reported by others [8], indicate that the total recovery of SDD and its acetylated metabolite (NASDD) in the rat is too low for this compound to be used as a model substrate for acetylation *in vivo*. Furthermore, although Nwankwo *et al.* reported that chloroquine reduces the deacetylation of NASDD *in vivo*, we were unable to detect any deacetylated compound after the administration of NASDD [7].

In summary, chloroquine caused only slight reductions in NAT activity when added *in vitro*, and had no detectable influence when animals were pretreated with it for 4 days. This would suggest that the previously reported reduced excretion of acetylated metabolites of INH and SDD following chloroquine pretreatment is not the result of inhibition of NAT. In contrast, we found that primaquine significantly ($P < 0.05$) reduced NAT activity when added *in vitro*, suggesting the need for further study with this agent.

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Comparison of the chromatographic characteristics of metabolites of tacrine hydrochloride in human serum and urine with those of *in vitro* metabolic products from hepatic microsomes

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Tacrine hydrochloride (THA) is a centrally acting cholinesterase inhibitor under investigation as a treatment for Alzheimer's disease [1–3]. It is unclear whether the adverse effects reported [4–6] are attributable to the parent compound or to a metabolite. Further development of this line of agents may depend upon an understanding of their metabolism and the structure and pharmacology of their metabolites. Kaul [7] found four metabolites of THA in rat urine using paper chromatography. The ultraviolet spectra of two resembled THA, one of which in the infrared spectrum revealed a cyclic carbonyl group. He suggested the formation of this C=O function could be through oxidative deamination which in biological systems would involve pyridoxal as the co-factor.

Metabolic products of THA have been observed in human and animal studies [5, 8, 9] using HPLC with ultraviolet or fluorescence detection. Their serum concentrations in patients receiving THA have been documented by this technique [10, 11]. Hsu *et al.* [12] have measured three metabolites in rat plasma which they postulate to be hydroxylated products.

Summers *et al.* [5] described a rapid conversion *in vivo* of THA to an apparent 1-hydroxy metabolite in rat, monkey and man. Hendrickson *et al.* [9] incubated rat hepatic microsomes with THA and added NADPH. One of the metabolites produced was identified tentatively as 9-hydroxylamine 1,2,3,4-tetrahydroacridine by *in situ* electrochemical characterization.

We describe here the metabolites found in human serum and urine and our attempt to produce corresponding metabolites *in vitro* from rat hepatic microsomes.

Materials and Methods

A Shimadzu liquid chromatography pump and RF535 fluorescence HPLC monitor (Dyson Instruments, Hetton, U.K.) and a Pye-Unicam CDP4 computing integrator (Philips Scientific, Cambridge, U.K.) were used with a 250 × 4.6 mm i.d. Shandon Hypersil 5ODS column (HPLC Technology, Macclesfield, U.K.) fitted with a Rheodyne 7125 valve (Dyson Instruments) and 100 μL loop. The excitation and emission wavelengths were 330 and 365 nm. The mobile phase was methanol–distilled water

(24.75:74.25; v/v) with 1% (v/v) triethylamine adjusted to pH 8.5 with AnalR orthophosphoric acid (15.9 M) at a flow rate of 1.5 mL/min.

An MSE homogenizer, MSE Mistral, HS18 centrifuges and a 55 m ultracentrifuge (Fisons Instruments, Loughborough, U.K.) were used in microsomal preparations.

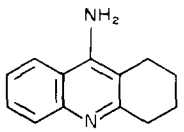
THA (9-amino-1,2,3,4-tetrahydroacridine) and acridanone (1,2,3,4-tetrahydro-9-acridanone) were from the Aldrich Chemical Co. (Gillingham, U.K.). Authenticated 1-hydroxy-THA was from the Astra Neuroscience Research Unit (London) (see Fig. 1). Triethylamine, Tris–base, NADPH and pyridoxal-5-phosphate were from the Sigma Chemical Co. (Poole, U.K.). HiPerSolv methanol, HiPerSolv chloroform, AnalR sodium sulphate and AnalR orthophosphoric acid were from B.D.H. (Poole, U.K.).

Patients' samples. Serum samples were collected from six patients with Alzheimer's disease taking part in a double blind trial of THA against placebo. Six hour urine collections were made before each treatment period and following administration of the trial therapies. The serum and urine were stored at −20° prior to analysis.

