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HYDROXYLATION AND FORMATION OF ELECTROPHILIC METABOLITES OF TIENILIC ACID AND ITS ISOMER BY HUMAN LIVER MICROSOMES

CATALYSIS BY A CYTOCHROME P450 IIC DIFFERENT FROM THAT RESPONSIBLE FOR MEPHENYTOIN HYDROXYLATION

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Abstract—Tienilic acid (TA) is metabolized by human liver microsomes in the presence of NADPH with the major formation of 5-hydroxytienilic acid (5-OHTA) which is derived from the hydroxylation of the thiophene ring of TA. Besides this hydroxylation, TA is oxidized into reactive metabolites which covalently bind to microsomal proteins. Oxidation of an isomer of tienilic acid (TAI), bearing the aroyl substituent on position 3 (instead of 2) of the thiophene ring, by human liver microsomes, gives a much higher level of covalent binding to proteins. Both covalent binding of TA and TAI metabolites are almost completely suppressed in the presence of glutathione. These three activities of human liver microsomes (TA 5-hydroxylation, covalent binding of TA and TAI metabolites) seem dependent on the same cytochrome P450 of the IIC subfamily, since (i) antibodies against human liver cytochromes P450 IIC strongly inhibit these three activities, (ii) there is a clear correlation between these activities in various human liver microsomes, and (iii) TA acts as a competitive inhibitor for TAI activation into electrophilic metabolites ($K_i \cong 25\ \mu M$) and TAI inhibits TA 5-hydroxylation. However cross inhibition experiments indicate that tienilic acid hydroxylation and mephenytoin hydroxylation, a typical reaction of some human liver P450 IIC isoenzymes, are not catalysed by the same member of the P450 IIC subfamily.

Although very much is known about the metabolic activation of benzene-containing compounds as well as on the toxicological consequences of this activation, very few data are presently available about the oxidative metabolism of thiophene compounds and about the toxicological consequences of this metabolism.

Thiophene itself was found to be metabolized in rabbits into a mercapturic acid arising presumably from a possible thiophene-2,3-oxide [1]. Moreover, recently, several drugs containing a thiophene ring were reported to be hydroxylated at position 5 of the thiophene ring. This is the case for tienilic acid [2] and suprofen [3] in rat and man, morantel in cattle [4] and tenoxicam in rat [5].

Tienilic acid (TA), an uricosuric diuretic drug was found devoid of any direct hepatotoxic effect but led to rare cases (1 for 10,000 patients) of hepatitis of the immunoallergic type [6]. In some patients having taken tienilic acid and suffering from hepatitis, circulating anti-organelle autoantibodies have been detected. They are directed against a component of human liver endoplasmic reticulum and have been called anti-LKM_2 antibodies [6]. It has been shown [7] that the anti-LKM_2 antibodies only recognized one protein from human liver microsomes, a cytochrome P450 of the P450 IIC subfamily [8] originally called P450-8. Interestingly, this cytochrome is mainly responsible for the 5-hydroxylation of TA by human liver microsomes [7], which is the major oxidative pathway implicated in the metabolism of TA *in vivo* in man [2].

More recently, a study of the metabolic activation of TA and of its isomer bearing the arylketo group on position 3 of the thiophene ring, TAI, by rat liver microsomes showed that TA was hydroxylated at position 5 of the thiophene ring and that electrophilic metabolites (or intermediates) able to bind covalently to microsomal proteins were formed upon microsomal oxidation of TA and TAI (Fig. 1) [9]. Although 5-hydroxylation of TA was the major microsomal reaction observed with TA, covalent binding of reactive metabolites to proteins was the major pathway observed with TAI and rat liver microsomes [9].

This paper describes the metabolic activation of TA and TAI by human liver microsomes and provides first results leading to a characterization of the major human cytochrome(s) P450 implicated in these reactions.

