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HYDROXYLATION OF THE THIOPHENE RING BY HEPATIC MONOOXYGENASES

EVIDENCE FOR 5-HYDROXYLATION OF 2-AROYLTHIOPHENES AS A GENERAL METABOLIC PATHWAY USING A SIMPLE UV-VISIBLE ASSAY

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Abstract—The 5-hydroxylation of tienilic acid by rat liver microsomes was measured by a new, simple method involving the detection of 5-hydroxytienilic acid by UV-visible spectroscopy. This assay allowed continuous detection of this metabolite and could be easily used to determine the kinetic parameters of the reaction (V_{max} and K_m being respectively 1 ± 0.2 nmol product formed/mg protein/min and 14 ± 2 μM for liver microsomes from phenobarbital-treated rats). This activity was found to be dependent on NADPH and to be inhibited by CO, SKF 525A and metyrapone, indicating that it is dependent on cytochromes P-450. This UV-visible assay is based on intrinsic properties of 5-hydroxy 2-aroylthiophenes which exist as highly conjugated anions at physiological pH and exhibit large ε values around 390 nm. Its application to other 2-aroylthiophenes like suprofen, 2-parachlorobenzoylthiophene and a series of 2-aroylthiophenes with various substituents on the aroyl group showed that, in general, thiophene compounds bearing a 2-arylketo substituent appear to be hydroxylated at position 5 by rat liver microsomes. The kinetic parameters of the 5-hydroxylation of suprofen and 2-parachlorobenzoylthiophene by liver microsomes from phenobarbital-treated rats were determined and found to be similar to those for tienilic acid hydroxylation.

Very little is known on the metabolism of thiophene derivatives. The only metabolites of thiophene identified so far are thienyl mercapturic acids deriving presumably from reaction of glutathione with an intermediate thiophene-2,3-oxide [1]. For some drugs containing a thiophene ring, data only on their *in vivo* metabolism have been reported. Thus, the *in vivo* formation of metabolites deriving from the hydroxylation of the thiophene ring of tienilic acid [2], suprofen [3], morantel [4], tiquizine [5] and tenoxicam [6] has been described.

Data on the *in vitro* metabolism of thiophene derivatives have appeared only recently and all concerned tienilic acid. This drug was found to be hydroxylated at position 5 of its thiophene ring by human liver microsomes [7, 8] as well as by rat liver microsomes [8]. The formation of 5-hydroxy-tienilic acid was followed by HPLC after oxidation of radioactive tienilic acid by liver microsomes [7, 8]. This method is sensitive, thanks to the use of radioactivity detection of the 5-OH metabolite, but it suffers from three main disadvantages: (i) it requires a relatively long time of analysis because of the necessary separation of tienilic acid and its metabolites, (ii) it is not a continuous method allowing reaction kinetics to be followed directly, and (iii) as such, it is only applicable to radioactive substrates.

In this paper, we describe a simple method to follow the hydroxylation of tienilic acid by liver microsomes using the detection of its 5-hydroxy metabolite by UV-visible spectroscopy. This assay allows a continuous detection of this metabolite and was used to determine the kinetic parameters of the reaction which appeared dependent on cytochrome P-450. It was applied to many other 2-aroylthiophenes and allowed us to show that all tested thiophene compounds with a 2-arylketo substituent were hydroxylated at position 5 by rat liver microsomes.

MATERIALS AND METHODS

Chemicals. All the chemical reagents were of the highest quality commercially available. Tienilic acid (TA†), [^{14}C]tienilic acid, 5-hydroxy-tienilic acid (5-OHTA), were given to us by Laboratories Anphar Rolland (Chilly Mazarin, France). 5-Hydroxy-tienilic acid was prepared as described [2] and [^{14}C]TA (label in the keto group, 25 Ci/mol, radiochemical purity higher than 98%) by CEA Saclay (France) [9]. SKF 525A (diethylaminoethyl 2,2-diphenylvalerate) was a gift of Dr Fort (SKF Laboratories, France). Suprofen (SU) and 5-hydroxy-suprofen (5-OHSU) were gifts from Laboratories Janssen and from Prof. Y. Mori (University of Gifu, Japan) [10]. 2-Parachlorobenzoylthiophene (CBT) was prepared as described previously [11] and 2-(2′,3′-dichloro,4′-methoxy)-benzoylthiophene (MBT) [12] was a gift

