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# Liquid chromatographic determination of tacrine and its metabolites in rat bile microdialysates

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**Abstract**

A microbore liquid chromatographic method using a phenyl stationary phase was developed for the determination of 9-amino-1,2,3,4-tetrahydroacridine (tacrine, THA) and its metabolites in microdialysis samples of bile. Analysis of microdialysis samples requires analytical methods with low detection limits and small sample volume requirements. The method uses a 1-mm I.D. phenyl column and fluorescence detection. A detection limit of 0.25 ng/ml in a 5-$\mu$l sample was achieved for THA. This method was then used to determine THA and THA-1-ol in the bile dialysate of a rat. Because of the small sample volume requirements, a 10-min temporal resolution was achieved for the microdialysis experiment. The low detection limit allowed the THA concentration in the bile to be monitored for more than 4 h following a 1.0 mg/kg i.v. dose of THA.

## 1. Introduction

Microdialysis sampling *in vivo* is gaining use for the determination of pharmacokinetics [1] and the study of xenobiotic metabolism [2,3]. Microdialysis sampling is performed by implanting a small dialysis fiber at the site of interest in an experimental animal. The fiber is perfused at a low flow-rate, typically 1 $\mu$l/min. Low-molecular-mass compounds in the extracellular fluid can diffuse across the membrane and into the probe lumen. These compounds are flushed through the probe and collected for analysis. Although a wide range of analytical methods can be coupled to microdialysis sampling, liquid chromatography has been the most commonly used because typically the dialysis samples are aqueous, the analytes are relatively hydrophillic low-molecular-mass compounds, and several analytes can be monitored simultaneously. Although the microdialysis sampling stage and the analysis stage do not have to be physically coupled in an on-line manner, the sample requirements of the analytical method (both concentration and volume) determine the minimum sampling interval which can be achieved. It is the minimal sampling interval that then determines the temporal resolution that can be achieved for the experiment. For example, at a perfusion rate of 1 $\mu$l/min, to achieve a 10-min resolution only 10 $\mu$l of sample are available. For this reason it is necessary to minimize the sample volume requirement of the analytical method while maintaining the concentration detection limits. The

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use of microbore columns results in significantly less sample dilution and is therefore often the technique of choice for microdialysis sampling.

9-Amino-1,2,3,4-tetrahydroacridine (tacrine, THA) is a potent cholinesterase inhibitor being investigated as a therapeutic agent for the treatment of Alzheimer's dementia [4,5]. Several techniques for the determination of THA and its metabolites have been reported. These methods are typically based on a chromatographic separation followed by a sensitive detection method. Liquid chromatographic separations of THA have been reported using octadecyl silyl- (ODS) [6], phenyl- [7], and cyano- [8,9] based stationary phases coupled to UV absorbance [7], fluorescence [6,8,9], or electrochemical [10] detection. Detection limits for THA using these methods vary between 0.2 ng/ml and 1 ng/ml. However, to achieve these detection limits sample volumes greater than 100 $\mu$l were required.

In this report, a liquid chromatographic method with fluorescence detection is described for the detection of THA and its metabolites in bile dialysates. The method utilized a phenyl microbore column to achieve detection limits comparable to the best previously reported values but using only 10 $\mu$l of sample. This method was used to analyze bile dialysates to determine the pharmacokinetics of THA in the bile of rats.

## 2. Experimental

### 2.1. Materials

THA (9-Amino-1,2,3,4-tetrahydroacridine hydrochloride) and THA-1-ol (9-amino-1,2,3,4-tetrahydroacridin-1-ol) were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were reagent grade and used as received. Deionized water (Nanopure water purification system, Barnstead, Dubuque, IA, USA) was used for all solutions throughout the study.

Buffers were prepared by mixing the appropriate acid and ammonium hydroxide until the desired pH and concentration were obtained. All mobile phases were filtered through a 0.2-$\mu$m nylon filter (Xpertek, St. Louis, MO, USA) prior to use. A mixture of 150 mg/kg ketamine-HCl (Aveco Co., Fort Dodge, IA, USA) and 9.74 mg/kg xylazine (Rompun, Mobay, Shawnee, KS, USA) was used to anesthetize the animals used for these experiments. Cuprophan (CUP) dialysis fibers (regenerated cellulose) were a gift from I.N. Mefford of the National Institute of Mental Health.

