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Metabolic disposition of tacrine in primary suspensions of rat hepatocyte and in single-pass perfused liver: *in vitro/in vivo* comparisons

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1. Incubations of tacrine (1,2,3,4-tetrahydro-9-acridinamine monohydrochloride monohydrate, THA) with a primary suspension of rat hepatocytes for 2 min resulted in formation of the 1-hydroxy derivative as the major metabolite with smaller amounts of the 2- and 4-hydroxy metabolites.

2. Apparent V_{max} and K_m for THA metabolism were 12·4 ± 3·3 nmol/min/g liver and 0·98 ± 0·34 μM respectively.

3. Incubations of THA for longer time-periods (> 10 min) resulted in irreversible binding of THA-derived radioactivity to hepatocellular protein. The apparent maximal rate of irreversible binding (B_{max}) was 76·7 ± 30·5 pmol equivalents bound/h/mg cell protein, whereas the apparent K_b for binding was 2·8 ± 1·4 μM.

4. The kinetic parameters, V_{max} and K_m, were used to predict steady-state extraction ratios (ER_{SS}) for various THA input concentrations (C_{in}) in single-pass perfused rat liver. At low input concentrations (0·72–0·85 μM; $C_{in} < K_m$), ER_{SS} of THA was approximately 1. For higher C_{in} (14·05, 20·72, 20·88 μM; $C_{in} \gg K_m$), the calculated ER_{SS} was markedly decreased with 0·300, 0·296 and 0·261, respectively.

5. The intrinsic clearance of THA (Cl_i) estimated from *in vitro* hepatocyte data was 6·7 ml/min/g liver while the apparent oral THA clearance (Cl_{oral}) calculated from *in vivo* rat data was 6·6 ml/min/g liver.

Introduction

Tacrine, (1,2,3,4-tetrahydro-9-acridinamine monohydrochloride monohydrate, THA) is an acetylcholinesterase inhibitor recently shown to be efficacious in treatment of senile dementia of the Alzheimer's type (Farlow *et al.* 1992, Knapp *et al.* 1994). During the course of clinical investigations, elevation of serum ALT levels have been observed in 20–50% of the patients suggestive of hepatic insult (O'Brien *et al.* 1991, Watkins *et al.* 1994). These elevations of liver enzymes were reversible upon cessation of THA administration (Farlow *et al.* 1992, Watkins *et al.* 1994). The mechanism(s) by which THA elevates liver marker enzymes is not known, but may involve formation of hepatotoxic metabolites, covalent binding, formation of active oxygen species, or a drug-induced hypersensitivity reaction (Horvath 1989, Neuberger and Williams 1989, Pessayre 1993).

Disposition studies in rat and dog have shown that THA undergoes extensive metabolism primarily to the monohydroxy alicyclic derivatives 1-, 2- and 4-OH-THA (Hsu *et al.* 1990, Pool *et al.* 1991, 1992a). In clinical trials, these alicyclic hydroxy metabolites have also been observed in plasma as well as urine (Hartvig *et al.* 1990, Pool *et al.* 1992b). However, in man the majority of THA-derived

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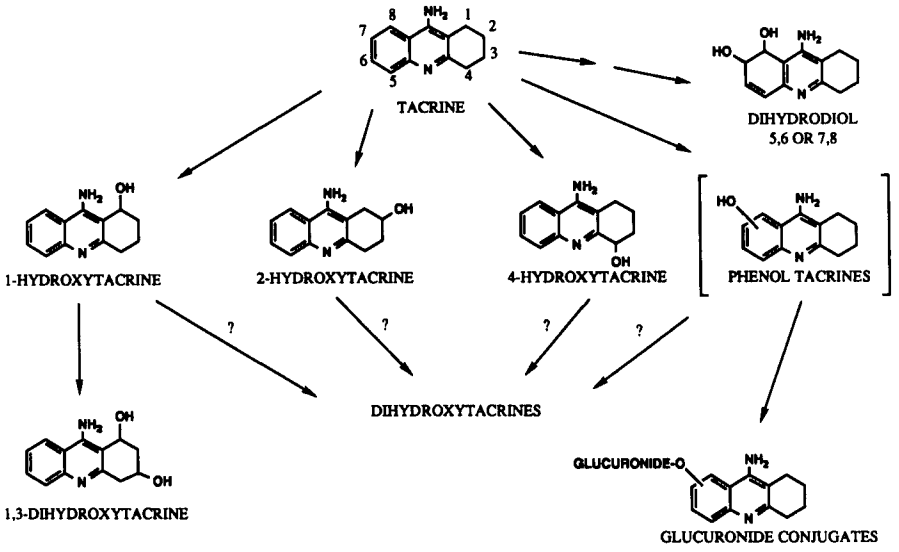

Figure 1. Proposed metabolic pathway for THA.

metabolites were excreted in the form of dihydroxylated derivatives and phenol glucuronides (Pool *et al.* 1992b). The metabolism of THA is depicted in figure 1.