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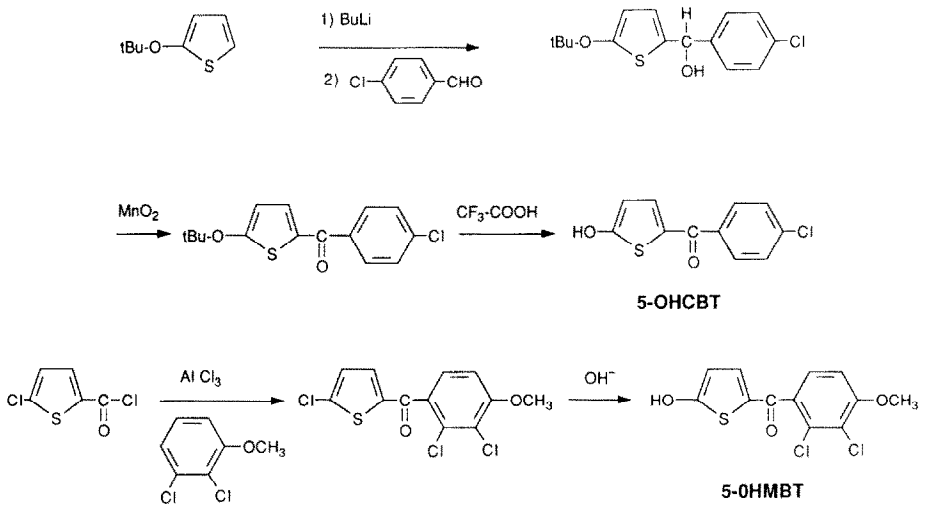
† Abbreviations: TA, tienilic acid; 5-OHTA, 5-hydroxy-tienilic acid; SKF 525A, diethylaminoethyl 2,2-diphenyl valerate; SU, suprofen; CBT, 2-parachlorobenzoylthiophene; MBT, 2-(2′,3′-dichloro 4′-methoxy)-benzoylthiophene; PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCN, 16α-cyanopregnenolone; 5-OHSU, 5-hydroxy-suprofen; 5-OHCBT, 5-hydroxy CBT; 5-OHMBT, 5-hydroxy MBT.



Scheme 1. Synthesis of 5-OHCBT and 5-OHMBT.

from Laboratories Anphar Rolland. The various 2-aroylthiophenes indicated in Table 4 [13] were kindly provided by Dr Andrieu (University of Caen, France).

^{1}H NMR spectra were recorded on a Brucker WM 250 spectrometer and mass spectra on a Nermag R 1010 spectrometer [direct introduction, electron impact (EIMS) or chemical ionization technique using NH_3 (CIMS)].

Synthesis of 5-hydroxy 2-parachlorobenzoylthiophene (5-OHCBT). This compound was prepared from 2-*t*-butoxythiophene by a procedure analogous to that previously described for the synthesis of 5-OHTA [2] (Scheme 1).

2-*t*-Butoxythiophene was prepared by a previously described procedure [14]. To 3.12 g (0.02 mol) of this compound in 10 mL ether, 9.6 mL (0.025 mol) of a solution of BuLi in ether were slowly added. After about 1 hr of reflux until complete formation of butane, the mixture was cooled to 0°. A solution of 3.51 g (0.025 mol) of 4-chlorobenzaldehyde in 20 mL of ether was added dropwise. After stirring for 3 hr at room temperature, 100 mL H_2O were added. After solvent evaporation and flash chromatography on silica (CH_2Cl_2 as eluent), 3 g (50% yield) of 2-t-*butoxy 5-(4′-chlorophenyl hydroxymethyl) thiophene* were obtained. This compound was characterized by ^{1}H NMR (δ in $CDCl_3$: 7.3 (m, 4H), 6.46 (d, J = 3.6 Hz, 1H), 6.16 (d, J = 3.6 Hz, 1H), 5.81 (s, 1H), 2.61 (s, 1H), 1.33 (s, 9H) and mass spectrometry: EIMS, 296 (M^+, 2%), 240 (M^+-C_4H_8, 30%), 223 (12%), 139 (15%) and 57 (100%).