MATERIALS AND METHODS

Chemicals. All the chemicals were of the highest quality commercially available. Tienilic acid,

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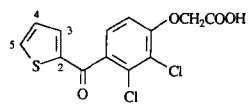
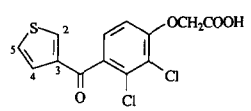
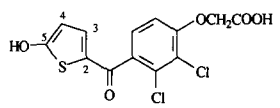


Tienilic Acid (TA)



Tienilic Acid Isomer (TAI)



5-Hydroxy-tienilic Acid (5-OHTA)

Fig. 1. Formula of TA, TAI and 5-OHTA.

[^{14}C]tienilic acid, 5-hydroxy-tienilic acid, the isomer of tienilic acid (TAI) and [^{14}C]TAI were given to us by laboratories Anphar-Rolland (Chilly-Mazarin, France). 5-hydroxy-tienilic acid was prepared as described [2] and [^{14}C]TA and [^{14}C]TAI (label on the keto group, 25 Ci per mol) were respectively prepared by CEA Saclay (France) [10] and by Amersham (Bucks, U.K.). HPLC analysis of [^{14}C]TA and [^{14}C]TAI showed a radiochemical purity higher than 98%. (±) Mephenytoïn was a gift of U.A. Meyer (Basel, Switzerland).

Human liver microsomes. Human livers were obtained from renal transplant donors after accidental death. Documentation of medical history and medication were unavailable. Livers H97 and F503 were from female donors and livers F504, F510 and SP1 from male donors. Liver KDL was from a poor metabolizer for mephenytoïn [11] and was kindly provided by U.A. Meyer (Basel, Switzerland). Human liver microsomes were prepared as previously described [12]. Their cytochrome *b*5 and P450 contents were determined by the method of Omura and Sato [13] and their protein concentration was measured by the method of Lowry *et al.* [14].

Human sera. Eleven serum samples containing anti-LKM$_2$ antibodies were obtained from patients suffering from hepatitis and having been treated with tienilic acid. They were given to us by Prof. J. C. Homberg (CHU Saint-Antoine, Paris). Their anti-LKM$_2$ titers by immunofluorescence [6] varied from 1:100 to 1:10,000 [7]. The titer of patient JOR was 1:500.

Sixteen control serum samples that did not contain anti-LKM$_2$ antibodies were obtained from blood donors or patients (see also Ref. 7). Eleven of them were from individuals who had never taken tienilic acid. Five of the control sera were from patients treated with tienilic acid for more than one year but not suffering from hepatitis [7].

Measurement of TA 5-hydroxylation using a UV-visible method. Kinetics of TA 5-hydroxylation was followed at 390 nm by a previously described procedure [15] using human liver microsomes (0.4–1 μM cytochrome P450) and 100 μM TA ($\Delta\varepsilon_{390}$ = 28,000).

Measurement of TA (or TAI) oxidation using radioactive TA (or TAI). Incubations were made as previously described [9] in a final volume of 150 μL 0.1 M phosphate buffer pH 7.4 containing 0.1 to 1 mg microsomal proteins, 100 μM [^{14}C]TA and a NADPH-generating system.

After 20 min incubation at 37°, a 50 μL aliquot was spotted on a glass fiber filter (GFB) Whatman, for covalent binding measurements. Then, 150 μL of methanol containing 3 nmol of unlabeled 5-OHTA was added to stop the reaction. HPLC analysis for identification of 5-OHTA as the major metabolite

Table 1. Metabolic activation of tienilic acid and its isomer (TAI) by microsomes from various human livers

	5-OHTA formation*	Covalent binding of TA†	Covalent binding of TAI†
H97	3.3 ± 0.3 (6.6)	0.53 ± 0.1 (1.1)	5.2 ± 0.6 (10.4)
H97-NADPH generating system	<0.1	<0.02	<0.02
H97 + GSH (6 mM)	3.7 ± 0.02	0.05 ± 0.02	0.3 ± 0.1
KDL	3.3 (5.5)	0.5 (0.83)	7.5 (12.5)
F 503	2.2 (7.3)	0.33 (1.1)	2.9 (9.5)
F 504	2.3 (8.2)	0.5 (1.8)	4.5 (16)
F 510	2.2 (6.8)	0.32 (1)	3.3 (10.4)
SP 1	1.5 (7.4)	0.28 (1.4)	3.0 (15.1)

* 5-OH TA measured by the HPLC technique, in nmol per mg protein per 20 min.