In vitro studies using human and rat liver microsomal preparations found THA to be a good substrate for NADPH-dependent oxidative metabolism (Kukan *et al.* 1992, Madden *et al.* 1993, Woolf *et al.* 1993). Similarities in stable metabolite formation were observed between rat and human preparations with 1-OH-THA as the predominate metabolite (Kukan *et al.* 1992, Madden *et al.* 1993, Woolf *et al.* 1993). During the course of these investigations, irreversible binding of THA-derived radioactivity to microsomal protein was found. THA metabolism and extent of THA-derived irreversible binding appeared to be related to P450 1A2 content and activity (Woolf *et al.* 1993). The mechanism(s) by which THA is activated to a specie(s) capable of irreversibly binding to hepatocellular proteins has not been clearly defined. However, mechanistic studies suggest involvement of a quinone–methide type reactive intermediate(s) (Woolf *et al.* 1993, Spaldin *et al.* 1994).

The present study was undertaken to investigate THA metabolism and irreversible binding in primary suspensions of rat hepatocytes and in the single-pass perfused rat liver. The utility of these models in predicting *in vivo* THA oral clearance in rat was also assessed.

Materials and methods

Materials

THA, 1,2,3,4-tetrahydro-9-acridinamine monohydrochloride monohydrate (78·5% parent); 1-OH-THA, 9-amino-1,2,3,4-tetrahydroacridin-1-ol (>99% pure); 2-OH-THA, 9-amino-1,2,3,4-tetrahydroacridin-2-ol (99% pure); 4-OH-THA, 9-amino-1,2,3,4-tetrahydroacridin-4-ol (>99% pure); and were prepared by published methods (Woolf *et al.* 1993). [^{14}C]-THA, 1,2,3,4-tetrahydro-9[^{14}C]-acridinamine monohydrochloride monohydrate (7·02 or 11·47 mCi/mmol, radiochemical purity >99% by hplc) was prepared by the Radiochemistry Section at Parke-Davis Pharmaceutical Research and will be published elsewhere. Collagenase was purchased from USOL (Prague, Czech Republic). Hplc grade acetonitrile and water were obtained from Fischer Scientific (FairLawn, NJ, USA). Ready gel® was purchased from Beckman Inst (Fullerton, CA, USA) and scintillation cocktail for radioactivity

monitoring was from Packard Inst (Downers Grove, IL, USA). All other chemicals used were the highest quality commercially available.

Single-dose oral pharmacokinetics in rat

Four male Wistar rats were surgically implanted with jugular cannulas. Following an overnight fast, each animal received a single oral solution dose (8 mg/kg, 50 μCi, 7·02 mCi/mmol) of [^{14}C]-THA by gavage. Blood samples (1 ml) were collected into heparinized tubes predose, and at 0·25, 0·5, 1, 1·5, 2, 4, 6, 8 and 12 h post-dose. Plasma was separated from blood by centrifugation and stored at − 20°C until analysis.

Preparation and incubation of isolated hepatocytes

Male Wistar, non-fasted rats, weighing 300–380 g, were used throughout the study. Hepatocytes were isolated according to the two-step method of Seglen (1976). Cell viabilities for all experiments were > 90%, as estimated by trypan blue dye exclusion test. The number of cells utilized for incubations was determined by counting an aliquot of viable cells in a Buerker chamber and correcting for dilution. Incubations of [^{14}C]-THA at various substrate concentrations and hepatocytes (4×10^6 cells/ml) were conducted in an oxygenated Krebs–Henseleit bicarbonate buffer (pH 7·4) fortified with glucose (10 mM final concentration) along with Hepes (25 mM final concentration). Experiments were performed in either round-bottomed flasks (10 ml reaction volumes) or scintillation vials (2 ml volumes) in triplicate. Incubations were conducted in a shaker style water bath operated at 37°C under atmospheric pressure. Reactions were initiated by addition of substrate. At designated time points, either a 0·5-ml aliquot or the whole 2-ml reaction incubate were removed. Reactions were terminated by adding 3 ml ice-cold absolute ethanol. A 0·05-ml aliquot of the quenched sample was taken for determination of total ^{14}C. Quenched samples were cooled to − 70°C and thawed three times to lyse cell membranes, and then centrifuged at 3500 rpm for 5 min. The ethanolic extracts were removed and evaporated under a gentle nitrogen stream at 37°C. Residues were dissolved in 0·2 ml 0·1 M ammonium acetate buffer (pH 4) prior to analysis by hplc-radioactivity detection.

To assess THA-derived irreversible binding to hepatocellular macromolecules, incubations of [^{14}C]-THA with hepatocytes were conducted using the same condition described above but for 40-min time periods. Incubations of [^{14}C]-THA (2 μM) with hepatocytes were conducted over a 60-min time course to assess THA-derived irreversible binding. Irreversible binding was determined by the exhaustive extraction method of Woolf *et al.* (1993). The Bio-Rad assay method was used to measure protein concentrations with bovine serum albumin (BSA) as the protein standard (Spector 1978).

