapid uptake of radiolabel, which

reached a maximum accumulation of 521 ± 14 pmol/g (71 ± 3% of dose) by 30 min. The half-life of uptake was 6.8 min. In kidney, the maximum concentration was achieved at 3 min (90 ± 7 pmol/g, 4.6 ± 0.6% of dose) followed by a decline in concentration until 20 min. The amount of radiolabel in gut reached a maximum at 30-40 min. In lung and carcass, the maximum amounts were measured at 3 min, after which the concentration declined. Spleen and heart did not contain measurable amounts of radioactivity. The distribution of radiolabel to tissues with time, presented as percent of injected dose, is shown in Table 1. The overall recovery of radiolabel ranged from 74 to 101% of dose. The partition coefficients or distribution ratios (R_t, ratio of mean concentration of toxin equivalents in tissue to that in plasma at steady state, 30 min) were 206, 4, and 11 for liver, kidney, and intestine, respectively, indicating accumulation of radiolabel in these tissues (R_t was >1), but not in carcass.

Excretion of [^{3}H]MCYST-LR in urine and feces. Six days post exposure to 35 μg/kg [^{3}H]MCYST-LR, 23.7 ± 1.7% of the radiolabel had been excreted (Fig 3, Panel A); 9.2 ± 1.0% and 14.5 ± 1.1% via urine and feces, respectively. The percent of dose that appeared in feces or urine per collection interval (midpoint) is given in Fig. 3, panels B and C, respectively. The rate of excretion via feces was 0.9 and 0.5% per hr for 6 and 12 hr, and ca. 1% per day for the remaining 6 days. Within the first 12 hr,

74% of cumulative urinary excretion had occurred.

Analysis of urinary and fecal metabolites. Urine samples collected 6 and 12 hr after toxin exposure were analyzed for metabolites by C-18 HPLC. At 6 hr, 6 ± 1% of the injected radiolabel was found in urine; and 63 ± 2% (n=7) of that was parent toxin, rt 9.4 min (Fig. 4, panel B). The major portion of the remaining radiolabel (30 ± 4%) was associated with a peak of rt 4.5 min. There were also several minor peaks detected in some of the samples. Between 6 and 12 hr, 1.5 ± 0.4% of the radiolabel was excreted in urine. In the two samples that contained sufficient radioactivity to be analyzed, the profile was similar to that seen at 6 hr; 35% at rt 4.5 min and 63% as parent. Later time points did not contain sufficient radiolabel for analysis.

Feces collected 6 hr post injection contained 5 ± 1% of the injected radiolabel, 69 ± 3% of which was recovered for HPLC analysis. A typical radioactive profile for a 6-hr fecal sample is shown in Fig. 4, panel C. Parent toxin, rt 9.4 min, comprised 63 ± 8% (n=6) of the radioactivity with the residual in five other peaks (legend of Fig. 4). Between 6 and 12 hr, 2.9 ± 0.3% of the dose appeared in feces; 52 ± 4% was recovered for HPLC analysis, of which 76 ± 8% (n=6) was associated with parent toxin. Later time points did not contain sufficient radiolabel for analysis.

HPLC analysis of hepatic-cytosolic radiolabel. The total amount of liver-sequestered radiolabel did not change from 1 hr (67 ± 4%, n=6) to 6 days (66 ± 2%, n=3) post-exposure, while carcass-associated radiolabel, comprised of all tissues except liver, decreased from 15 ± 2% at 1 hr to trace amounts by 4 days. The subcellular distribution of hepatic radiolabel also remained unchanged over 6 days; the 10,000 x *g* pellet, consisting of unbroken cells, mitochondria and plasma membrane, contained 13 ± 1%; the 100,000 x *g* pellet, composed mainly of microsomes, residual plasma membrane, and mitochondria, contained 12 ± 1%; and the cytosol contained 70 ± 2% of the hepatic radioactivity. After heat denaturation, pronase treatment and C-18 Sep Pak extraction, the Sep Pak-eluted radioactivity was separated into five radioactive, HPLC peaks of rt 4.9, 5.6, 6.6, 8.1, and 9.4 min, the latter being the rt of [^{3}H]MCYST-LR (Fig. 4, Panels D-F). The percent distribution of hepatic-cytosolic radiolabel with respect to time is presented in Table 2. The portion of radiolabel that remained bound to the C-18 Sep Pak resin plus that which remained insoluble decreased between 12 hr and 4 days, as did the 6.6 min HPLC peak. The 8.1-min fraction steadily increased and the peaks of rt 4.9- and 5.6-min appeared at 12 and 6 hr, and disappeared after 2 and 6 days, respectively.

Analysis of hepatic cytosol under native and denaturing conditions by desalting chromatography. Pronase digestion of hepatic cytosol was the only method we found to be effective in

separating radiolabel from cytosolic protein (Pace *et al.*, 1990c). To further explore this observation, hepatic cytosol was analyzed by desalting chromatography under native conditions. From 1 hr through 6 days post injection, all cytosolic radiolabel eluted in the void volume of a DG10 desalting column suggesting association with high molecular weight components, while free [^{3}H]MCYST-LR eluted as a lower molecular weight component (Fig. 5). To determine if radiolabel was covalently bound to the high molecular weight-cytosolic components, the samples were first denatured with 6 M urea and 1% SDS and then chromatographed. [^{3}H]MCYST-LR eluted in fractions 4 and 5, as did a portion of the cytosolic radiolabel (Fig 6). The remaining radiolabel eluted in fractions 2 and 3 indicating it was covalently bound to cytosolic components. The amount of radiolabel that was covalently bound from 1 hr through 6 days post exposure is given in Table 3. The percent bound did not change from 1 hr through 1 day. At 2 days post exposure the amount of covalently bound radioactivity began to decrease to a value of 42% ± 11 by 6 days.