Oxidation of this compound (0.119 g, 0.4 mmol) by MnO_2 (0.174 g, 2 mmol) in 5 mL CH_2Cl_2 was performed at room temperature over 48 hr. This gave 0.05 g (42% yield) of 2-*t*-butoxy 5-(4′-chlorobenzoyl)thiophene after thin-layer chromatography of the crude mixture on silica (CH_2Cl_2 as eluent): m.p. = 99°; ^{1}H NMR (δ in CD_3CN: 7.69 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 8.5 Hz, 2H), 7.33 (d, J = 4.5 Hz, 1H), 6.43 (d, J = 4.5 Hz, 1H); mass spectrum: CIMS, 295 (MH^+, 100%), 256 (20%), 239 (M^+-C_4H_8, 85%) and 223 (5%).

Treatment of this compound (0.02 g, 68 μmol) by 1 mL of CF_3COOH gave 15 mg of 5-*hydroxy* 2-*parachlorobenzoylthiophene* (m.p. = 187°) which was found to be more than 95% pure by HPLC and ^{1}H NMR spectroscopy. It was fully characterized by elemental analysis (C, H, Cl), ^{1}H NMR (δ in CD_3CN: 7.6 (d, J = 8.5 Hz, 2H), 7.46 (d, J = 8.5 Hz, 2H), 7.37 (d, J = 5 Hz, 1H) and 6.18 (d, J = 5 Hz, 1H) and mass spectrometry: EIMS, 238 (M^+, 25%), 222 (20%), 139 (100%), 127 (12%), 111 (35%).

Synthesis of 5-hydroxy 2-(2′,3′-dichloro 4′-methoxy)-benzoyl thiophene (5-OHMBT). $AlCl_3$ (15 mmol) were progressively added to a mixture of 12 mmol of the freshly prepared chloride of 2-carboxy 5-chlorothiophene and 12 mmol of 2,3-dichloro 1-methoxybenzene. The reaction mixture was stirred at 0° for 3 hr, hydrolysed by 30 mL H_2O and extracted by CH_2Cl_2. 5-Chloro 2-(2′,3′-dichloro 4′-methoxy)-benzoyl thiophene was obtained as a crystalline solid after chromatography on silica gel (CH_2Cl_2 as eluent) and two recrystallizations from EtOH (60% yield): m.p. = 162°; ^{1}H NMR (δ in $CDCl_3$: 7.32 (d, J = 8.5 Hz, 1H), 7.28 (d, J = 4.2 Hz, 1H), 6.94 (d, J = 4.2 Hz, 1H), 6.9 (d, J = 8.5 Hz, 1H) and 3.97 (s, 3H); mass spectrometry [EIMS, 320 (M^+, 30%), 285 (10%), 203 (90%) and 145 (100%)].

This compound (0.2 mmol) in 15 mL EtOH was treated by 35 mmol of KOH in 15 mL H_2O for 16 hr at 60°. After filtration and acidification to pH 1, the crude mixture was extracted with 200 mL diethyl ether. The 5-hydroxyderivative formed in this reaction was purified by reverse phase HPLC (5μ Nucleosil C18 column, elution by an acetonitrile-ammonium acetate buffer 0.1 M, pH 7.4, 0–60% in 15 min, 1 mL/min). Light yellow crystals of 5-OHMBT were obtained (6% yield after purification). Its ^{1}H NMR and mass spectra were in complete agreement with the proposed structure: ^{1}H NMR δ in CD_3CN: 7.3 (d, J = 8.5 Hz, 1H), 7.18 (d, J = 5 Hz, 1H), 7.05 (d, J = 8.5 Hz, 1H), 6.09 (d, J = 5 Hz, 1H) and 3.95 (s, 3H). Mass spectrum (after methylation by CH_2N_2,); CIMS: 317 (MH^+, 100%), 285 (25%) and 159 (50%).