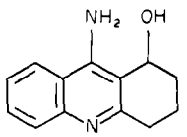
Serum samples from people not taking THA were spiked with authenticated 1-hydroxy-THA, acridanone and THA for direct comparison of chromatographic retention times.

Microsomal incubations. Two untreated male Sprague–Dawley rats were killed by CO_2 suffocation. The livers were removed immediately, weighed and immersed in 1.15% KCl solution at 4°. Each was homogenized on ice with 2 volumes 0.1 M KCl–phosphate buffer (pH 7.4). The microsomes were then isolated by differential centrifugation at 10,000 *g* which removed nuclei, mitochondria, lysosomes and cell debris. The supernatant was divided into eight 2 mL samples. These were centrifuged further at 100,000 *g* for 90 min at 4°. The pellets rich in microsomes were separately resuspended in 2 mL of 0.1 KCl–phosphate buffer (pH 7.4) and kept on ice prior to incubation.

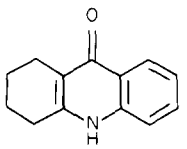
Solutions of 100 μg/mL THA in 0.1 M KCl–phosphate buffer (pH 7.4), 25 mg NADPH (co-factor) in 2 mL 150 mM $MgCl_2$ buffer, and 100 mg pyridoxal-5-phosphate (co-factor) in 10 mL 150 mM $MgCl_2$ buffer were prepared.



THA (9-amino-1,2,3,4,-tetrahydroacridine)



1-Hydroxy-THA



1,2,3,4,-Tetrahydro-9-acridanone

Fig. 1. Chemical structures.

The THA and the co-factors were added to each of the eight samples of resuspended microsomes to initiate the reaction as follows:

300 μL phosphate buffer only (blank);

200 μL phosphate buffer + 100 μL NADPH co-factor solution (control);

200 μL phosphate buffer + 100 μL pyridoxal co-factor solution (control);

100 μL phosphate buffer + 100 μL NADPH co-factor solution + 100 μL pyridoxal co-factor solution (control);

200 μL phosphate buffer + 100 μL THA solution;

100 μL phosphate buffer + 100 μL NADPH co-factor solution + 100 μl THA solution;

100 μL phosphate buffer + 100 μL pyridoxal co-factor solution + 100 μL THA solution;

100 μL NADPH co-factor solution + 100 μL pyridoxal co-factor solution + 100 μL THA solution.

Incubations were then carried out at 37° for 1 hr. Incubated microsomal mixture (100 μL) was removed for extraction and the remaining suspension immediately frozen at −20°.

Extraction procedure. The incubated microsomes (100 μL) were reconstituted to 1 mL with 0.1 M KCl–PO_4 buffer and extracted using the method of Forsyth *et al.* [10]. Urine samples taken from patients both before and after THA administration were diluted 1/20 with distilled water and 1 mL was extracted from each sample. Serum samples from patients on both placebo and active THA treatment were extracted in 1 mL aliquots.

Results

The retention times of authenticated 1-hydroxy-THA, THA and acridanone were 7.8, 23.7 and 32.5 min, respectively. Typical chromatograms of serum and urine from one patient taking either placebo or THA together with those of rat microsomes incubated with THA and NADPH are shown in Fig. 2. There were five peaks (retention times—5.4, 6.4, 7.0, 7.8 and 8.2 min) plus that for THA in patients on treatment and none whilst on placebo. In urine, the first four peaks corresponded to those in serum but the compound with a retention time of 8.2 min was not separable from the adjacent peak. All the peak areas, except that attributable to THA, were much greater in urine than in serum. It was noted that one of the peaks corresponded to that of 1-hydroxy-THA whereas none corresponded to acridanone. Following microsomal incubation with NADPH co-factor, four chromatographic peaks were detected but were not evident in the corresponding control samples and were barely visible in the sample incubated with THA only. The first peak in the human samples was not detected in the microsomal preparations but all the subsequent peaks corresponded by retention time. The addition of pyridoxal-5-phosphate alone did not result in the appearance of any chromatographic peaks and when combined with NADPH did not affect the chromatographic profile.

Discussion

We conclude that the five chromatographic peaks found in human serum and the four in human urine represent metabolites of THA, as none were present in control samples. The fourth peak is identified as 1-hydroxy-THA by retention time. The peak area ratio to parent THA suggests that this is the major metabolite and accounts for the unknown metabolic products described previously [11].