Discussion

One of the objectives of this study was to measure excretion and hepatic metabolism of [^{3}H]MCYST-LR. We chose a dose of the hepatotoxin which resulted in minimal toxic effects to avoid inhibition of these processes. The only indication of toxicity was a transient rise in several serum enzymes. Histologically, livers appeared normal 1 min through 6 days post injection.

The kinetic parameters defined in this study indicate that plasma elimination did not reflect toxicity, as liver, the target organ, accumulated the toxin. Hepatic accumulation of radiolabeled MCYST toxins has been observed by several groups (Pace *et al.*, 1990c; Robinson *et al.*, 1989; Brooks and Codd, 1987; Falconer *et al.*, 1986); however, this is the first time that the long-term hepatic retention of radiolabel has been documented. Runnegar *et al.* (1986), who studied liver retention 24 hr post injection of various doses of [^{125}I]MCYST-YM, concluded that livers that were relatively normal by histological examination excreted almost all of the hepatic radioactivity, while those that exhibited more extensive damage retained the labeled toxin. It is not clear whether the discrepancy between their study and the data presented here is due to the difference in toxin variants or to the nature of the radiolabel attached to the compound. We found that the amount of radiolabel sequestered by liver peaked at 30 min and did not change thereafter over the 6-day study. All of the sequestered hepatic-cytosolic radiolabel appeared to be bound to high molecular weight components, most

likely protein, as evidenced by a) co-precipitation of radioactivity with protein after addition of TCA or organic solvents (data not shown), b) elution of radioactivity in the void volume of a DG10 desalting column under native conditions (Fig. 5), and c) requirement for protease treatment prior to HPLC analysis. A substantial portion of the radiolabel was covalently bound to the cytosolic component(s) evidenced by elution of radiolabel in fractions 2 and 3 of desalting columns under denaturing conditions. The V_β, >300 ml/kg, was also indicative of extravascular tissue binding. Hepatic protein binding could explain the large liver-to-plasma partition coefficient, as only unbound or free toxin could influence partitioning (Pang, 1983). Also, protein-bound toxin or metabolite would probably not follow typical xenobiotic elimination pathways, which could explain the long hepatic retention.

Fecal elimination, after the first 12 hr, was constant at ~1% per day; but the amount of hepatic radiolabel did not change over the course of the study. There was, however, a decrease in carcass (all tissues except liver) radioactivity over time, but it is not clear whether the radiolabel was eliminated directly or via bile. It is possible that toxin was taken up by gut (8.6 ± 0.7% at 1 hr) and subsequently excreted directly into the intestinal lumen and eliminated in feces.

The major portion of urinary excretion occurred early and accounted for 9% of the dose. The limited urinary excretion is likely due to the rapid decline in plasma radiolabel. The half-

life of the β phase of the plasma concentration curve and the hepatic uptake half-life were identical, ~7 min, suggesting that hepatic tissue binding was a major factor in the kinetics of plasma elimination of [^{3}H]MCYST-LR. This is not surprising, as extravascular tissue binding is known to be a principal determinate of the apparent half-life of xenobiotics if V_β is >100 ml/kg (Gibaldi and Perrier, 1982). Drugs such as rifampicin that inhibit hepatic uptake of toxin (Pace *et al.*, 1990a; Runnegar *et al.*, 1981) increase its plasma half-life and thus may increase urinary excretion.

Unlike the hepatic-cytosolic radiolabel, urine and fecal radiolabel did not appear to be protein bound since pronase treatment was not required for sample preparation. Most of the radioactivity in both urine and feces was parent toxin (approximately 60%) and the metabolites that were detected did not appear to correlate with those seen in liver.

In a previous study, (Pace *et al.* 1990c) we described two radioactive fractions other than parent (rt 5.5 min) in hepatic cytosol isolated from rat liver perfused 1 hr with toxic levels of [^{3}H]MCYST-LR; the C-18 Sep Pak-bound plus insoluble species and a radioactive HPLC peak of rt 5.0 min. The reason for the difference in the retention times reported in this study is that the HPLC mobile phase was modified to allow greater separation between parent toxin and metabolites. Analysis of hepatic cytosol from perfused liver under the modified HPLC conditions (data not shown) yielded the same retention times as reported in

Table 2 for the 1 hr time point.

