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Note

High-performance liquid chromatographic method for the determination of 3-methylcholanthrene in channel catfish plasma

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Oxidative metabolism of xenobiotics is often the first step in biotransformation. Hepatic microsomal oxygenases, in both mammalian and non-mammalian groups, are the dominant catalysts for oxidative transformation of xenobiotics. This enzyme system is regulated by various environmental and biological variables. 3-Methylcholanthrene (3MC) is one of the efficient inducing agents of hepatic microsomal oxygenases in mammals and fish [1,2]. The degree of induction depends on the concentration of 3MC in blood/liver of the animal as well as the time of exposure. In order to characterize the pharmacokinetics and pharmacodynamics of 3MC in channel catfish, a sensitive method of analysis of 3MC was needed.

Of the methods currently available for measuring 3MC, few are suitable for pharmacokinetic studies. A high-performance liquid chromatographic (HPLC) method for the analysis of 3MC in human stools was reported by Duane *et al.* [3], however, the procedure was not suitable for 3MC analysis in plasma. Stoming and Gerardot [4] employed a reversed-phase linear gradient HPLC method for 3MC determination, but the retention time was too long for practical applica-

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tions. Yasuhira and Takahashi [5] reported a fluorometric measurement of 3MC, but the procedure was applicable for the analysis of 3MC in organic solvents only. The primary objective of this study was to develop a sensitive and reproducible HPLC method suitable for analysis of 3MC in catfish plasma samples. Preliminary plasma concentration–postinfusion data of 3MC in channel catfish are also presented.

EXPERIMENTAL

Materials and reagents

3MC and β-naphthoflavone (βNF), used as the internal standard, were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A). All other chemicals were analytical grade.

Chromatography

The HPLC system consisted of a single-piston, reciprocating pump which delivers solvent to the column via a flow controller, pulse damper, pressure transducer (Varian 5000 liquid chromatograph) and a variable-wavelength UV detector (Varian UV-50, Varian, Palo Alto, CA, U.S.A.). The stationary phase was a 5-μm Ultramex ODS packed in a 250 mm × 4.6 mm I.D. column (Phenomenex, Rancho Palos Verdes, CA, U.S A.). Analysis was performed using a mobile phase of acetonitrile at a flow-rate of 1.5 ml/min at room temperature (*ca.* 23°C). Samples of 50 μl were injected manually through a fixed-loop injector into the HPLC system and the eluent was detected at a 295-nm wavelength. The electronic signal was recorded and integrated by a Waters 740 data module integrator (Waters Assoc., Milford, MA, U.S.A.). A 3 cm × 4.6 mm I.D. MCH-10 guard column (Varian) was connected between the injector and analytical column to remove impurities from solvent and samples.

Sample preparation

A solid-phase extraction (SPE) column was used to purify the plasma sample for the analysis of 3MC. Reversed-phase octadecyl (C_{18}, 0.5 g, 3 ml) SPE column (Bakerbond SPE, J.T. Baker, Phillipsburg, NJ, U.S.A.) was conditioned under positive pressure with two 0.5-ml portions of a mixture of water–isopropyl alcohol (80·20) followed by two 0.5-ml portions water using vacuum manifolds (Baker SPE-10 column processing system, J. T. Baker). A specified volume (0.1–0.4 ml) of plasma was placed on top of the SPE column material and a partial vacuum was applied, followed by a specified volume of 8 μg/ml βNF solution in acetonitrile. Vacuum was applied and after drawing through to waste, the column was washed with two 0.5-ml portions of a mixture of water–isopropyl alcohol (80:20) and dried under suction. 3MC and βNF were eluted with four 0.5-ml portions of methylene chloride The eluent was drawn through under suction and

the organic solvent was evaporated under a gentle stream of nitrogen. The residue was reconstituted with acetonitrile.

Assay calibration

The assay was calibrated by the addition of known amounts of 3MC, internal standard and blank control plasma on top of the SPE column and extracting by the procedure described above. Standard calibration curves were established for 3MC concentrations ranging from 0.04 to 10.0 μg/ml. The internal standard was used at a fixed concentration of 2.0 μg/ml in all plasma samples. The final sample concentrations were calculated by determination of the peak-height ratios of 3MC to internal standard.

Recovery and assay precision

The absolute recoveries of 3MC from plasma were determined by comparing the peak height of assayed plasma to those of unextracted standard solution of 3MC at three concentrations (0.5, 1.5, 5.0 μg/ml). Day-to-day and within-day precision were determined using five replicate analyses of spiked plasma at five different concentrations.

Pharmacokinetic experiments with catfish

A 3MC solution was prepared by dissolving 3MC in a mixture of water–dimethylacetamide (10:90) yielding a final concentration of 10 mg/ml. Channel catfish, *Ictalurus punctatus*, acclimated to 23°C with a mean body weight of 0.450 kg, were fitted with a cannula in the dorsal aorta according to the procedure described by Smith and Bell [6]. The following day, the 3MC solution was infused at a rate of 200 μl/h to deliver a dose of 10 mg/kg body weight. The time of infusion varied between 2.0 and 2.5 h, depending on the body weight of the fish. The infusion was delivered through a Syringe Infusion Pump 22 (Harvard Apparatus, South Natick, MA, U.S.A.). At the end of the infusion the cannula was flushed several times with heparinized saline to remove any trace of 3MC. Serial blood samples (0.15–0.5 ml) were collected at various time intervals, ranging from 10 min to 60 h postinfusion, and placed into microcentrifuge tubes. Blood samples were centrifuged immediately and the plasma was separated. Plasma samples were stored at −4°C until analyzed for 3MC.