### 2.2. Equipment

A Shimadzu SCL-6A pump controller (Shimadzu Scientific Instruments, Colombia, MD, USA) was used to set the flow-rate on a Shimadzu LC-6A pump. When the flow-rate on the controller was set at less than 100 $\mu$l/min the actual flow-rate was determined gravimetrically. For the methods using the microbore columns, the flow-rate was set at 50 $\mu$l/min and was determined to be $45.4 \pm 0.1$ $\mu$l/min gravimetrically. A Rheodyne 9125 injection valve with a 5-$\mu$l sample loop was used for all samples. A variety of columns were used to evaluate methods previously described in the literature. Normal analytical scale columns had dimensions of 15 cm $\times$ 4.6 mm I.D. and were packed with either 5-$\mu$m Hypersil ODS or Spherisorb phenyl from Alltech (Deerfield, IL, USA). The microbore columns used in this study were 10 cm $\times$ 1 mm I.D. SepStiks from Bioanalytical Systems (West Lafayette, IN, USA) packed with 8-$\mu$m cyano or 5-$\mu$m phenyl stationary phase. Literature methods were followed as closely as possible depending on the equipment available in our laboratory. Detection was accomplished with a Chrompack fluorescence detector (Raritan, NJ, USA) with the excitation wavelength set at 330 nm and the emission wavelength set at 365 nm.

### 2.3. Chromatography

In order to determine the best chromatographic method for the separation of THA and its metabolites consistent with the small volume requirements of microdialysis sampling, several

previously published methods were evaluated [6–9]. Forsyth *et al.* [6] used a $C_{18}$ reversed-phase column with a mobile phase of methanol–water–triethylamine (49.5:49.5:1, v/v/v). Hsu *et al.* [7] used a phenyl reversed-phase column with a mobile phase of acetonitrile–0.02 *M* ammonium formate buffer pH 2.8 (70:30, v/v). Telting-Diaz and Lunte [9] reported a method using a 100 × 1 mm I.D. cyano column with a mobile phase of acetonitrile–0.05 *M* ammonium phosphate buffer pH 6.9 (25:75, v/v). The method ultimately adopted for the analysis of THA and its metabolites in the bile used a 100 × 1 mm I.D. phenyl column. The mobile phase composition was optimized to methanol–acetonitrile–0.05 *M* ammonium phosphate buffer pH 2.5 (10:5:85, v/v/v). The column flow-rate was 45.4 ± 0.1 μl/min. Evaluation and optimization of the various chromatographic methods was performed using a pooled rat bile dialysis sample collected between 30 min and 60 min after a 1.0 mg/kg i.v. dose of THA. This time range was selected because all metabolites are present and significant THA still remains in the samples. Earlier samples do not contain sufficient metabolites and later samples do not contain sufficient THA for optimization.

### 2.4. Microdialysis

Flow-through dialysis probes were constructed in-house as described previously [10]. The perfusion fluid was delivered by a CMA 100 microinjection pump from Bioanalytical Systems. The perfusion flow-rate was 1.0 μl/min as determined gravimetrically. The sample inlet of the flow-through probe was connected either to a CMA 100 syringe pump for calibration, or surgically cannulated to the bile duct of a male Sprague–Dawley rat for *in vivo* experiments.

### 2.5. Probe calibration

Control rat bile was obtained by cannulating the bile duct of male Sprague–Dawley rats and collecting bile for six hours. The probe was calibrated *in vitro* using control rat bile spiked with THA and THA-1-ol to yield a final concentration of 100 ng/ml. The probe was connected to a syringe pump and calibrated by pumping the spiked rat bile through the sample inlet at a flow-rate of 20 μl/min while maintaining the temperature at 37°C in a thermostatted water bath. This flow-rate corresponds to the normal rate of bile flow in rats [11–14]. The dialysis fiber was perfused with Ringer's solution (155 m*M* NaCl, 5.5 m*M* KCl, 2.3 m*M* $CaCl_2$) at a flow-rate of 1.0 μl/min and dialysate samples collected over 10-min intervals. The bile dialysates were injected directly into the chromatographic system. The relative recovery (*R.R.*) of the probe was calculated as: $R.R. = [THA]_d / [THA]_s$, where the subscripts d and s refer to dialysate and a 100 ng/ml standard prepared in Ringer's solution respectively. A 5.7-cm CUP dialysis fiber perfused at 1.0 μl/min with Ringer's solution gives a 75.6 ± 2.4% ($n = 5$) relative recovery for THA and a 80.3 ± 4.3% ($n = 5$) relative recovery for THA-1-ol from rat bile.