Liver perfusion

Surgical techniques used to perform the hepatectomy and prepare the isolated liver for perfusion experiments were essentially the same as previously described (Bezek *et al.* 1990, Kukan *et al.* 1990). This procedure involved a slight modification of the method reported by Sies (1978). The perfusion medium consisted of a Krebs–Henseleit bicarbonate buffer (pH 7·4) fortified with glucose (10 mM final concentration). Single-pass experiments were performed for 60 min with specified concentrations of [^{14}C]-THA at a fixed perfusate flow rate of 20 ml/min/liver into the portal vein of the rat. One-hour perfusions were performed based on preliminary experiments in which steady-state THA clearance was achieved at approximately 45 min. The mean THA concentrations in hepatic outflow after 45, 50, 55 and 60-min perfusions was considered the steady-state drug outflow concentration (C_{out}). Bile samples were collected at these steady-state time points. After perfusion, 0·2-ml aliquots of perfusate and whole volumes of bile were counted for ^{14}C. Individual livers were blotted and weighed. For hplc-radioactivity profiling of perfusate, 1-ml aliquots were diluted with 2 ml absolute ethanol, concentrated under a nitrogen stream, and reconstituted in 0·2 ml 0·1 M ammonium acetate buffer (pH 4) prior to injection.

Analytical procedures

Aliquots of the post-reaction incubates and sample workup fractions were counted directly in 15 ml Ready Safe® by liquid scintillation counting (LSC) using external standardization for quench correction. Each vial was counted for 10 min or 2% sigma error, whichever occurred first.

Tacrine assay

Plasma aliquots (0·5 ml) and internal standard were basified (50 μl, 5 N sodium hydroxide) and extracted in *n*-propanol:chloroform (1:9; 4 ml). The resulting mixtures were shaken, centrifuged, and the organic phase removed and evaporated to dryness at 45°C under nitrogen. Residues were reconstituted in water (100 μl), vortexed, and aliquots (70 μl) injected on to the hplc system.

THA and internal standard were resolved using a Waters Nova-Pak® (Milford, MA, USA) C-18 column (5 μm, 3·9 id × 75 mm). The mobile phase consisted of 10% acetonitrile in buffer. The buffer consisted of 0·1 M sodium acetate with phosphoric acid (5·6 ml), TEA (5 ml), and glacial acetic acid (5 ml) added per litre. THA and internal standard were eluted at a flow rate of 0·5 ml/min with fluorescence detection (excitation, 240 nm; emission, 270 nm). Plasma THA concentrations were quantified by regressing peak-height ratios as a function of THA concentrations. A weighting factor of 1/concentration was used in the linear regression analysis.

Table 1. Kinetic parameters of THA metabolism in rat hepatocyte.

Experiment no.	V_{max} (nmol/min/10^6 cells)	K_m (μM)
1	1·22 ± 0·05	1·36 ± 0·11
2	1·16 ± 0·06	1·03 ± 0·22
3	0·55 ± 0·02	0·34 ± 0·07
4	1·18 ± 0·04	1·17 ± 0·13
5	1·03 ± 0·08	0·99 ± 0·28
Mean	1·03	0·98
SD	0·28	0·38

Various concentrations of THA were incubated for either 30 min (expt no. 1, 2 μM initial THA concentration) for 2 min (expt no. 2–5, 0·25–11 μM) with rat hepatocyte as described in Materials and methods. Values are mean ± SD of estimated parameters.

Metabolic profiling

Hplc radioactivity profiling was accomplished using a Waters μBondapak® (Milford, MA, USA) C-18 column (3·9 id × 300 mm) in series with an Upchurch Uptight® (Oak Harbor, WA, USA) precolumn. The mobile phase consisted of acetonitrile and 0·1 M ammonium acetate adjusted to pH 4·0 with glacial acetic acid (10:90 v/v). THA and its metabolites were eluted at a flow rate of 1·0 ml/min with UV detection at 325 nm prior to online radioactivity monitoring. The flow rate of scintillant (Flo-Scint III) was 3 ml/min.

Treatment of data

Apparent V_{max} and K_m describing THA metabolism in isolated hepatocytes were calculated using either the differential form of the Michaelis–Menten equation

$$-\frac{dC}{dt} = \frac{V_{max} \times C}{K_m + C} \tag{1}$$

or by fitting the metabolic rates of THA to the hyperbolic equation,

$$v = \frac{V_{max} \times C}{K_m + C}, \tag{2}$$

where C is substrate concentration, t is time, and v is velocity of metabolism. PCNONLIN (Statistical Consultants Inc 1986) was used in the estimation procedure. V_{max} and K_m were determined by fitting the data from experiment 1 to the differential form of Michaelis–Menten equation (equation 1) and in the case of experiments 2–5 to the hyperbolic function (equation 2) and are shown in table 1. Apparent B_{max} and K_b for irreversible binding were determined using an analogous expression to equation 2.

The hepatic extraction ratio at steady-state, ER_{SS}, for the single-pass experiments was taken as the difference between the input and output concentrations at steady state divided by the input concentration.

$$ER_{SS} = \frac{C_{in} - C_{out}}{C_{in}}. \tag{3}$$

For prediction of THA C_{out} in perfusion studies, equation 4 ('well-stirred' model) or equation 5 ('parallel-tube' model) were used (Pang and Rowland 1977).

$$v = Q \times (C_{in} - C_{out}) = \frac{V_{max} \times C_{out}}{K_m + C_{out}} \tag{4}$$

$$v = Q \times (C_{in} - C_{out}) = (Q \times K_m) \ln\left(\frac{C_{out}}{C_{in}}\right) + V_{max}, \tag{5}$$

where Q is the rate of perfusate flow through the liver in ml/min. V_{max} for whole liver was estimated by assuming 1 g liver contains $1·2 \times 10^7$ parenchymal cells (Zhalten and Stratman 1974). Equation 4 was solved as a simple quadratic equation. For equation 5 no explicit solution exists for $C_{in} > K_m$, therefore a second order implicit approximation of Runge–Kutta type was used for calculation of C_{out}.