RESULTS AND DISCUSSION

In the course of development of an extraction procedure for 3MC from plasma, the more traditional liquid–liquid extraction by organic solvents was employed. Plasma proteins were precipitated by addition of acetonitrile and the supernatant was extracted with petroleum ether. The petroleum ether layer was then evaporated and the residue was reconstituted in a small volume of mobile phase before injection into the HPLC system. This procedure was time-consuming and the minimum detection limit was 0.4 μg/ml.

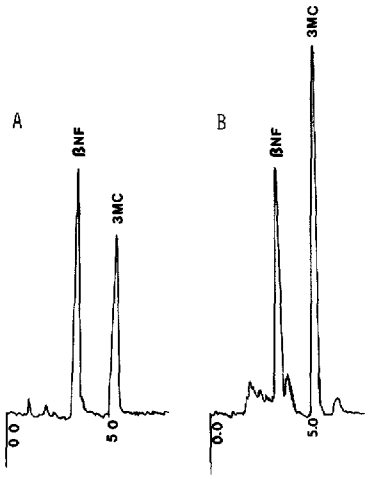

Fig. 1 Chromatogram obtained after a 50-μl injection of (A) blank plasma containing 2.0 μg/ml βNF and 0.5 μg/ml 3MC and (B) plasma of 3MC-treated fish containing 2.0 μg/ml βNF

SPE techniques offer a more rapid and efficient methodology. Sorbents of the same types as that in analytical columns, but with a much greater particle size (usually 40 μm instead of 3–10 μm) are packed at the bottom of a syringe-like small column between two inert frits. A sorbent is chosen which has a high affinity for the compound of interest. In this case C_{18} material was selected. The plasma passes through the material, and 3MC was bound to the sorbent. The sorbent was then washed with a mixture of water–isopropyl alcohol (80:20) to remove any impurities from the plasma. This fraction was collected and extracted with methylene chloride and analyzed for the presence of 3MC. 3MC was not detected in the organic extract. This indicates that 3MC is quantitatively bound

TABLE I

DAY-TO-DAY AND WITHIN-DAY REPRODUCIBILITY OF 3MC IN PLASMA

Concentration (μg/ml)	Coefficient of variation (n = 5) (%)	
	Within-day	Day-to-day
0.04	4.8	6.3
0.2	3.4	4.5
0.5	4.2	5.9
1.0	3.2	5.4
2.0	3.9	4.6
5.0	2.1	3.6
10.0	2.7	4.2

TABLE II

RECOVERY STUDY FROM CATFISH PLASMA

Concentration of 3MC (μg/ml)		Recovery (mean ± S.D , n = 5) (%)
Added	Found	
0 50	0 482	96.5 ± 5 47
1 50	1.429	95.3 ± 4 71
5 00	4 717	94 2 ± 3 33

to the packing material. 3MC and the internal standard were then removed from the sorbent using methylene chloride.

A chromatogram which illustrates the analysis of 3MC in catfish plasma is shown in Fig. 1. The retention times of βNF and 3MC were 3.6 and 5.7 min, respectively. There were no interfering peaks. The calibration curve (peak-height ratio *versus* concentration) was linear (r = 0.992) over the concentration range investigated (0.04–10.0 μg/ml).

The precision data for the assay are shown in Table I. The coefficient of variation was less than 7% in all cases for both within-day and day-to-day analysis. The recoveries of 3MC by SPE was approximately 95% for the three concentrations tested (Table II).

Data from the pharmacokinetic study

A typical mean plasma concentration–postinfusion time profile for three fish after intraaortic infusion of 10 mg/kg 3MC is shown in Fig. 2. The analytical

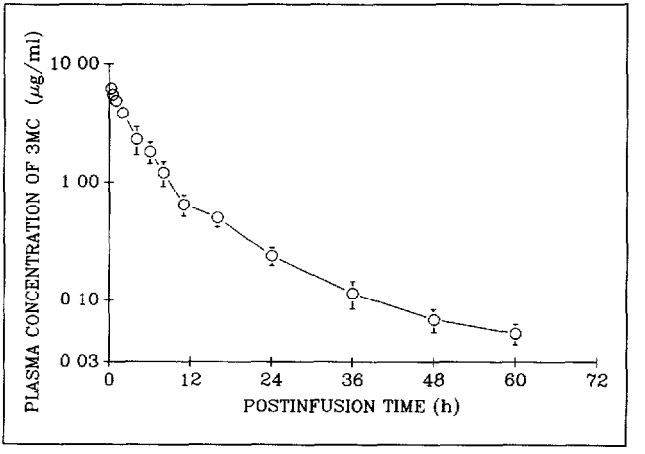

Fig 2 Mean plasma concentration–postinfusion time profile for three catfish after infusion of 10 mg/kg body weight of 3MC

method was sufficiently sensitive to enable 3MC quantitation from plasma consistently up to 60 h after the termination of the infusion. The data appear to show a biexponential decline of 3MC concentration as a function of time.

CONCLUSION

A simple, rapid and reproducible assay has been developed for the determination of 3MC from catfish plasma. Sample preparation requires minimal time and labor, and chromatography is complete within 10 min for each run.

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