The *in vivo* recovery of the microdialysis probe was determined by externalizing the bile flow. In these experiments, the sample inlet of the dialysis probe was implanted into the anterior side of the common bile duct while the outlet side was externalized. The bile from the sample outlet was collected in 1.5-ml microcentrifuge tubes and extracted using the method described below. The actual concentration of THA and THA-1-ol in the bile was compared with that in dialysate samples collected at the same time. There was good agreement between the recoveries determined *in vitro* and those determined *in vivo*. Therefore, for pharmacokinetic experiments, the outlet end of the flow-through microdialysis probe was inserted into the distal end of the bile duct. In this manner, bile flow in the experimental animal was maintained at normal rates and the bile acids recirculated in a normal manner. The recoveries determined *in vitro* were used to determine the concentration of THA and THA-1-ol in the bile.

### 2.6. Bile assay

The total concentrations of THA and THA-1-ol were determined by liquid–liquid extraction from the rat bile. In a 1.5-ml microcentrifuge

tube 100 μl of rat bile was mixed with 50 μl of 0.5 *M* NaOH. The mixture was vortex-mixed for one minute to ensure thorough mixing. THA was extracted by adding 500 μl of ethyl acetate and vortex-mixing for 2 min. The mixture was centrifuged at 12 000 *g* for 30 s to insure the separation of the two phases. The organic layer was pipetted off into a test tube and the ethyl acetate was evaporated at 55°C under argon. The residue was reconstituted in 100 μl of Ringer's solution and injected onto the chromatographic system for analysis. The concentration of THA recovered was determined by comparing the peak heights of the reconstituted extracts with a standard calibration curve. The working curve for THA had a slope of 1025 ± 6 rfu/ng/ml (rfu = relative fluorescence units) and an intercept of − 551 ± 349 rfu, while the working curve for THA-1-ol had a slope of 506 ± 2 rfu/ng/ml and an intercept of 104 ± 273 rfu, in both cases $r = 0.999$. Using spiked rat bile standards, the extraction procedure yielded a recovery of 91.3 ± 7.8% for THA and 96.5 ± 3.5% for THA-1-ol over a concentration range of 1 to 200 ng/ml.

## 3. Results and discussion

Typical chromatograms of bile dialysates using each of the methods described above are shown in Fig. 1. Each of the chromatograms resulted from a 5-μl injection of a bile dialysate. The $C_{18}$ method (Fig. 1A) was not suitable for the resolution of THA metabolites because all of the metabolites are not satisfactorily resolved. Fig. 1B shows a separation of a bile dialysate collected in the 30–60 min pool using the method developed by Hsu *et al.* [7] with a standard sized column (I.D. = 4.6 mm). As shown, good resolution exists between THA and its metabolites. However, the detection limit for THA was only 5 ng/ml when limited to a 5-μl sample. In order to follow the pharmacokinetics of THA over the desired period, an order a magnitude improvement in detection limits was required. Because of the excellent resolution of this method, the same mobile phase was evaluated using a 1-mm

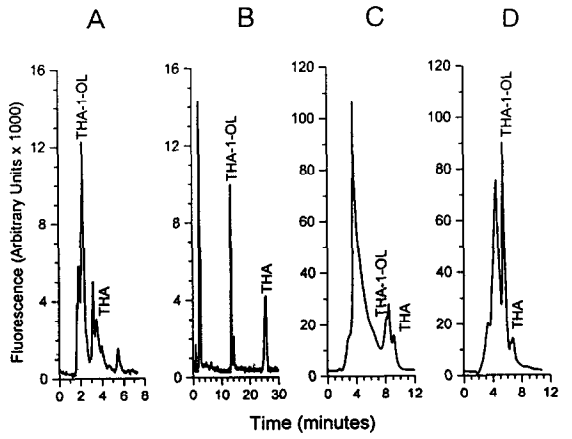


Fig. 1. Chromatograms of pooled bile dialysates. (A) Method $C_{18}$; (B) method Phenyl; (C) method μPhenyl; (D) method μCyano.