The corresponding four peaks after rat microsome incubation are likely to be due to the same metabolic products. The major metabolite produced was not 1-hydroxy-THA in accordance with the findings of Hsu *et al.* [12]. They found the concentration of 1-hydroxy-THA in rat plasma to be intermediate in position amongst the metabolic products and suggested that three metabolites were hydroxylated products by comparing retention times

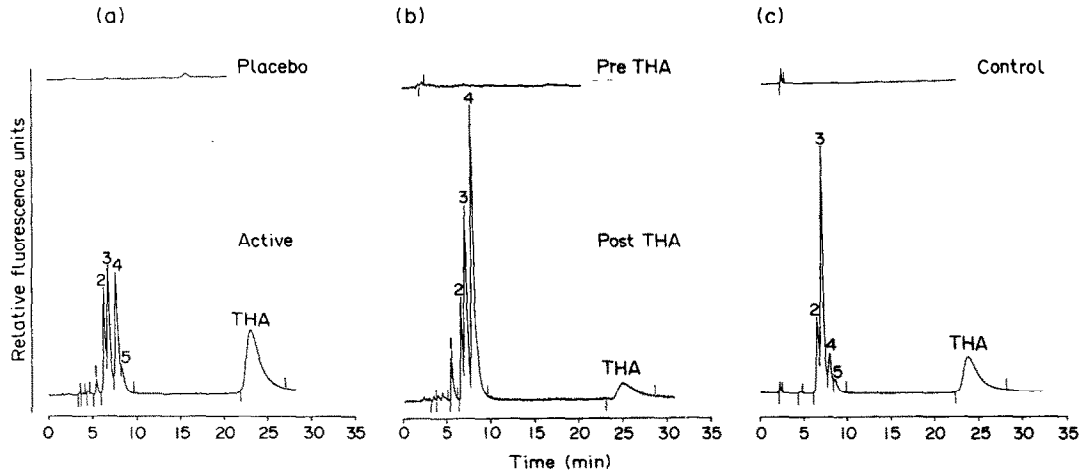


Fig. 2. (a) Chromatograms from serum of a patient taking placebo and THA. (b) Chromatograms from urine of same patient before and after THA. (c) Chromatograms of isolated hepatic microsomes and control incubated with THA and NADPH.

Table 1. Characteristics of chromatographic peaks obtained following administration of THA in humans and after incubation of isolated rat hepatic microsomes with THA and added NADPH

		Peak area ratio to THA		
		Human		Isolated microsomes
Peak	Retention time (min)	Serum	Urine	NADPH
1	5.4	0.04	1.46	—
2	6.4	0.31	2.13	0.36
3	7.0	0.43	5.12	1.33
4	7.8	0.46	9.2	0.24
5	8.2	0.1	—	0.10

to those of synthesized compounds. The close concordance between the chromatograms obtained from rat microsomes and human urine suggests that these metabolites are all products of Phase I metabolism.

Hendrickson *et al.* [9] suggested that one of the metabolites is 9-hydroxylamine-1,2,3,4-tetrahydroacridine. This cannot be verified without an authentic standard compound. Kaul [7] suggested the presence of a cyclic carbonyl group which may be due to oxidative de-amination involving pyridoxal as a co-factor. However, oxidative de-amination of THA would produce an acridanone. The absence of a peak corresponding to authentic 1,2,3,4-tetrahydro-9-acridanone, even with the addition of pyridoxal-5-phosphate as co-factor, lends no support to this theory.

In summary, there may be as many as five metabolites of THA in man, four corresponding to products in the rat. The study provides some evidence that one of major metabolites is 1-hydroxy-THA but lends no support to the putative oxidative deamination pathway.