† In nmol metabolites bound per mg protein per 20 min. Values in parentheses are in nmol per nmol cytochrome P450 per 20 min.

Conditions as in Materials and Methods, with incubations containing 0.2 nmol cytochrome P450 in a final volume of 150 μL. Cytochrome P450 contents in microsomes (nmol P450 per mg protein) were H97:0.5; KDL:0.6, F 503:0.3; F 504:0.28; F 510:0.32; SP1:0.2. Values indicated for liver H97 were means from seven determinations ± SD; for the other livers, values are means from two determinations. Values for incubations performed with H97 microsomes either in the absence of a NADPH-generating system or in the presence of GSH were means from four experiments ± SD.

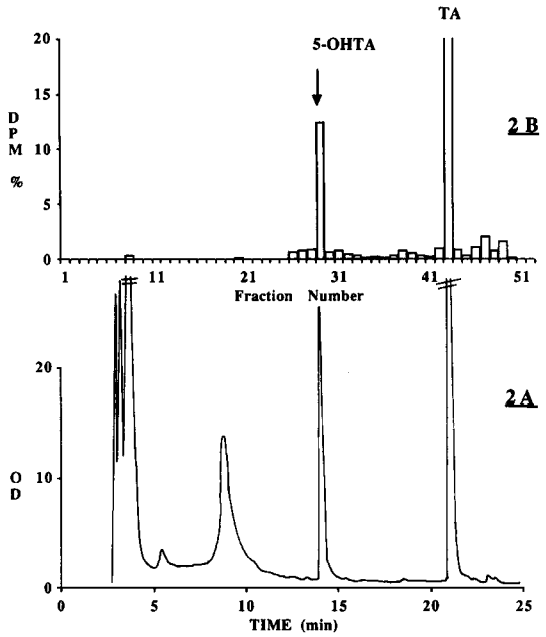


Fig. 2. Reverse-phase HPLC profile of tienilic acid metabolites formed in the presence of human liver microsomes. (A) UV detection at 310 nm (O.D. × 10^3). (B): radioactivity detection (dpm per fraction of 0.5 min). Analysis done after 20 min incubation of 100 μM [^{14}C]TA with 7 mg microsomal human liver protein (liver SP1:0.2 nmol P450 per mg protein) per mL. Conditions indicated in Materials and Methods.

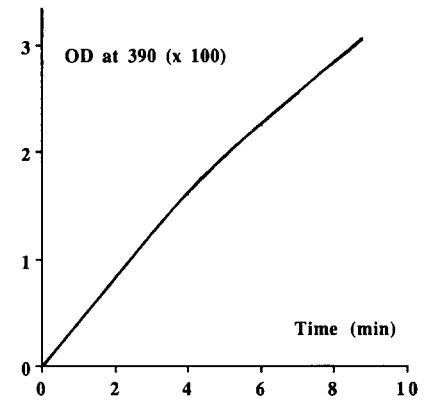


Fig. 3. Formation of 5-hydroxytienilic acid by human liver microsomes followed by UV-visible spectroscopy. Incubation of 100 μM TA with human liver microsomes (liver H97:0.5 nmol P-450 per mg protein) (0.48 nmol P450 per mL incubate).

of TA and for quantification of 5-OHTA was done as reported previously [9].

Incubations of microsomes with antibodies were made for 30 min at 4°, then after warming at 37°, reaction was started by addition of the NADPH-generating system and the substrate [7].

Covalent binding studies. These measurements were done according to previously described procedures [16, 17] adapted to the particular cases of TA and TAI [9].