Cl_i of THA was calculated by taking the estimated V_{max} for the entire liver and apparent K_m and multiplying by THA's free fraction (f_b) in blood. In rat, f_b of 0·53 was used in calculations (Parke-Davis, unpublished data).

$$Cl_i = \frac{V_{max}}{K_m} \times f_b. \tag{6}$$

The apparent oral clearance values (Cl_{oral}) for THA in rat was calculated according to

$$Cl_{oral} = \frac{Dose}{AUC_{(0-\infty)}}, \quad (7)$$

where AUC is area under the THA plasma concentration–time profile after oral administration of THA. For *in vitro/in vivo* correlations, Cl_{oral} was expressed in units of ml/min/g liver. A liver weight of 48 g/kg rat body weight (mean value observed in the present study) was utilized.

Results

Metabolic disposition of [^{14}C]-THA in rat hepatocyte

Metabolic profiling of 2-μM [^{14}C]-THA incubates with primary suspensions of rat hepatocyte showed extensive THA metabolism over a 30-min sampling period. THA was transformed to several polar metabolites including the alicyclic monohydroxy derivatives 1- (major), 2-, and 4-OH-THA (figure 2). The identities of these monohydroxylated metabolites was confirmed by chromatographic and mass spectral comparisons with synthetic reference agents (data not shown). At later times, additional polar metabolites were produced (data not shown). The relatively low amounts of these metabolites precluded accurate structural identification. In control incubations with boiled hepatocytes, no evidence for [^{14}C]-THA biotransformation was found (figure 2, upper). The time course for THA disappearance in this initial study is shown in figure 3. While THA displayed rapid removal from the incubate, a semilogarithmic plot of the data (figure 3, upper) suggests saturable elimination. Fitting of this time course data, assuming a Michaelis–Menten kinetic model (figure 3, lower) allowed an approximation of apparent V_{max} and K_m as $1{\cdot}22 \pm 0{\cdot}05$ nmol/min/10^6 cells and $1{\cdot}36 \pm 0{\cdot}11$ μM respectively.

Based on the non-linearity found in the above time-course study, subsequent determination of V_{max} and K_m were made by incubating [^{14}C]-THA (0·25–11 μM) in primary hepatocyte suspensions for 2-min time periods. Under these conditions only 1-, 2- and 4-OH-THA were observed (figure 2, middle), thus allowing the initial rate of substrate metabolism to be determined. A representative plot of the rates of THA metabolism in primary hepatocyte suspensions is depicted in figure 4. The rate of THA metabolism was saturable at substrate concentrations > 3 μM. V_{max} and K_m are summarized in table 1.

Although THA was well resolved from the alicyclic monohydroxy metabolites, this chromatographic method did not allow for complete base line separation of 1-, 2- and 4-OH-THA derivatives. Therefore, it was not possible to determine individual metabolite formation kinetics accurately.

Irreversible binding of [^{14}C]-THA-derived radioactivity

Incubations of [^{14}C]-THA with isolated rat hepatocytes for > 10 min resulted in measurable levels of irreversible (presumably covalent) binding of [^{14}C]-THA-derived radioactivity as determined by exhaustive solvent extraction. Following an initial lag period, a linear time course of [^{14}C]-THA-derived radioactivity binding was observed (figure 5). A representative plot of the rate of irreversible binding at various THA concentrations is shown in figure 6. Examination of this plot suggests that irreversible binding of THA also follows Michaelis–Menten-type kinetics. Mean apparent B_{max} and K_b parameters for THA-derived irreversible binding were 76·7 pmol/h/mg cell protein and 2·8 μM (table 2). No evidence of irreversible binding

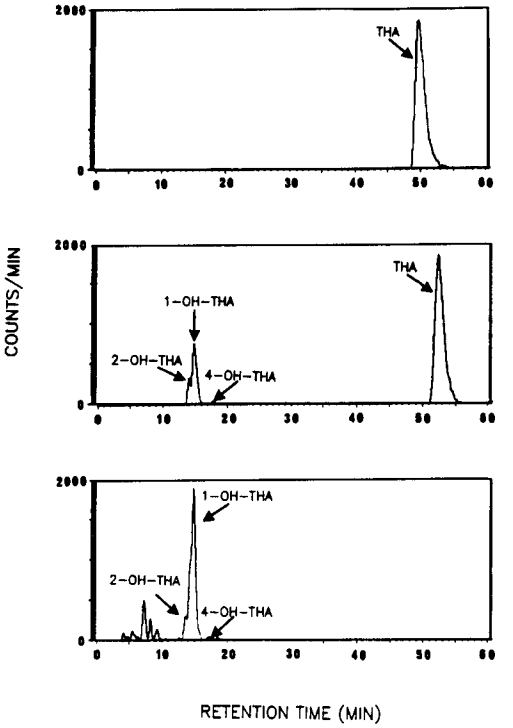

Figure 2. Hplc radioactivity profiles obtained after incubations of 2 μM [^{14}C]-THA with a suspension of primary rat hepatocyte. (upper) A 30-min incubate with boiled hepatocytes; (middle) a 2-min incubation; and (bottom) a 30-min incubation. Hplc radioactivity conditions are described in Materials and methods. Metabolite assignments were based on retention time comparisons with synthetic standards.