Measurement of the 5-hydroxylation of 2-aroylthiophenes by rat liver microsomes (UV-visible assay): typical procedure. Liver microsomes were suspended

Table 1. UV-visible characteristics of thiophene derivatives, and pK_a values of various 5-hydroxy 2-aroylthiophenes*

	UV-visible		
	λm (nm)	ε (mM^{-1} cm^{-1})	pK_a
TA	272	11	
	306	14	
5-OHTA	266	3.6	
	384	28	2.5
SU	276	12	
	300	14	
5-OHSU	276	6.7	
	386	27	2.9
CBT	267	7.7	
	297	8.2	
5-OHCBT	260	6	
	385	28	2.5
MBT	274	8.8	
	309	15	
5-OHMBT	264	6.3	
	382	29	2.5

* In 0.1 M phosphate buffer pH 7.4.

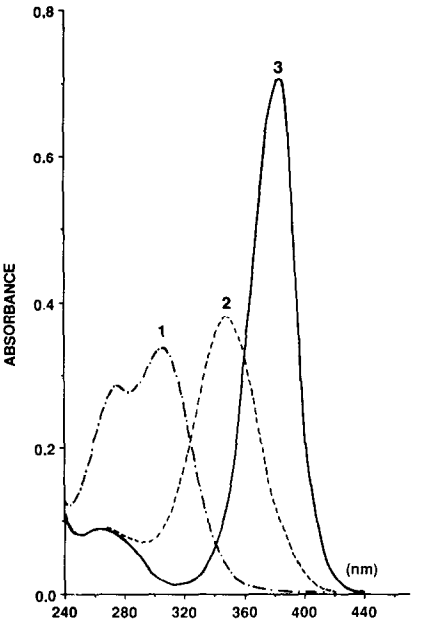


Fig. 1. UV-visible spectra of tienilic acid and 5-hydroxy-tienilic acid. (1) 25 μM TA in Tris–HCl (0.1 M) buffer pH 7.4; (2) 25 μM 5-OHTA in H_2O at pH 1 (HCl + KCl 0.1 M); (3) 25 μM 5-OHTA in carbonate (0.05 M) buffer pH 11.

in a 0.1 M phosphate buffer pH 7.4 (0.2–1.5 mg protein/mL and most often 1 nmol cytochrome P-450/mL) in a 1 cm cuvette. A NADPH-generating system consisting in 10 mM glucose-6 phosphate, 1 mM NADP and 2 units/mL of glucose-6-phosphate dehydrogenase was added to the suspension. The mixture was divided between two cuvettes and equilibration at 37° ensured by incubation for 2 min. The reaction was started by the addition of the substrate to the sample cuvette (an equal volume of vehicle was added to the reference cuvette) and followed at 390 nm in a double beam spectrophotometer (Kontron 820) at 37°. The reaction can also be followed in a single sample cuvette by following the ΔA between 390 and 490 nm on a dual wavelength spectrophotometer (Aminco Chance DW2). TA and SU were added as solutions in Tris–HCl buffer pH 7.4, and the other substrates or inhibitors as CH_3OH solutions.

Measurement of 5-hydroxylation of TA by rat liver microsomes by HPLC was performed as previously reported [7, 8]. The same HPLC technique was used to confirm the formation of 5-OHSU by oxidation of SU by liver microsomes.

Rat liver microsomes. Male Sprague–Dawley rats (200 g) (Iffa Credo, France) were maintained on a standard laboratory chow (UAR, France). Induction with phenobarbital (1 g/L in drinking water) was done for 5 days. Induction by 3MC was achieved by i.p. injection of solution in corn oil (5 mg/mL) at the dose of 25 mg/kg/day for 3 days. Induction by PCN was achieved by i.p. injection of a solution in corn oil (10 mg/mL) at the dose of 50 mg/kg/day for 5 days. After induction, rats were fasted overnight and killed by cervical dislocation. Livers were removed, perfused with saline and microsomes prepared as described previously [15]. Their cytochrome P-450 content was determined by the method of Omura and Sato [16] and protein concentrations were measured by the method of Lowry *et al.* [17].