RESULTS

Metabolic activation of TA by human liver microsomes

Oxidation of TA by human liver microsomes was followed by two techniques previously described for TA oxidation by rat liver microsomes [9, 15]. The HPLC method already used to study TA oxidation by rat liver microsomes [9] was employed to follow the oxidation of radioactive TA (0.1 mM, ^{14}C on the keto group) with human liver microsomes in the presence of NADPH. HPLC analysis of the reaction mixture using either a UV-visible or radioactivity detection showed the formation of 5-OHTA as a major metabolite (Fig. 2). Identification of this metabolite was done by comparison with an authentic sample of 5-OHTA using several columns, and by isolation of the metabolite by HPLC, methylation by diazomethane and analysis by gas chromatography coupled to mass spectrometry according to a previously described procedure [2, 9].

Oxidation of TA by human liver microsomes was also followed by a recently described UV-visible technique based on the detection of 5-OHTA at 390 nm [15]. This assay allowed one to directly follow the enzymatic reaction and to measure initial rates (Fig. 3). Data obtained by this technique with five different adult human microsomes gave an activity of 0.8 ± 0.4 nmol 5-OHTA formed per min per nmol P450. However, the UV-visible assay consumed at least five times more microsomes than the HPLC end point assay. Thus, for all reactions described in the following, conditions identical to those previously described for rat liver microsomes [9] were used, and quantitation was performed by HPLC analysis. The 5-hydroxylase activities found with this technique (Table 1) were in agreement with those obtained by the UV-visible assay. When expressed per nmol P450 per 20 min, these activities were similar to those described for liver microsomes from phenobarbital-pretreated rats [9] (around 7 nmol per nmol P450 per 20 min).

Irreversible binding of reactive metabolites of TA to microsomal proteins also occurred upon incubation of TA with human liver microsomes (Table 1). Both this irreversible binding and 5-hydroxylation of TA required NADPH and O_2 in the incubation mixture, whereas only the irreversible binding of TA metabolites to microsomal proteins was almost completely inhibited in the presence of 6 mM glutathione (Table 1). These properties of TA oxidation by human liver microsomes are very similar to those previously observed for rat liver microsomes [9]. Thus, as it has been shown that the oxidative activation of TA by rat liver microsomes was catalysed by cytochrome(s) P450, it is likely that 5-hydroxylation of TA as well as its oxidation into electrophilic alkylating metabolites by human microsomes are also cytochrome P450-dependent.

Figure 4A shows the correlation between 5-hydroxylation of TA and covalent binding of TA

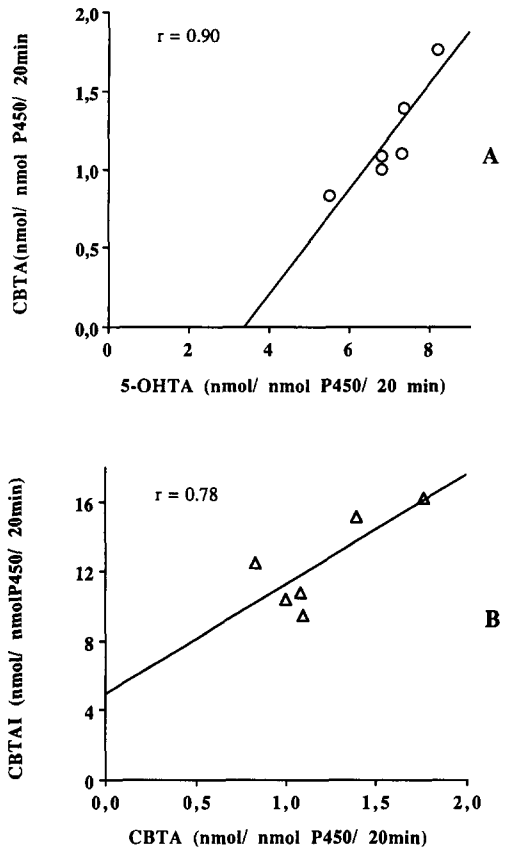


Fig. 4. Correlation between metabolic activation of TA into electrophilic metabolites with 5-hydroxylation of TA (curve A) or with metabolic activation of TAI into electrophilic metabolites (curve B), by different human liver microsomes. Data concerning the various activities (covalent binding of TA or of TAI: CBTA and CBTAI; 5-hydroxylation of TA: 5-OHTA) are those of Table 1. Regression lines were calculated using a Cricket Graph program on a Mackintosh microcomputer.

metabolites to microsomal proteins observed for six different human liver microsomes. The observed r value (0.9) indicates that these two activities are dependent on the same enzyme(s).