This study is the first to characterize over time the hepatic biotransformation of [^{3}H]MCYST-LR. The C-18 Sep Pak bound plus insoluble radiolabel could not be further analyzed with the techniques employed, so it is not clear if this fraction is composed of multiple chemical species. As this fraction and the 6.6-min peak decreased, between 12 hr and 4 days, three new radioactive peaks appeared, two transiently (rt = 4.9 and 5.6 min), and the third (rt of 8.1 min) increasingly. By 6 days the 8.1 min peak accounted for 60% of the cytosolic radiolabel. The parent peak did not change substantially during the study. It is unknown whether the biotransformed radiolabel described at 1 hr represents the toxic compound or if it is a detoxication product and residual parent is the toxic component. However, since Pace *et al.* (1990c) reported toxic effects in livers perfused for 1 hr with lethal doses of [^{3}H]MCYST-LR and described only the insoluble plus bound fraction, the 6.6 min rt peak, and parent compound, it appears likely that the new HPLC peaks detected in this study represent detoxication products of [^{3}H]MCYST-LR and are not involved in manifestation of toxicity.

The decrease with time in amount of radiolabel that was covalently bound may represent a pathway by which the radiolabel would finally be eliminated from liver. There was not, however a strong numerical correlation between the appearance of the 8.1 min HPLC peak (or the appearance/disappearance of any other HPLC peak) and the degree of covalent binding (Table 2 compared to

Table 3), but there was a trend that as the 8.1 min peak accumulated covalent binding decreased. It is possible that the biotransformation that produced the species of rt 8.1 min made the covalent bond more susceptible to cleavage. The covalent interaction demonstrated in this study needs to be further defined and may hold the key to the mechanism of action of MCYST toxins; e.g. a critical protein may be inactivated by the covalent interaction.

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Footnotes

[1]The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense (para. 4-3, AR 360-5). In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2]Portions of this data have been presented at the 1990 FASEB meeting and published in abstract form in FASEB J. 4: A753, 1990.

TABLE 1. **Distribution of radiolabel over time**[a]

Time (min)	% Dose				
	Liver	Kidney	Intestine	Carcass	Plasma
1	23 ± 5	2.0 ± 0.2	5.2 ± 0.9	30 ± 3	25.0 ± 4.0
3	56 ± 6	4.6 ± 0.6	4.6 ± 0.5	27 ± 4	8.0 ± 0.8
6	55 ± 2	2.1 ± 0.3	7.1 ± 0.5	20 ± 5	3.5 ± 0.7
10	50 ± 1	1.1 ± 0.1	4.7 ± 0.5	16 ± 4	2.7 ± 0.3
15	60 ± 5	1.2 ± 0.1	5.8 ± 0.7	17 ± 4	1.4 ± 0.3
20	66 ± 5	0.8 ± 0.2	6.1 ± 1.3	11 ± 3	0.5 ± 0.0
30	71 ± 3	0.7 ± 0.1	6.8 ± 0.7	3 ± 1	0.2 ± 0.0[b]
40	69 ± 5	0.9 ± 0.1	10.5 ± 1.0	6 ± 2	0.1 ± 0.0
60	67 ± 4	0.8 ± 0.1	8.6 ± 0.7	6 ± 2	T[c]

[a]Values are mean ± SE, n=6. Heart, lung, and spleen constituted <0.1% of dose at all time points.

[b]Plasma values at 30 and 40 min not significantly different.

[c]Trace amount.

TABLE 2. Distribution of hepatic-cytosolic radiolabel (% of total)[a]

Time	SEP PAK[b]	HPLC Retention Time (min) 4.9	5.6	6.6	8.1	9.4[c]
1 hr	35 ± 2	0	0	43 ± 3	0	16 ± 3
6 hr	41 ± 5	0	4 ± 1	41 ± 3	0	15 ± 1
12 hr	40 ± 3	T[d]	7 ± 1	34 ± 3	T	15 ± 2
1 day	30 ± 4	5 ± 0	12 ± 1	33 ± 3	7 ± 1	16 ± 1
2 day	19 ± 3	3 ± 2	9 ± 2	20 ± 1	25 ± 1	14 ± 1
4 day	10 ± 2	0	4 ± 1	14 ± 2	45 ± 2	21 ± 1
6 day	8 ± 1	0	T	5 ± 0	60 ± 2	17 ± 1
Control[e]	9 ± 1	0	0	0	0	90 ± 1

[a] Values are mean ± SE, n=3.

[b] % of hepatic-cytosolic radiolabel which pelleted after pronase digestion + that which remained bound to the Sep Pak C-18 resin.

[c] Retention time of [^{3}H]MCYST-LR.

[d] Trace amount detected.

[e] Control-liver cytosol was prepared, heat denatured and spiked with 30,000 dpm of [^{3}H]MCYST-LR. The samples were then processed as described in Methods.

TABLE 3. **Percent of hepatic cytosolic radiolabel covalently bound 1 hr to 6 days post injection of [^{3}H]MCYST-LR**[a]

Time	% Radiolabel Covalently Bound[b]
1 hr	84 ± 6
6 hr	84 ± 1
12 hr	74 ± 6
1 day	83 ± 5
2 day	62 ± 3*
4 day	42 ± 10***
6 day	42 ± 11***

[a]Values are mean ± SE, n=3.

[b]Fractions 2 and 3 were added together and expressed as percent of total hepatic cytosolic radiolabel.

*P < 0.05 compared to 1 and 6 hr.

***P < 0.001 compared to 1, 6, 12 hr and 1 day.

Figure legends

Fig. 1. Semilog plot of plasma concentration of toxin equivalents versus time. The open circles represent the mean ± SE for six animals per time point. The solid line is the theoretical curve defined by the inset equation.