RESULTS

Physicochemical basis for the detection of 5-hydroxy 2-aroylthiophene metabolites

Figure 1 shows the UV-visible spectrum of tienilic acid (TA) in Tris–HCl buffer pH 7.4 which exhibits a peak at 306 nm and an ε value around 14 mM^{-1} cm^{-1}. This spectrum does not change when the pH varies from 1 to 11 and is almost identical to that of TA in CH_2Cl_2. The spectrum of 5-hydroxy-tienilic acid (5-OHTA) in CH_2Cl_2 is characterized by a red-shifted peak at 350 nm and a similar ε value. This spectrum is very similar to that of 5-OHTA in water at pH 1 (in the presence of HCl) but very different from that of 5-OHAT in water at pH 11 (carbonate buffer) which shows an intense ($\varepsilon = 28\ mM^{-1}\ cm^{-1}$) more red-shifted peak at 384 nm (Fig. 1). Upon changing the pH of the solution, one observes a reversible change of the 5-OHTA spectrum with an isosbestic point at 360 nm and two peaks at 350 nm (predominant at low pH and corresponding to the acid form A of Fig. 2). and at 384 nm (predominant at high pH and corresponding to the anionic form B or C of Fig. 2). Thus, it was possible to determine the pK_a of the 5-OH function of 5-OHTA by measuring the absorbance of 5-OHTA around 384 nm as a function of pH (Fig. 3). Graphical evaluation gave a pK_a value of 2.5, in agreement with literature data reporting that 2-hydroxy-thiophenes containing electron-withdrawing substituents are much more acidic than the corresponding phenols [18]. We verified that ionization

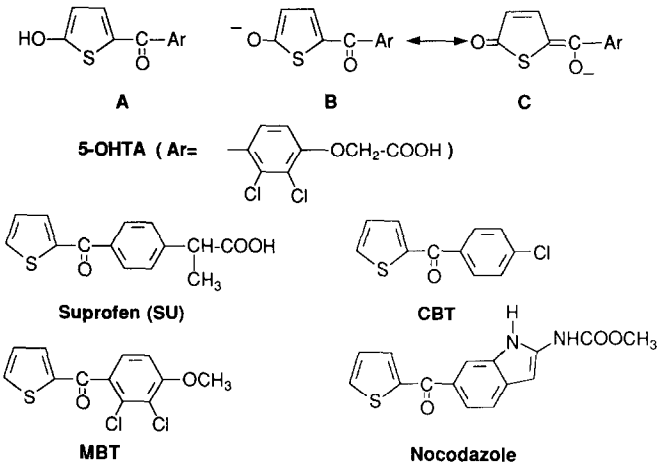


Fig. 2. Formula of 5-hydroxy-tienilic acid (acidic and basic forms at the level of 5-OH) and of other 2-aroylthiophenes used in this study.

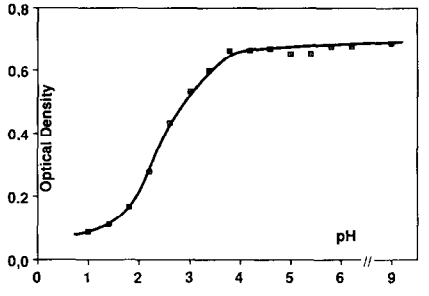


Fig. 3. Determination of the pK_a of the 5-OH function of 5-OHTA. Absorbance of 5-OHTA at 390 nm as a function of pH (5-OHTA 25 μM).

of the COOH function of 5-OHTA did not interfere with this pK_a determination since the spectra of the methyl ester of 5-OHTA at pH 1 and at pH 11 were almost identical to those of 5-OHTA under identical conditions.

Very similar results were obtained for three other 2-aroylthiophenes [suprofen SU, 2-parachlorobenzoyl thiophene CBT and 2-(2',3'-dichloro 4'-methoxy)-benzoyl thiophene MBT], and their 5-hydroxy derivatives. All exhibited UV-visible spectra at pH 1, 7.4 and 11 very similar to those of TA and 5-OHTA, respectively (Table 1). The pK_a values of their 5-OH function were all around 2.5. These data suggest that, in general, it should be possible to detect the formation of 5-hydroxy 2-aroylthiophenes as possible metabolites of 2-aroylthiophenes by UV-visible spectroscopy around 390 nm.