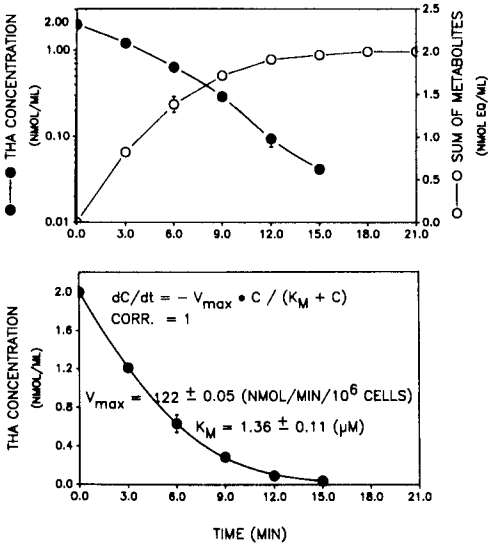

Figure 3. Time-course of [^{14}C]-THA metabolism in primary suspension of rat hepatocyte. (top) A semilogarithmic plot of the time course for 2 μM [^{14}C]-THA metabolism and total metabolite formation in a suspension of primary rat hepatocytes. The data represents the mean of triplicate incubations ± SD from a single hepatocyte isolation experiment. (bottom) The resultant plot from fitting of the data to the differential form of the Michaelis–Menten equation (equation 1; Materials and methods).

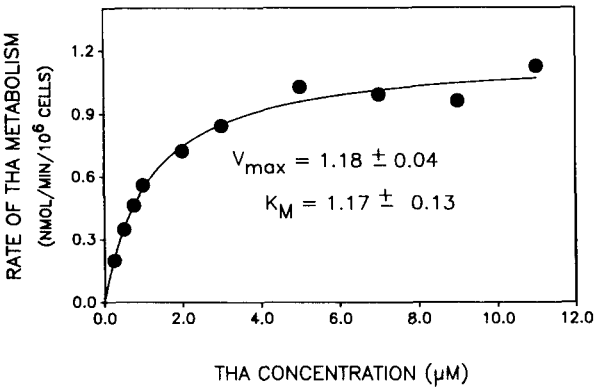

Figure 4. Rate of THA metabolism in a suspension of primary rat hepatocyte. Concentrations of [^{14}C]-THA ranged from 0·25 to 11 μM and incubations were conducted for 2-min time periods as described in Materials and methods. The rates of THA metabolism from a single hepatocyte experiment are shown.

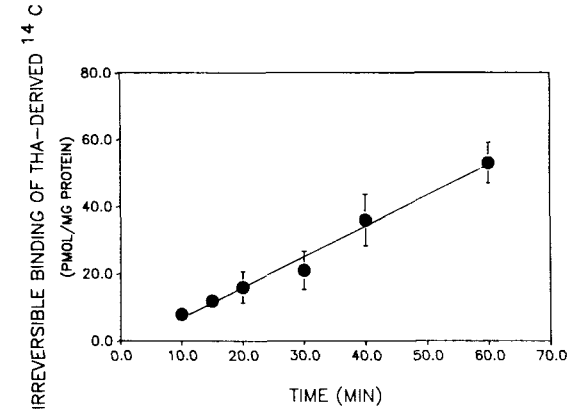

Figure 5. Irreversible binding time-course for [^{14}C]-THA-derived radioactivity in primary rat hepatocyte incubations. Initial THA concentration was 2 μM. Values represent means from triplicate incubates ± SD. Results are from a single hepatocyte isolation experiment.

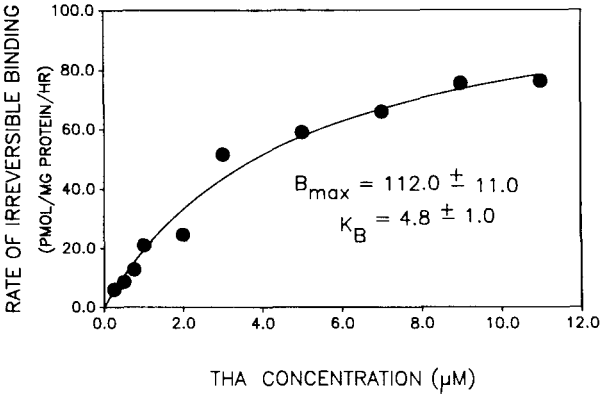

Figure 6. Rate of irreversible binding of [^{14}C]-THA-derived radioactivity in primary rat hepatocyte incubations. Concentrations ranged from 0·25 to 11·0 μM [^{14}C]-THA with incubations conducted for 40-min time periods. The rates of THA-derived radioactivity irreversible binding from a single hepatocyte isolation experiment are shown.