Fig. 2. Semilog plot of tissue concentration of toxin equivalents versus time. The values presented are the mean ± SE for six animals per time point. The symbols represent the following tissues: (○) liver, (●) kidney, (▲) carcass, (■) lung, and (△) gut. Heart and spleen did not contain measurable quantity of radiolabel.

Fig. 3. Appearance of radioactivity in urine and feces. Panel A is the cumulative urinary and fecal appearance of radioactivity. Panel B and C are the percent of injected dose that appeared in feces or urine per collection period, respectively. Values are mean ± SE, n = 9-13 and are plotted at the midpoint of the collection period.

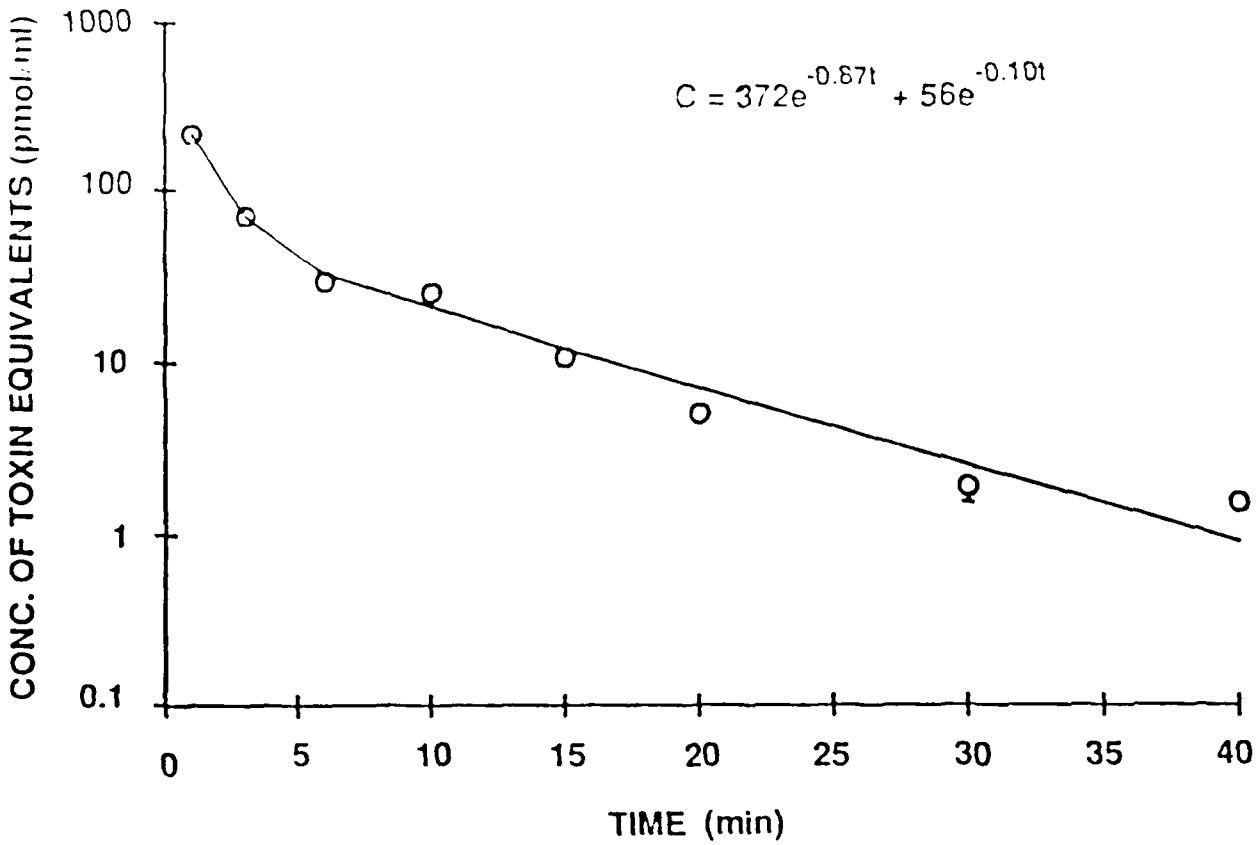
Fig. 4. C-18 HPLC profile of radiolabel in urine, feces, and hepatic cytosol. Panel A: [^{3}H]MCYST-LR in control fecal extract. Control spiked urine or control, heat-denatured hepatic cytosol that was spiked with [^{3}H]MCYST-LR prior to pronase treatment yielded identical results. Panel B: representative 6-hr urine,