Hydroxylation of tienilic acid by rat liver microsomes

After addition of 0.1 mM TA to a suspension of liver microsomes from rats pretreated by phenobarbital (around 0.4 mg of protein/mL) containing a NADPH-generating system, the absorbance of the suspension at 390 nm increased linearly as a function of time for at least 10 min allowing direct measurement of the initial rate of the 5-hydroxylation of TA (Fig. 4A). This rate expressed in nmoles of 5-OHTA formed per min increased linearly with the amount of microsomal protein used at least between 0.3–2 mg protein/mL (Fig. 4B). Formation of 5-OHTA as a major metabolite during these experiments was checked by analysis of the reaction mixtures by reverse phase HPLC as described previously [7]. Identification of 5-OHTA was achieved by coinjection of an authentic sample of 5-OHTA (identical HPLC retention times for authentic 5-OHTA and the metabolite), and GLC-MS after methylation by CH_2N_2 according to a previously described procedure [2] (data not shown). Moreover, the amounts of 5-OHTA formed in the enzymatic assay calculated from the UV-visible technique were in very good agreement with those estimated by the HPLC analysis.

As shown in Table 2, 5-hydroxylation of TA did not occur with boiled microsomes or in the absence of NADPH. NADH could not replace NADPH for the hydroxylation to occur. It did not seem to be catalysed by a flavine monooxygenase as heating microsomes at 45° for 5 min, a treatment described to inactivate this monoxygenase [19], had no effect. Classical inhibitors of cytochrome P-450-dependent monooxygenases such as CO, SKF 525A and metyrapone inhibited this activity up to about 80%. Finally, liver microsomes from control rats exhibited an activity very similar to that of microsomes from phenobarbital-pretreated rats. However, pretreatment of rats by other classical inducers of cytochromes P-450, 3-methylcholanthrene and 16α-cyanopregnenolone, led to a marked decrease of 5-hydroxylation of TA.

Figure 5 shows the variation of the 5-hydroxylation rate as a function of TA concentration for liver microsomes from phenobarbital-pretreated rats, which gave kinetic parameters for the reaction of K_m 14 μM and V_{max} around 1 nmol 5-OHTA formed/mg protein/min (0.42 nmol/nmol P-450/min). It is noteworthy that these kinetic parameters determined by the UV-visible method are in good agreement with those previously estimated by an HPLC method using radioactive tienilic acid (V_{max} around 0.5 nmol/nmol P-450/min, and K_m around 20 μM) [8].

Hydroxylation of 2-aroylthiophenes analogous to tienilic acid

Microsomal oxidation of the three 2-aroylthio-

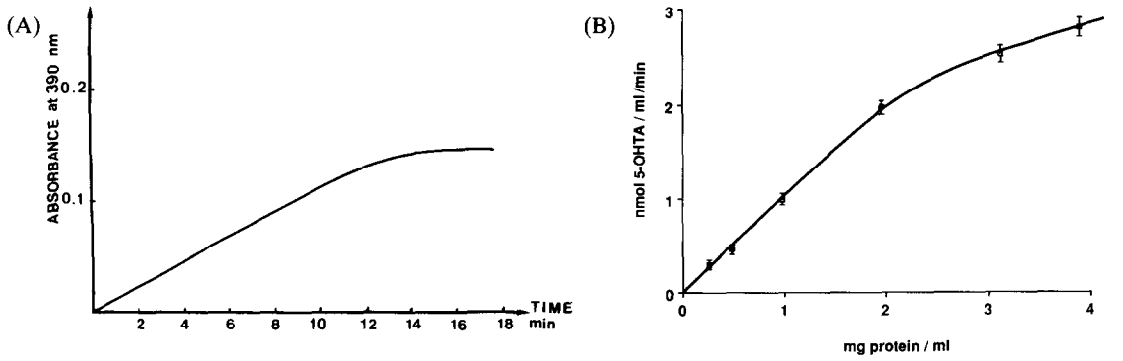


Fig. 4. Formation of 5-OHTA by liver microsomes as a function of time (A) and protein concentration (B). (A) Absorbance at 390 nm of a liver microsomal suspension (0.4 mg protein/mL) from PB-treated rats (2.4 nmol P-450/mg protein) after addition of 0.1 mM TA and a NADPH-generating system (see Materials and Methods). (B) rates of 5-hydroxylation of 0.1 mM TA in nmol product formed/min/mL under the same conditions as (A) but with various microsomal protein concentrations. Values shown are means ± SE from four determinations.