Table 2. Kinetic parameters of irreversible binding of [^{14}C]-THA-derived radioactivity to proteins of rat hepatocyte.

Experiment no.	B_{max} (nmol/h-/mg protein)	K_b (μM)
1	6·25 ± 8·8	2·8 ± 1·0
2	42·9 ± 4·0	1·5 ± 0·5
3	112·8 ± 10·8	4·8 ± 1·0
4	88·8 ± 9·5	2·2 ± 0·6
Mean	76·7	2·8
SD	30·5	1·4

Various concentrations of THA (0·24–11 μM) were incubated for 40 min as described in Materials and methods. Values are mean ± SD of estimated parameters.

Table 3. Effect of input concentrations of THA[a] on its hepatic extraction ratio at steady-state, ER_{SS}, in a single-pass perfused rat liver.

Experiment no.	C_{in} (μM)	ER_{SS} Measured[b]	ER_{SS} Predicted[c] WSM	ER_{SS} Predicted[c] PTM	Rate into bile[d] (%/min of input)
1	20·72	0·296[e] ± 0·030[f]	0·333	0·338	3·38 ± 0·39
	0·72	1·000 ± 0·000	0·874	0·999	3·83 ± 0·34
2	3·30	0·732 ± 0·034	0·841	0·991	3·77 ± 0·16
	14·05	0·300 ± 0·030	0·497	0·515	2·32 ± 0·40
3	0·85	1·000 ± 0·000	0·890	0·999	5·26 ± 1·70
	20·88	0·261 ± 0·038	0·387	0·393	3·55 ± 0·64

[a] Constant THA input concentrations, C_{in}'s, were delivered at constant hepatic perfusate flow (20 ml/min/liver) for 60 min to each liver preparation.

[b] Measured using THA output concentrations, C_{out}'s, at steady-state and calculated from

$$ER_{ss} = \frac{(C_{in} - C_{out})}{C_{in}}.$$

[c] Predicted from isolated hepatocytes assuming 'well-stirred' model (WSM) or 'parallel-tube' model (PTM) (for details see Materials and methods).

[d] Rate into bile represents excretion of metabolites of tacrine.

[e] Mean.

[f] SD of mean.

of [^{14}C]-THA-derived radioactivity could be detected in incubations with boiled hepatocyte preparations (data not shown).

THA metabolism in single-pass perfused rat liver

In an exploratory single-pass rat liver perfusion study, [^{14}C]-THA extraction, as determined by total ^{14}C output in perfusate and bile, reached steady-state after a 45-min perfusion period. Because of the length of time to achieve steady-state, it was only possible to examine two THA input concentrations ($C_{in} < K_m$ and $C_{in} \gg K_m$) in each individual liver preparation. The hplc profile of the perfusate leaving the liver was similar to that observed in the 2-min hepatocyte incubations described above (figure 2). Although polar metabolites were present, 1-, 2- and 4-OH-THA accounted for > 90% of the metabolite radioactivity (data not shown).

Measured values for the ER_{SS} of [^{14}C]-THA, as well as predicted values using the well-stirred (WSM) or parallel-tube (PTM) models, are shown in table 3.

Whereas at low input concentrations the PTM gave results slightly closer to measured, the WSM was better at higher input concentrations. Therefore, both models appear to predict the experimentally determined ER_{SS} under the conditions studied. Experimentally, it was observed that the order of the study, namely, the initial $C_{in} < K_m$ as in perfusion experiment 1, or $C_{in} > K_m$ as in experiment 3, had no effect on the ER_{SS} of [^{14}C]-THA. For THA $C_{in}s \gg K_m$, ER_{SS} was markedly < 1. Predicted ER_{SS} based on kinetic data from hepatocyte studies and those measured in single-pass perfused liver were in close agreement.

Rate of biliary excretion of THA-derived radioactivity was low and accounted for 2·3–5·3%/min of the [^{14}C]-THA liver input rate.

Intrinsic clearance

The Cl_i for THA was 6·7 ml/min/g liver following substitution of mean V_{max} (estimated for total liver) and K_m into equation 6 (see Materials and methods).

Discussion

Metabolism of THA in rat hepatocytes

Incubations of [^{14}C]-THA with primary suspensions of rat hepatocytes for 2-min time periods resulted in formation of 1-, 2- and 4-OH-THA metabolites, with 1-OH-THA as the major metabolite. Incubations of [^{14}C]-THA with rat hepatocytes for longer time periods resulted in the formation of several unidentified polar metabolites, although in relatively minor amounts compared with the 1-, 2- and 4-OH-THA metabolites. Prior *in vitro* metabolism studies using rat liver microsomal preparations also found 1-(major), 2- and 4-OH-THA to be the principal stable metabolites formed (Madden *et al.* 1993, Woolf *et al.* 1993). During *in vivo* THA studies in rat, these same monohydroxylated alicyclic metabolites were identified as urinary excretion products with 1-OH-THA as a major metabolite (Hsu *et al.* 1990, Pool *et al.* 1992c). Therefore, THA metabolism by isolated rat hepatocytes produces a metabolic profile similar to that observed *in vivo* in rat.