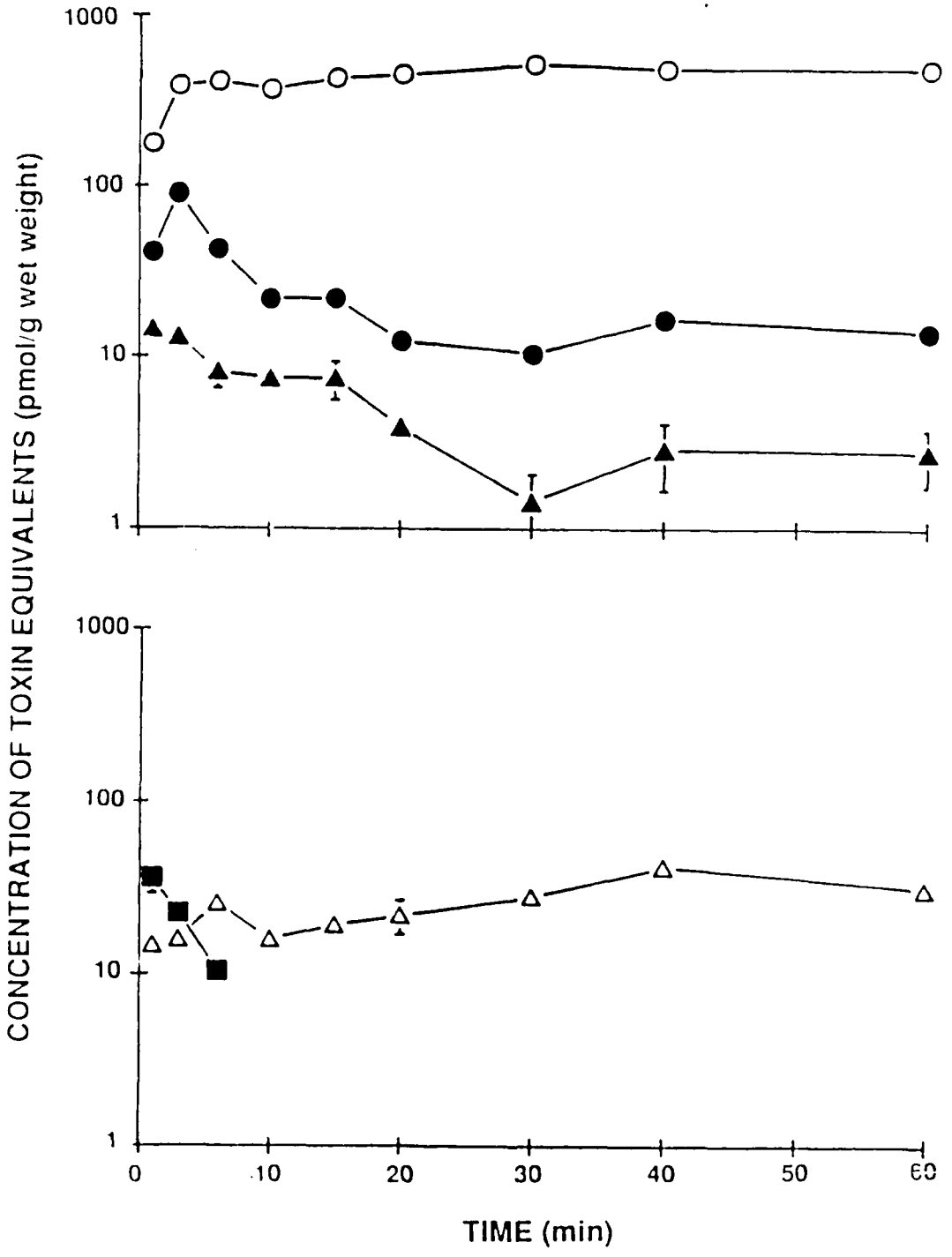
analysis was performed directly. Panel C: representative 6 hr fecal extract prepared as described in Methods. The fecal extracts were darkly colored, which appeared to affect counting efficiency of the Radiomatic Flow I β counter. The following peaks at the indicated rt were detected (mean % of total radioactivity ± SE) in n out of six samples: 4.0 min, 13% (n=2); 4.6 min, 9 ± 2% (n=4); 5.3 min, 6.5% (n=2); 6.3 min, 20 ± 4% (n=5); 8.4 min, 11 ± 3% (n=4); and [^{3}H]MCYST-LR, 9.3 min, 63 ± 8% (n=6). Panels D-F: representative hepatic cytosol profiles 6 hr, 1 day and 6 days post injection, respectively. Cytosols were prepared for analysis as described in Methods. The [^{3}H]MCYST-LR peak is indicated by <- M.