Table 2. Effects of various factors on the 5-hydroxylation of TA by rat liver microsomes

	5-OH Tienilic acid (% of control)
Complete system*	100
Boiled microsomes†	<3
Microsomes heated 5 min at 45°	96
− NADPH‡	<3
− NADPH + NADH 0.7 mM	<3
+ NADH	140
− O_2	<3
+ CO§	26
+ Metyrapone 0.01 mM	40
+ Metyrapone 0.1 mM	25
+ SKF 525A 0.1 mM	22
Rat pretreatment‖	
no	108
3MC	65
PCN	60

* The complete system consisted of liver microsomes from PB-treated rats (1 nmol P-450/mL, 2.4 nmol P-450/mg protein) in 0.1 M phosphate buffer pH 7.4, 0.1 mM TA and a NADPH-generating system as described in Materials and Methods. Its activity measured by the UV technique described above was 1 nmol 5-OHTA formed/mg protein/min (0.42 nmol 5-OHTA/nmol P-450/min).

† Microsomes heated 5 min at 100°.

‡ Complete system without the NADPH-generating system.

§ Using a $CO:O_2$ mixture 4:1 (v/v).

‖ Microsomes from rats treated by various inducers were used; treatments were as described in Materials and Methods; cytochrome P-450 contents for control, 3MC- and PCN-treated rats were 0.8, 1.5 and 1.5 nmol P-450/mg protein, respectively.

Results are means from four replicate determinations.

phenes analogous to tienilic acid, the physico-chemical properties of which have been described above (see Table 1), SU, CBT and MBT, was studied by using the same UV detection at 390 nm. The three compounds gave rise to metabolites hydroxylated on position 5 of the thiophene ring as shown by a clear time-dependent appearance of a UV-visible spectrum characterized by a maximum around 390 nm with a shape identical to that of the spectrum of

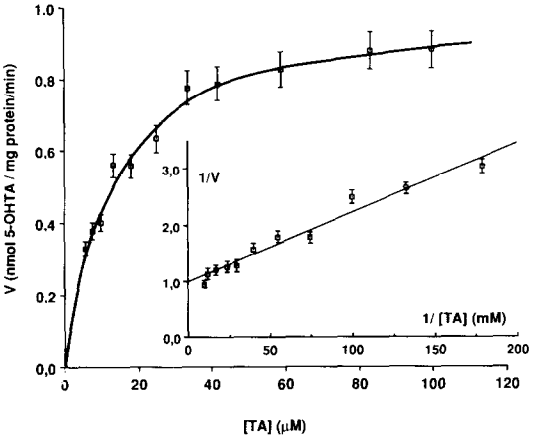


Fig. 5. Kinetics of 5-hydroxylation of TA by rat liver microsomes. Initial rates (nmol 5-OHTA/nmol P-450/min) as a function of TA concentration. Conditions as in Fig. 4A. Values shown are means ± SE from four experiments. Insert: $1/v = f(1/[TA])$.

authentic samples of 5-OHSU, 5-OHCBT and 5-OHMBT, respectively (Table 1). Formation of 5-OHSU, 5-OHCBT and 5-OHMBT was verified by a reverse-phase HPLC analysis of the incubates which showed retention times for the major microsomal metabolite of SU, CBT and MBT identical to those of authentic 5-OHSU, 5-OHCBT and 5-OHMBT, respectively. Identification of 5-OHSU as a microsomal metabolite was confirmed by a GLC-MS analysis of the incubate after methylation by CH_2N_2 (spectrum identical to that described for an authentic sample by Mori *et al.* [3], data not shown).

The V_{max} and K_m values for the 5-hydroxylation of SU, CBT and MBT by liver microsomes from phenobarbital-treated rats were determined. They are similar to those found for tienilic acid, with V_{max} between 1 and 3 nmol product formed/mg protein/min and K_m between 9 μM for CBT and 33 μM for suprofen (Table 3) (Fig. 6).