Observation of non-linearity in THA clearance at a concentration of 2 μM in rat hepatocytes is suggestive for involvement of a '*high affinity–low capacity*' metabolic clearance process. Kinetic analysis of these data along with a concentration ranging study resulted in an estimated K_m slightly < 1 μM and a corresponding V_{max} of 12·4 nmol/min/g liver.

Irreversible binding of [^{14}C]-THA-derived radioactivity

Incubation of [^{14}C]-THA with rat hepatocytes resulted in THA-derived metabolites binding irreversibly to intracellular proteins. Binding required viable cells as no binding occurred in boiled hepatocyte preparations.

The process of irreversible binding of THA-derived radioactivity was shown to follow Michaelis–Menten kinetics and to be saturable. Saturation of binding occurred over the same substrate concentration range found to saturate THA metabolism. The low μM value for K_b and the relatively low rate for B_{max} is also consistent with a high affinity–low capacity metabolic activation process.

THA elimination in single-pass perfused rat liver

Biliary excretion of THA metabolites as well as a high partition coefficient of THA between perfusate and liver are possible explanations for the relatively long perfusion time (45 min) required to achieve steady-state in the single-pass perfused

Table 4. Individual and mean plasma THA pharmacokinetic parameters in rat following a single oral dose (8 mg/kg).

Rat	C_{max}	T_{max}	$t_{\frac{1}{2}}$	λz	$AUC(0-\infty)$
1	209	1·0	2·4	0·294	448
2	199	0·5	3·6	0·190	380
3	118	1·0	1·4	0·500	346
4	238	1·0	1·5	0·466	520
Mean	191	0·9	2·2	0·363	424
SD	51·4	0·3	1·0	0·146	77·1

C_{max} is maximum plasma concentration (ng/ml).
T_{max} is time of C_{max} (h).
$t_{\frac{1}{2}}$ is terminal phase elimination half-life (h).
λz is apparent terminal phase elimination-rate constant (h^{-1}).
$AUC(0-\infty)$ is area under the concentration versus time curve from time zero to infinity (ng·h/ml).

liver (unpublished data). The metabolite profile in perfusate was similar to that produced in hepatocyte incubations with approximately 90% of the metabolized THA being accounted for as 1- (major), 2- and 4-OH-THA.

The calculated V_{max}/K_m THA intrinsic clearance predicted that in single-pass liver perfusions THA clearance would be essentially complete under conditions in which $C_{in} < K_m$. Under perfusion conditions in which THA input is $> K_m$ ($C_{in} \gg K_m$), the predicted ER_{SS} should be markedly lower reflecting saturation of THA metabolism. The results in table 3 are in agreement with the predicted ER_{SS}.

In vitro/in vivo *clearance comparisons*

The ratio of V_{max} to K_m multiplied by free fraction of drug in blood allows calculation of a Cl_i for hepatic THA metabolism. Calculated Cl_{oral} normalized per g liver should be comparable with Cl_i when substrate clearance is governed by hepatic metabolism, and oral absorption is not an issue (Wilkinson and Shand 1975). Cl_i calculated from rat hepatocyte data was 6·7 ml/min/g liver, which is in agreement with Cl_{oral} of 6·6 ml/min/g liver found for THA *in vivo* in rat (table 4). Therefore, hepatic metabolism appears to be the primary site for oral THA clearance in rat.

In conclusion, the major route of THA metabolic elimination in isolated rat hepatocytes and perfused liver is hydroxylation. The metabolism and bioactivation of THA in rat appears to be mediated by a high affinity–low capacity process. Cl_i calculated from rat hepatocyte experiments was in close agreement to the estimated Cl_{oral} found *in vivo* rat. This agreement in clearance values indicates the utility of rat hepatocyte and perfusion models in assessing factors that may influence THA metabolic clearance in intact rat.

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References

Bezek, Š., Kukan, M., Kállay, Z., Trnovec, T., Štefek, M., and Piotrovskiy, L. B., 1990, Disposition of ethimizol, a xanthine-related nootropic drug, in perfused rat liver and isolated hepatocytes. *Drug Metabolism and Disposition*, **18**, 88–95.

Farlow, M., Gracon, S. I., Hershey, L. A., Lewis, K. W., Sadowsky, C. H., and Eolan-Ureno, J., 1992, A controlled trial of tacrine in Alzheimer's disease. *Journal of the American Medical Association*, **268**, 2523–2529.

HARTVIG, P., ASKMARK, H., AQUILONIUS, S. M., WIKLUND, L., and LINDSTROM, B., 1990, Clinical pharmacokinetics of intravenous and oral 9-amino-1,2,3,4-tetrahydroacridine, tactrine. *European Journal of Clinical Pharmacology*, **38,** 259–263.

HORVATH, T., 1989, Drug metabolism in drug-induced liver diseases: pathogenetic role of active metabolites. *Acta Physiologica Hungarica*, **73,** 293–304.

HSU, R. S., SHUTSKE, G. M., DILEO, E. M., CHESSON, S. M., LINVILLE, A. R., and ALLEN, R. C., 1990, Identification of the urinary metabolites of tacrine in the rat. *Drug Metabolism and Disposition*, **18,** 779–783.