Metabolic activation of an isomer of TA by human liver microsomes

Incubation of radioactive TAI, the isomer of TA with the keto group on position 3 of the thiophene ring (Fig. 1), with several human liver microsomes in the presence of NADPH and O_2 were performed under conditions identical to those used previously for TA. As shown in Table 1, covalent binding of TAI to microsomal proteins exhibited characteristics similar to that of TA metabolites. It only occurred upon incubation with NADPH and was greatly inhibited in the presence of glutathione. However, microsomal oxidation of TAI led to a much higher level of covalent binding to proteins than the oxidation of TA (around 5 nmol bound per mg protein per 20 min instead of 0.5).

Figure 4B shows the existence of a correlation between two activities measured in six different human liver microsomes: the activation of TAI into electrophilic metabolites which bind covalently to proteins, and the 5-hydroxylation of TA ($r = 0.78$).

Moreover, TA was found as a good inhibitor of the activation of TAI into metabolites able to bind covalently to proteins by human liver microsomes. A Dixon plot analysis of the data (TA between 0 and 100 μM, TAI between 50 and 200 μM) gave a K_i value of about 25 μM (Fig. 5). Treatment of these data by a Lineweaver–Burk analysis showed that TA acted as a competitive inhibitor (data not shown). Finally, TAI was found to inhibit TA 5-hydroxylation by human liver microsomes (40% inhibition when using 100 μM TAI and 100 μM TA). These data suggest that the microsomal oxidative activation of TA by human liver microsomes, leading to its 5-hydroxylation and its transformation into reactive metabolites, and that of TAI, are mediated either by the same enzyme or by very closely related isoenzymes.

Nature of the cytochromes P450 involved in TA and TAI oxidation by human liver microsomes

It was previously reported [7] that the 5-hydroxylation of TA was strongly inhibited by a polyclonal antibody raised in rabbits against human liver cytochrome P450-8 [20], a member of the P450 IIC subfamily, as well as by human anti-LKM_2 antibodies which specifically recognize cytochrome P450-8 [8]. In fact, the almost complete inhibition of microsomal TA-hydroxylation observed with anti-LKM_2 antibodies showed that this activity of human liver microsomes was catalysed by cytochrome P450-8 or a closely related isoenzyme.

Figure 6 shows that anti-P450-8 antibodies also inhibit TA and TAI activation by human liver microsomes and the covalent binding of their metabolites to microsomal proteins. Human sera containing anti-LKM_2 antibodies greatly inhibited these reactions of TA and TAI whereas control sera not containing anti-LKM_2 antibodies either had no significant effect or even exhibited a small stimulatory effect (Fig. 6). The effects of 11 human sera containing anti-LKM_2 antibodies and of 16 sera not containing anti-LKM_2 antibodies from individuals either having taken TA or having not taken TA, on the covalent binding of TA or TAI metabolites to human liver microsomal proteins were compared (Fig. 7). All the used sera not containing anti-LKM_2 antibodies failed to inhibit TA and TAI activation, the ID_{50} (amount of serum leading to a 50% inhibition of TA or TAI activation, in μL of serum per nmol microsomal P450) being almost always above 350. On the contrary, all sera containing anti-LKM_2 antibodies inhibited TA and TAI oxidative activation with ID_{50} between 1 and 45 μL serum per nmol P450. Moreover, a relatively good correlation was found between the ID_{50} determined for these sera and their titer which was previously determined for their reaction with liver or kidney slices by immunofluorescence techniques [6] (data not shown: $r = 0.84$ for the inhibition of TA activation and covalent binding; $r = 0.82$ for the inhibition of TAI activation).