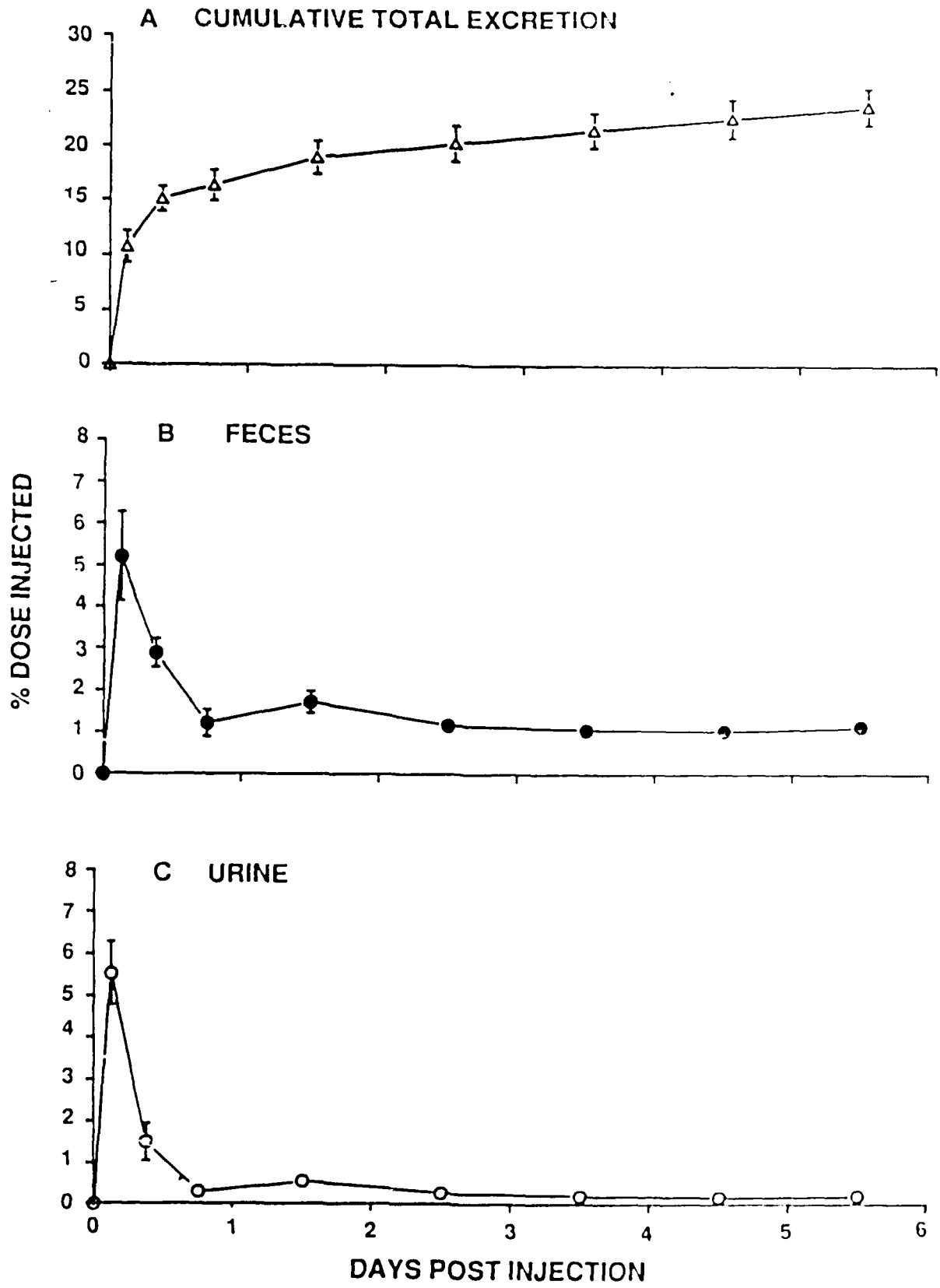
Fig. 5. Econo-Pac 10DG desalting column profile of hepatic-cytosolic radiolabel under native conditions. Samples (0.8 ml) of liver cytosol were chromatographed on a 10-ml Bio-Gel P-6 column equilibrated with 100 mM Tris-Cl, pH 7.2 and 1-ml fractions were collected. The solid line is a representative elution pattern from cytosol 2 days post injection. All the time points, 1 hr through 6 days, yielded similar results. The dashed line is the elution profile of [^{3}H]MCYST-LR. V_o indicates the void fraction of the column measured with blue dextrin.

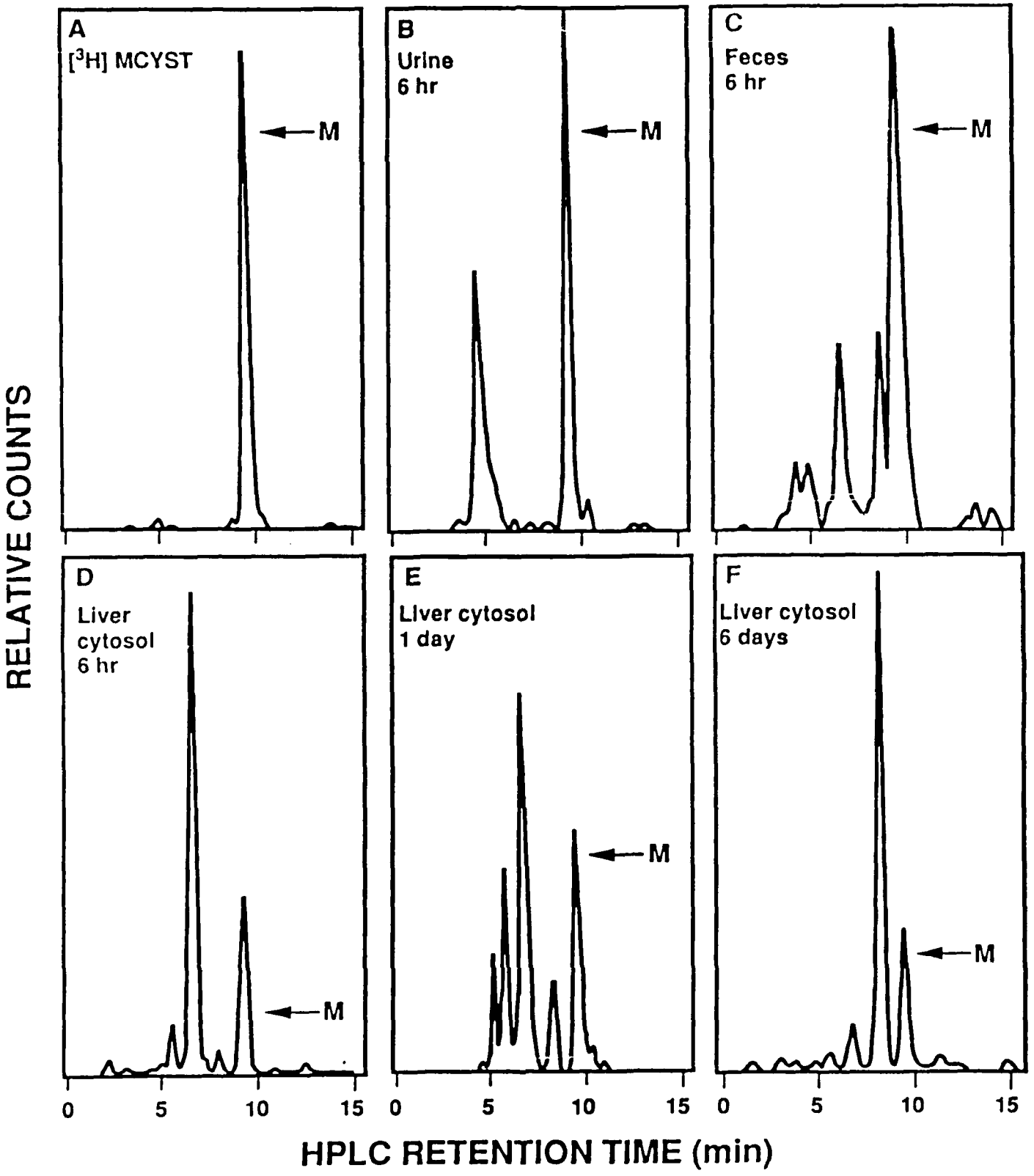
Fig. 6. Econo-Pac 10DG desalting column profile of hepatic-cytosolic radiolabel under denaturing conditions. Samples (0.5 ml) were denatured with urea and SDS (6 M and 0.1%, respectively)