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Inhibitory potencies of fish oil hydroxy fatty acids on cellular lipoxygenases and platelet aggregation

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Recent epidemiological studies suggest that the low incidence of thrombosis in Greenland Eskimos may be due to the large amounts of two polyunsaturated fatty acids (PUFAs)* in their diet, i.e. eicosapentaenoic acid [EPA, 20:5 (n-3)] and docosahexaenoic acid [DHA, 22:6 (n-3)] [1]. Most mammalian cells can lipoxygenate these PUFAs and some of the biological effects of these PUFAs are probably due to the effects of their metabolites. Platelets have been reported to convert EPA and DHA to 12-hydroxyeicosapentaenoic acid (12-HEPE) and 14-hydroxydocosahexaenoic acid (14-HDHE), respectively, whereas leukocytic 5- and 15-lipoxygenases (LOs) produce 5-HEPE and 15-HEPE and 7-HDHE and 17-HDHE, respectively [2–5]. Previous studies have shown that different hydroxyeicosatetraenoic acids (HETEs), derived from arachidonic acid (AA), can modulate cellular LOs and certain platelet functions [6, 7]. The present study examined the relative potencies of isomeric HEPEs and HDHEs on (a) exogenous AA metabolism in human and rat platelets and in human polymorphonuclear (PMN) leukocytes, and (b) aggregation of platelets from these species.

Materials and Methods

5(*S*)-HEPE, 12(*S*)-HEPE and 20:5 (n-3) were purchased from BioMol Research Laboratories, Inc. (Plymouth Meeting, PA). 14(*S*)-HDHE, 15(*S*)-HETE, 15(*S*)-HEPE, and 17(*S*)-HDHE were prepared as previously described [3, 8–10]. 22:6 (n-3) was obtained from Nuchek Prep, Inc. (Elysian, MN) and collagen from the Chrono-Log Corp. (Havertown, PA). Young adult male Wistar rats, approximately 300 g, were fed diets for 7 days. The corn oil (CO) diet contained 5% (w/w) CO plus basal diet (all other necessary nutrients; Teklad, Madison, WI), whereas the menhaden oil (MO)-supplemented diet contained 4% MO (Zapata-Haynie Corp., Reedville, VA) plus 1% CO by weight [11]. Human PMN leukocytes and platelets and rat platelets were isolated as previously described [6, 12, 13].

The effects of hydroxy fatty acids (HOFAs) on [^{14}C]AA metabolism in PMN leukocytes (2×10^7/mL) and platelets (5×10^7/mL) were determined by previously described methodologies [6, 13]. Aggregation studies were performed with gel-filtered platelets that had been recalcified with 1 mM $CaCl_2$ following centrifugation [12].

Results and Discussion

Human platelets (5×10^7/mL) metabolized exogenously added [^{14}C]AA (16 μM) to [^{14}C]12-HETE (29 ± 3.8%, N = 10), [^{14}C]12-hydroxyheptadecatrienoic acid (6.5 ± 0.63%, N = 9), and [^{14}C]thromboxane B_2 ([^{14}C]TXB_2) (2.6 ± 0.32%, N = 9) upon product separation and analysis by TLC. The identity of these metabolites was confirmed by comparison of HPLC retention times of authentic standards. When platelets were pretreated with various HEPEs, HDHEs or 15-HETE followed by the addition of [^{14}C]AA substrate, several of these HOFAs inhibited the platelet 12-LO as shown by decreased formation of [^{14}C]-12-HETE (Table 1). 15-HEPE was the most effective inhibitory HOFA tested, followed by 15-HETE and 17-HDHE, whereas the HOFAs produced by the 5- and 12-LOs did not appreciably inhibit the human platelet 12-LO at the concentrations tested (up to 50 μM). The relative inhibitory potencies of 15-HEPE and 17-HDHE contrast with the results reported by Mitchell *et al.* [9]. One possible explanation is that intact platelets, rather than a 105,000 *g* platelet supernatant, were used in the present studies. 17-HDHE, 15-HEPE and 15-HETE also inhibited the platelet cyclooxygenase pathway (measured as [^{14}C]TXB_2 formation) but this pathway was 3- to 10-fold less sensitive to the inhibitory effects of these acids than the 12-LO pathway. When the effects of these fatty acids were tested on the human PMN 5-LO, it was found that this enzyme was also less sensitive to inhibition than the 12-lipoyxgenase and that the order of inhibitory potencies was 15-HETE > 17-HDHE > 15-HEPE. Since the 5-LO pathway leads to leukotriene production [14], these findings indicate that fish oil HOFAs are less potent inhibitors of leukotriene production than the corresponding HOFA derived from

* Abbreviations: AA, arachidonic acid; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HOFA, hydroxy fatty acid; RP-HPLC, reverse-phase high pressure liquid chromatography; LO, lipoxygenase; MO, menhaden oil; PGE_2, prostaglandin E_2; PMN, polymorphonuclear; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; and TXB_2, thromboxane B_2.