These data clearly show that the 5-hydroxylation of TA and the activation of TA and TAI, with

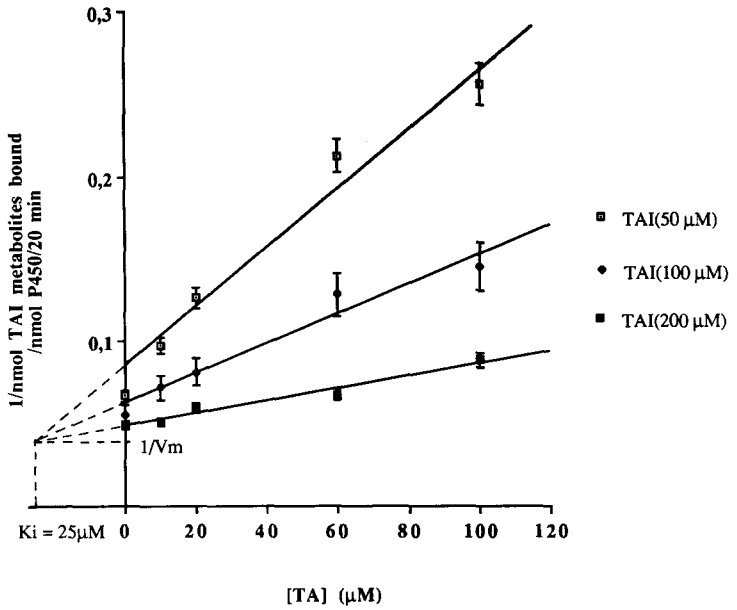


Fig. 5. Dixon plot analysis for the inhibition by TA of covalent binding of TAI metabolites to human liver microsomal proteins. Covalent binding of TAI metabolites to human liver (SP1) microsomal proteins was measured as described in Materials and Methods, on incubations of microsomes (1.4 nmol P450 per mL) with TAI (50, 100 or 200 μM) in the presence of various TA concentrations.

formation of metabolites able to bind covalently to proteins, by human liver microsomes, are all catalysed by cytochrome P450-8 or a closely related cytochrome P450 of the IIC subfamily.

Cytochromes P450 of the IIC subfamily have been shown to catalyse the hydroxylation of (*S*)-mephenytoïn [18, 19]. Polyclonal antibodies raised in rabbits against cytochrome P450-Meph, which catalyses (*S*)-mephenytoïn hydroxylation, and human anti-LKM_2 antibodies were found to inhibit TA hydroxylation by human liver microsomes [11, 7]. Thus, it seemed possible that (*S*)-mephenytoïn hydroxylation and TA hydroxylation could be catalysed by the same P450 IIC isoenzyme. In order to examine this possibility, the effect of mephenytoïn on TA 5-hydroxylation by human liver microsomes was studied. Table 2 shows that this activity was not inhibited by mephenytoïn used at concentrations as high as 200 μM (with TA either 20 or 100 μM). Table 2 also shows that the formation of electrophilic metabolites by oxidation of TA or TAI by human liver microsomes was not inhibited by mephenytoin. Moreover, TA concentrations as high as 400 μM were found without effect on (*S*)-mephenytoïn (20 or 100 μM) hydroxylation by human liver microsomes (U.A. Meyer, personal communication). These data indicate that 5-hydroxylation of TA and (*S*)-mephenytoïn hydroxylation are catalysed by different isoenzymes of the IIC subfamily. Accordingly, liver microsomes from a kidney transplant donor phenotyped as a slow hydroxylator of (*S*)-mephenytoïn (KDL), which presented a very low mephenytoïn hydroxylase activity [11], exhibited a usual TA 5-hydroxylation activity and activated TA and TAI as the other human liver microsomes (Table 1).