I.D. phenyl microbore column. Fig. 2C shows the separation of a bile dialysate taken from the 30–60 min pool using this method with a microbore column. Unfortunately, the resolution of THA and its metabolites could not be re-

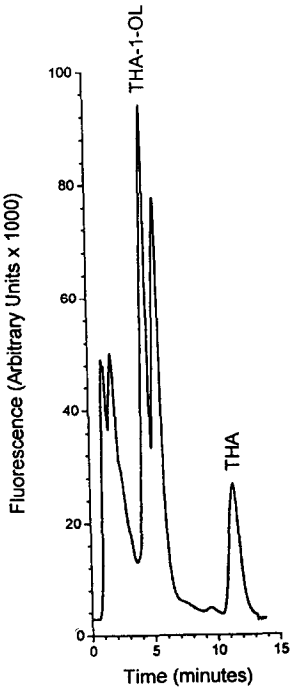


Fig. 2. Chromatogram of pooled bile dialysate using the optimized μPhenyl method.

produced. We have previously reported a microbore liquid chromatographic method for the determination of THA in brain dialysates which used a cyano-based stationary phase [9]. A typical chromatogram using this method is shown in Fig. 1D. While this method performed well for determining THA in brain dialysates it did not provide sufficient resolution for studying THA and its metabolites in the more complex bile dialysates.

Ultimately a mobile phase capable of resolving THA and its metabolites using a microbore phenyl column was developed through simplex optimization of the mobile phase composition. The chromatogram of a 5-$\mu$l injection of the 30–60 min bile dialysate pool is shown in Fig. 2. In addition to THA-1-ol, several other metabolites were detected. The identity of the other metabolites is unconfirmed at this point, however it is likely that they are the hydroxylated conjugates of THA as reported by Hsu *et al.* [7]. The peak volume for THA using a 4.6-mm I.D. column was 3 ml while the peak volume using a 1-mm I.D. column was only 100 $\mu$l. The much smaller dilution factor on the microbore column results in improved detection limits for dialysis samples. In addition, the retention time for THA was only 11 min on the microbore column *vs.* over 25 min on the standard sized column.

Table 1 lists the detection limits for THA along with the required sample volume reported in the literature as well as the detection limits obtained in this laboratory using a 5-$\mu$l injection volume for the various methods evaluated. The optimized method using a microbore phenyl column required far less sample volume to obtain equivalent detection limits. As a result, detection limits somewhat better than those obtained with the other techniques can be achieved with twenty times less sample volume.

Microdialysis sampling of the bile was validated by comparing the concentration of THA determined in dialysates to that in collected bile. As shown in Fig. 3, good agreement exists between the two methods. The plot had a slope of $1.04 \pm 0.02$ with an intercept of $2.64 \pm 1.90$ and $r^2 = 0.996$. Microdialysis sampling offers the advantage that the bile flow is not interrupted and the bile salts therefore are recirculated in a normal manner. This allows for longer experiments without dramatically altering the animals physiological state. In addition, the possibility exists to observe enterohepatic circulation with a single experimental animal.

Table 1
Detection limits of THA using various chromatographic methods

| Method | Volume injected ($\mu$l) | Detection limit[d] | |
|---|---|---|---|
| | | Concentration (ng/ml) | Mass (pg) |
| Forsyth *et al.*[a] | 100 | 0.2 | 20 |
| Forsyth *et al.*[c] | 5 | 5.0 | 25 |
| Hsu *et al.*[b] | N.R.[e] | 1 | N.R.[e] |
| Hsu *et al.*[c] | 5 | 5.0 | 25 |
| $\mu$Cyano | 5 | 0.25 | 1.25 |
| $\mu$Phenyl | 5 | 0.25 | 1.25 |

[a]From data published in ref. 3.
[b]From data published in ref. 4.
[c]Obtained in this laboratory using a 5-$\mu$l injection volume.
[d]Determined at S/N = 2.5.
[e]Not reported.

This improved microbore chromatographic method was then used for the analysis of bile dialysates following an i.v. dose of THA. A temporal resolution of 10 min could be achieved because of the low sample volume requirements

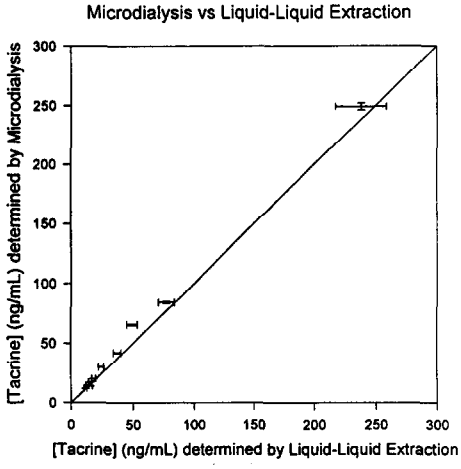


Fig. 3. Comparison of microdialysis sampling of bile to bile collection and extraction.