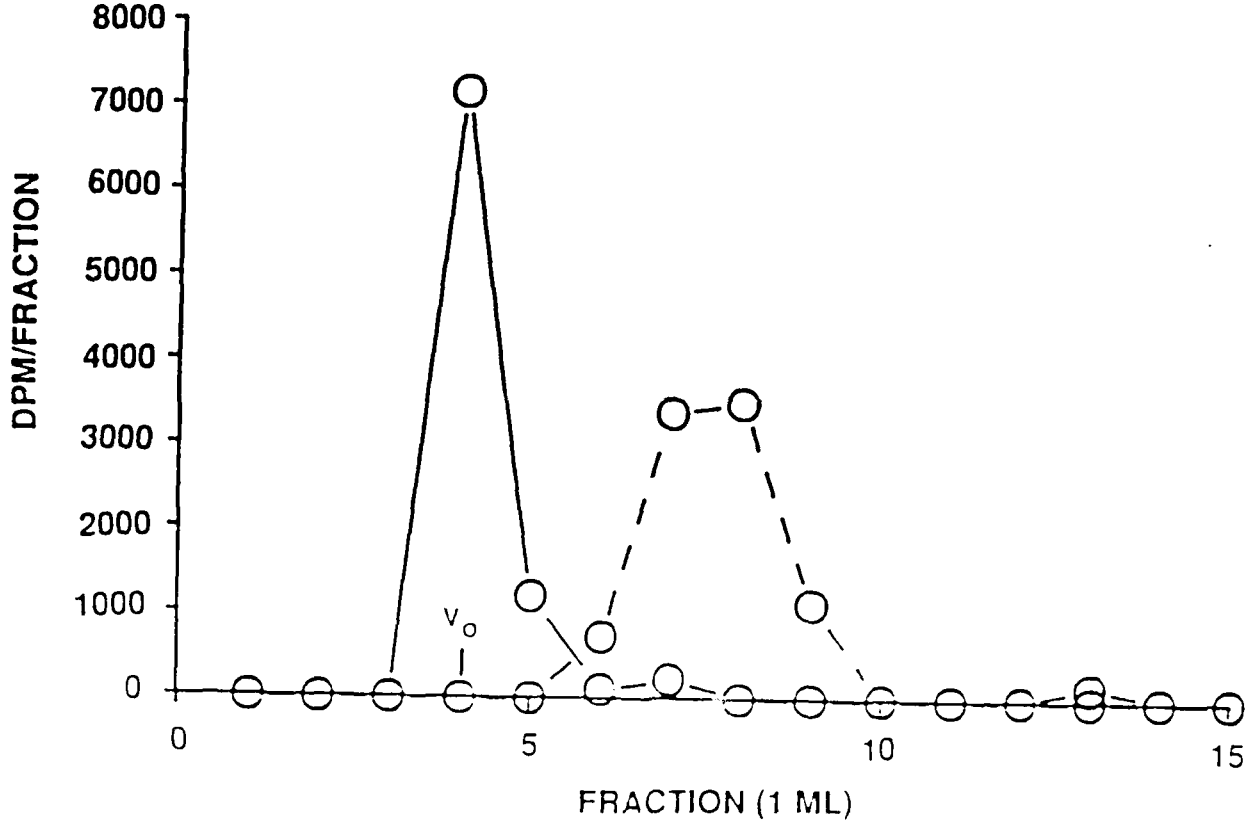
prior to being applied to a 10-ml Bio-Gel P-6 column equilibrated with 6 M urea and 0.1% SDS in 10 mM Tris-Cl, pH 7.2 and 1 ml fractions were collected. Panel A: [^{3}H]MCYST-LR radiolabel profile. Panel B: Hepatic cytosol radiolabel profile 2 day post injection, bars are mean ± SE, n=3.