DISCUSSION

The above results show that the oxidative metabolic activation of TA and TAI by human liver microsomes exhibits characteristics very similar to that of TA and TAI by rat liver microsomes [9]. In both cases, 5-hydroxylation of the thiophene ring of TA is the major route of TA oxidation, the formation of 5-OHTA by human liver microsomes amounting for more than 60% of the fate of TA (as deduced from a radioactivity balance). With both microsomes, TA and its isomer TAI are oxidized into electrophilic metabolites that covalently bind to microsomal proteins, this covalent binding being almost completely suppressed in the presence of glutathione. It is noteworthy that the amount of covalent binding is considerably higher with TAI than with TA in the case of human liver microsomes (Table 1) as in that or rat liver microsomes [9]. Finally, the three activities—i.e. 5-hydroxylation of TA, activation of TA into reactive metabolites that covalently bind to microsomal proteins and activation of TAI—are dependent on microsomal monooxygenases as shown by the absolute requirement of NADPH and O_2 for the reactions to occur (Table 1) and by the strong inhibitions observed with antibodies against human liver cytochromes P450 IIC (Fig. 6 and Ref. 7). The levels of these three activities in human liver microsomes are similar to those described in rat liver microsomes (about 7, 1 and 12 nmol per nmol P450 per 20 min for TA 5-hydroxylation, covalent binding of TA metabolites and covalent binding of TAI metabolites, respectively, for human microsomes, and 5, 2.5 and 12 for rat microsomes [9]). The only marked difference concerns the level of covalent binding of TA metabolites which is clearly higher in

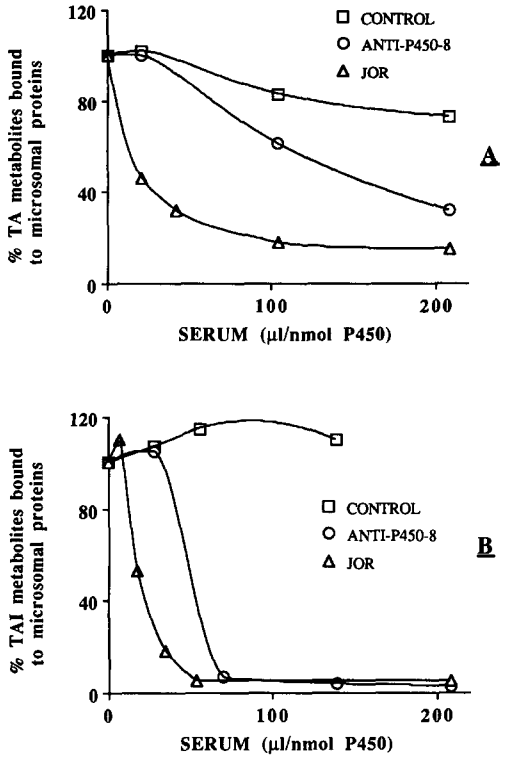


Fig. 6. Inhibition of human liver microsome-dependent covalent binding of TA (curves A) or of TAI (curves B) to proteins by anti-human liver cytochrome P450-8 or by sera containing or not containing anti-LKM_2 antibodies. Microsomes from liver H97 were used. 100% control activities: 1.1 nmol of TA metabolites bound to proteins per nmol P450 per 20 min (A); 10.4 nmol of TAI metabolites bound to proteins per nmol P450 per 20 min (B). □ control human serum not containing anti-LKM_2 antibodies; ○ rabbit serum containing anti-P450-8 antibodies obtained as described previously [20]; △ serum JOR containing anti-LKM_2 antibodies.