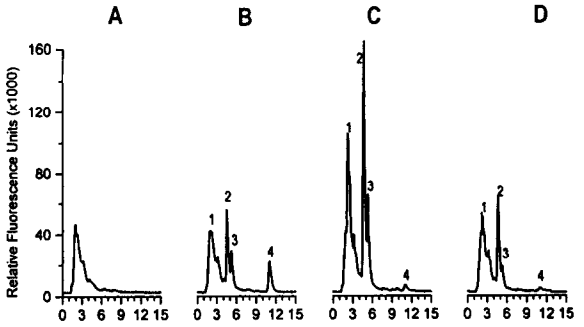


Fig. 4. Chromatograms of rat bile dialysate before and after a 1.0 mg/kg i.v. administration of THA. (A) Blank prior to dosing; (B) sample from 0 to 10 min; (C) sample from 50 to 60 min; (D) sample from 120 to 130 min. Peaks: 1 = metabolite 1; 2 = THA-1-ol; 3 = metabolite 2; 4 = THA.

coupled to the low detection limits of the microbore method. Typical chromatograms of bile microdialysates at various times following a 1.0 mg/kg i.v. dose of THA are shown in Fig. 4. The pharmacokinetic curves for THA and one of its major metabolites, THA-1-ol, obtained from a microdialysis experiment are shown in Fig. 5. Because of the low detection limits of the microbore chromatographic method, THA in the bile could be continuously monitored for more than 4 h using microdialysis sampling. The data in Fig. 5 show the elimination of THA from the bile. These data were fitted to a biexponential decay given by the formula

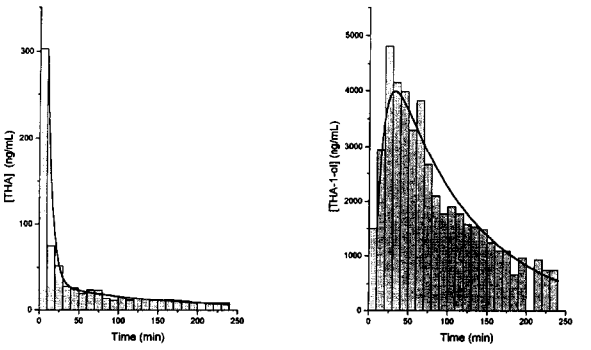


Fig. 5. Pharmacokinetic profiles for THA (left) and THA-1-ol (right) from the bile of an anesthetized Sprague–Dawley rat. (—) Non-linear regression best fit line. THA was dosed i.v. at $t = 0$.

Table 2
Pharmacokinetic parameters for THA and THA-1-ol in bile

| Parameter | THA[a] | THA-1-ol[a] |
|---|---|---|
| $t_{1/2}$ ($\alpha$) min | 6.32 ± 1.7 | 7.46 ± 2.2 |
| $t_{1/2}$ ($\beta$) min | 151.2 ± 54.2 | 64.2 ± 11.2 |
| AUC[b] ($\mu$g min/ml) | 8.34 ± 1.27 | 302.6 ± 152.3 |

[a]Values are mean ± S.D. ($n = 3$).
[b]Area under the curve.

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}.$$

The metabolic fate of the THA-1-ol is also shown in Fig. 5. In this case the data were fitted to the function

$$C(t) = Ae^{-\alpha t} - Be^{-\beta t}$$

The parameters for these expressions were derived by fitting the data using a non-linear least squares program (Origin for Windows, MicroCal Software, Northampton, MA, USA). The relevant pharmacokinetic parameters are given in Table 2.

## 4. Conclusion

The technique of microdialysis sampling using the flow-through probe design is well adapted for monitoring THA and its metabolites *in vivo* in the bile of an anesthetized rat. However, microdialysis sampling places constraints on the analytical method in terms of the available sample volume. The use of a microbore column (*i.e.* 1-mm I.D.) provides concentration detection limits equivalent to or better than those obtained with methods using standard sized columns while requiring significantly less sample. Further improvements can be anticipated using even smaller I.D. columns although specialized pumping and detection systems will be required. One-millimeter I.D. columns provide an order of magnitude improvement over standard columns and are still compatible with standard chromatographic systems.

## 5. Acknowledgement

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