KNAPP, M. J., KNOPMAN, D. S., SOLOMON, P. R.,. PENDLEBURY, W. W., DAVIS, C. S., and GRACON, S. I., 1994, A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. *Journal of the American Medical Association*, **271,** 985–991.

KUKAN, M., BEZEK, Š., ŠTEFEK, M., TRNOVEC, T., ĎURIŠOVÁ, M., and PIOTROVSKIY, L. B., 1990, The effect of enzyme induction and inhibition on the disposition of the xanthine-related nootropic drug ethimizol in perfused liver and hepatocytes of rats. *Drug Metabolism and Disposition*, **18,** 96–101.

KUKAN, M., BEZEK, Š., POOL, W. F., and WOOLF, T. F., 1992, *In vitro/in vivo* correlations in tactrine metabolism and its stimulation by 3-methylcholanthrene. Presented at the Fourth European ISSX Meeting, 3–6 July, Bologna, Italy (Bethesda, MD: ISSX).

MADDEN, S., POOL, W. F., WOOLF, and PARK, B. K., 1993, An investigation into the formation of stable, protein-reactive and cytotoxic metabolites from tacrine *in vitro*: studies with human and rat liver microsomes. *Biochemical Pharmacology*, **46,** 13–20.

NEUBERGER, J., and WILLIAMS, R., 1989, Immune mechanism in tienilic acid associated hepatotoxicity. *Gut*, **30,** 515–519.

O'BRIEN, J. T., EAGGER, S., and LEVY, R., 1991, Effects of tetrahydroaminoacridine on liver function in patients with Alzheimer's disease. *Age and Ageing*, **20,** 129–131.

PANG, K. S., and ROWLAND, M., 1977, Hepatic clearance of drugs. I. Theoretical considerations of a 'well-stirred' model and a 'parallel tube' model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *Journal of Pharmacokinetics and Biopharmaceutics*, **5,** 625–653.

PESSAYRE, D., 1993, Cytochromes P450 and formation of reactive metabolites. Role in drug-induced hepatotoxicity. *Therapie*, **48,** 537–548.

POOL, W. F., BJORGE, S. M., WINDSOR, B., BLACK, A., CHANG, T., and WOOLF, T. F., 1991, Comparison of brain to plasma metabolic distributions of tacrine and 1-hydroxytacrine in rat. Presented at the Society for Neuroscience 21st Annual Meeting, November 10–15, New Orleans, Louisiana (Bethesda, MD: ISSX).

POOL, W. F., BJORGE, S. M., REILY, M. D., CHANG, T., and WOOLF, T. F., 1992a, Metabolic disposition of the cognition activator tacrine in dog: identification of dihydroxy metabolites including a dihydrodiol. Presented at the Fourth North American ISSX Meeting, 2–6 November, Bal Harbour, Florida (Bethesda, MD: ISSX).

POOL, W. F., BJORGE, S. M., CHANG, T., and WOOLF, T. F., 1992b, Metabolic disposition of the cognition activator tacrine in man: identification of phenol glucuronide metabolites in urine. Presented at the Fourth North American ISSX Meeting, 2–6 November, Bal Harbour, Florida (Bethesda, MD: ISSX).

POOL, W. F., BJORGE, S. M., CHANG, T., and WOOLF, T. F., 1992c, Effect of 3-methyl cholanthrene induction of the disposition of the cognition activator tacrine in rat. Presented at the Fourth North American ISSX Meeting, 2–6 November, Bal Harbour, Florida (Bethesda, MD: ISSX).

SEGLEN, P. O., 1976, Preparation of isolated rat liver cells. *Methods in Cell Biology*, **13,** 29–83.

SIES, H., 1978, The use of perfusion of liver and other organs for the study of microsomal electron-transport and cytochrome P-450 systems. *Methods in Enzymology*, **52,** 48–59.

SPALDIN, V., MADDEN, S., POOL, W. F., WOOLF, T. F., and PARK, B. K., 1994, The effect of enzyme inhibition on the metabolic activation of tacrine by human liver microsomes. *British Journal of Clinical Pharmacology*, **38,** 15–22.

SPECTOR, T., 1978, Refinement of the coomassie blue method of protein quantitation. *Analytical Biochemistry*, **86,** 142–146.

WATKINS, P. B., ZIMMERMAN, H. J., KNAPP, M. J., GRACON, S. I., and LEWIS, K. W., 1994, Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *Journal of the American Medical Association*, **271,** 992–998.

WILKINSON, G. R., and SHAND, D. G., 1975, A physiological approach to hepatic drug clearance. *Clinical Pharmacology and Therapeutics*, **18,**, 377–390.

WOOLF, T. F., POOL, W. F., BJORGE, S. M., CHANG, T., GOEL, O. P., PURCHASE, C. F., SCHROEDER, M. C., KUNZE, K. L., and TRAGER, W. F., 1993, Bioactivation and irreversible binding of the cognition activator tacrine using human and rat liver microsomal preparations: species differences. *Drug Metabolism and Disposition*, **21,** 874–882.

ZHALTEN, R. N., and STRATMAN, F. W., 1974, The isolation of hormone-sensitive rat hepatocytes by a modified enzymatic technique. *Archives of Biochemistry and Biophysiology*, **163,** 600–608.