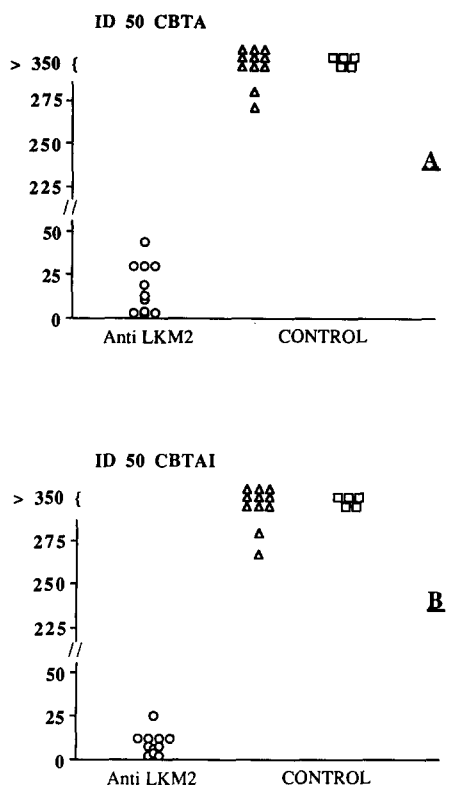


Fig. 7. Effects of different human sera containing or not containing anti-LKM_2 antibodies on the covalent binding of TA (A) or TAI (B) metabolites to human liver microsomal proteins. Incubation conditions as in Fig. 6. (○) Sera containing anti-LKM_2 antibodies. (△) Sera not containing anti-LKM_2 antibodies from humans having not taken TA. (□) Sera not containing anti-LKM_2 antibodies from humans having taken TA. ID_{50}: amounts of serum (μL per nmol P450) leading to 50% inhibition of the activity. For most control sera, 50% inhibition was never reached even with 300 μL of serum per nmol P450. Points at the >350 level correspond to sera giving less than 30% inhibition with 300 μL per nmol P450.

rat (covalent binding: 5-OHTA ratio of 0.5) than in human (ratio of 0.14) liver microsomes.

These three activities of human liver microsomes toward TA and TAI appear mainly dependent on the same cytochrome P450 of the IIC subfamily for the three main following reasons: (i) the strong inhibition of the three activities by antibodies against human liver cytochromes P450 of the IIC subfamily, either anti-cytochrome P450-8 or anti-LKM_2 antibodies (Figs 6 and 7 and Ref. 7). (ii) A clear correlation between TA 5-hydroxylation and covalent binding of TA metabolites ($r = 0.9$), and between covalent binding of TA and of TAI metabolites ($r = 0.78$) in six different human liver microsomes (Fig. 4); (iii) the competitive inhibition of TAI activation to reactive metabolites by TA ($K_i \cong 25$ μM) (Fig. 5), and the inhibitory effect of TAI toward TA 5-hydroxylation.

Human cytochromes P450 of the IIC subfamily have been associated with the para-hydroxylation of the phenyl ring of (*S*)-mephenytoïn [18, 19]. Accordingly, both anti-cytochrome P450-8 and anti-LKM_2 antibodies [11] have been shown to inhibit mephenytoïn hydroxylation but not (*S*)-mephenytoïn *N*-demethylation. However, since (i) mephenytoïn fails to inhibit TA 5-hydroxylation by human liver microsomes, (ii) TA fails to inhibit (*S*)-mephenytoïn hydroxylation, and (iii) human liver microsomes from a donor phenotyped as a slow hydroxylator of mephenytoïn exhibit a normal TA 5-hydroxylase activity, TA hydroxylation and (*S*)-mephenytoïn hydroxylation by human liver microsomes should be catalysed by two different cytochromes P450 of the IIC subfamily.

Definitive determination of the cytochrome P450 mainly responsible for TA 5-hydroxylation by human liver microsomes would require the use of purified human P450 isoenzymes of the IIC subfamily or of systems where the genes coding for each cytochrome P450, such as P450 IIC8, IIC9 or IIC10, would be expressed.

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Table 2. Effects of (±) Mephenytoin on the 5-hydroxylation of TA and on the covalent binding of TA or TAI metabolites to proteins by human liver microsomes

Mephenytoïn concentration (M)	5-OHTA formation (%)*	Covalent binding of TA (%)*	Covalent binding of TAI (%)*
0	100	100	100
10^{-4}	105 ± 5	101 ± 10	98 ± 11
2×10^{-4}	102 ± 5	100 ± 12	98 ± 12
2×10^{-4}†	98 ± 8	105 ± 10	

Conditions as in Table 1 using H97 microsomes. Values are means of four determinations ± SD.

* 100% activities were 3.3, 0.5 and 5.2 nmol per mg protein per 20 min for 5-OHTA formation, covalent binding of TA and covalent binding of TAI respectively.

† Identical conditions except that TA concentration was 20 μM instead of 100 μM. 100% values under these conditions were 2.1 nmol 5-OHTA and 0.3 nmol metabolites irreversibly bound to proteins formed per mg protein per 20 min.

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