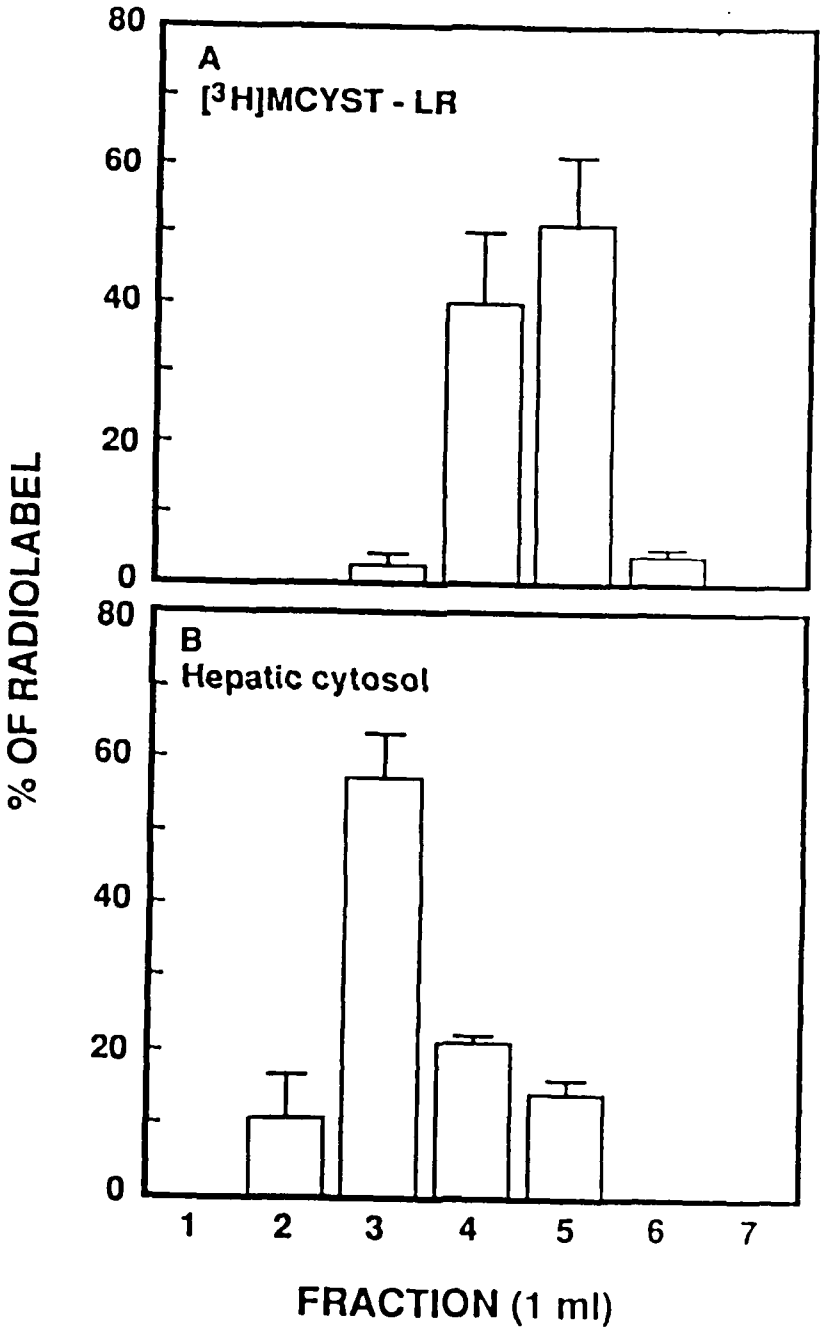

CONC. OF TOXIN EQUIVALENTS (pmol/ml)
1000
100
10
1
0.1
$C = 372e^{-0.87t} + 56e^{-0.10t}$
0
5
10
15
20
25
30
35
40
TIME (min)


1000
100
10
1
1000
100
10
1
0
10
20
30
40
50
60
CONCENTRATION OF TOXIN EQUIVALENTS (pmol/g wet weight)
TIME (min)


A CUMULATIVE TOTAL EXCRETION
30
25
20
15
10
5
0
B FECES
8
7
6
5
4
3
2
1
0
% DOSE INJECTED
C URINE
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
DAYS POST INJECTION


A
[3H] MCYST
M
B
Urine
6 hr
M
C
Feces
6 hr
M
D
Liver
cytosol
6 hr
M
E
Liver cytosol
1 day
M
F
Liver cytosol
6 days
M
RELATIVE COUNTS
0
5
10
15
HPLC RETENTION TIME (min)


8000
7000
6000
5000
4000
3000
2000
1000
0
DPM/FRACTION
V_O
0
5
10
15
FRACTION (1 ML)


A
[3H]MCYST - LR
B
Hepatic cytosol
% OF RADIOLABEL
80
60
40
20
0
1
2
3
4
5
6
7
FRACTION (1 ml)

Index terms

Microcystin, MCYST, pharmacokinetics, metabolism, hepatic retention, detoxication, tissue binding, covalent interaction, metabolites, mice