Microsomal oxidation of various 2-aroylthiophenes

In order to determine whether the 5-hydroxylation observed in the case of TA, SU, CBT and MBT was

Table 3. Kinetic parameters for the hydroxylation of TA, SU, CBT and MBT by liver microsomes from phenobarbital-treated rats

	K_m (μM)	V_{max} (nmol/nmol P-450/min)	V_{max} (nmol/mg protein/min)
TA	14 ± 2	0.45 ± 0.1	1.0 ± 0.2
SU	33 ± 5	1.1 ± 0.2	2.2 ± 0.3
CBT	9 ± 2	1.5 ± 0.3	3.1 ± 0.5
MBT	22 ± 3	1.4 ± 0.2	2.9 ± 0.4

Mean values ± SE for measurements performed as indicated in Figs 5 and 6 but with different preparations of liver microsomes from phenobarbital-treated rats (1.8–2.6 nmol P-450/mg protein): five independent experiments for TA, 3 for SU, CBT and MBT.

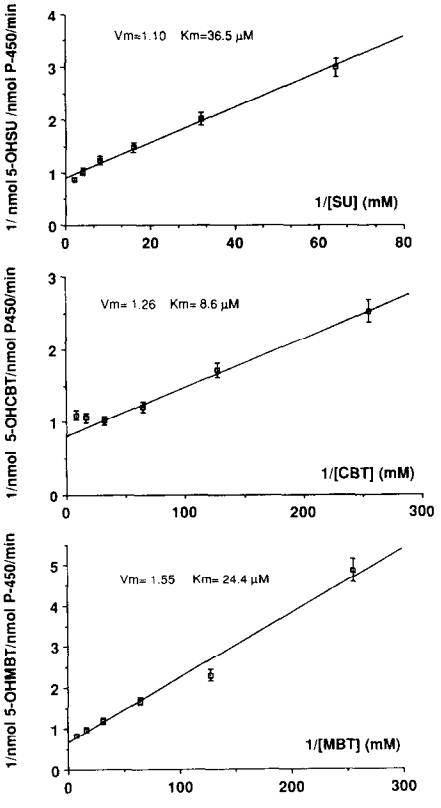


Fig. 6. Kinetics of 5-hydroxylation of SU, CBT and MBT by liver microsomes from PB-treated rats. 1/rate (nmol 5-OH product × 1/nmol P-450 × 1/min) = f(1/substrate concentration). Conditions as in Materials and Methods. Values shown are means ± SE from four determinations.

a general reaction, we have studied the oxidation of various 2-aroylthiophenes by liver microsomes of phenobarbital-treated rats. Table 4 shows preliminary results on ten 2-aroylthiophenes with various substituents on the aroyl group.

All these compounds exhibited behavior similar to that of TA, SU and CBT when incubated with liver microsomes and a NADPH-generating system. For all of them, a UV-visible spectrum characterized by a maximum around 390 nm appeared and increased linearly with time.

Although we did not have the 5-hydroxy derivatives of the compounds listed in Table 4, these preliminary results suggest that all of these 2-aroylthiophenes, as TA, SU and CBT, are hydroxylated at position 5 of their thiophene ring. The rates of their 5-hydroxylation given in Table 4 were calculated assuming that their 5-hydroxy-derivatives had ε values similar to those of 5-OHTA, 5-OHSU and 5-OHCBT and equal to 28,000. These rates determined under identical conditions (substrate 0.2 mM) were found to be very similar (between 0.7 and 1.7 nmol/nmol P-450/min).

Table 4. 5-Hydroxylation of various 2-aroylthiophenes by liver microsomes from PB-treated rats

Aroyl substituents X (*ortho*)	Y (*para*)	Rate (nmol 5-OH product/nmol P-450/min)
X=Y=H		1.4
X=H	Y=F	1.1
X=H	Y=Cl	1.5
X=H	Y=CH_3	0.9
X=OCH_3	Y=H	1.7
X=CH_3	Y=H	1.4
X=F	Y=H	1.6
X=Cl	Y=H	1.3
X=Br	Y=H	0.6
X=NO_2	Y=H	0.7

Rates measured by following the absorbance increase at 390 nm as a function of time and assuming an ε value of 28 $mM^{-1}\,cm^{-1}$. Conditions as in Materials and Methods with substrate concentrations of 0.2 mM and 0.5 mg microsomal protein/mL (2.4 nmol P-450/mg protein).

Values shown are means from two experiments.

DISCUSSION

The aforementioned data led to a new, efficient and simple technique for measuring the 5-hydroxylation of TA by rat liver microsomes. As expected, the kinetic data and the effects of co-factors and inhibitors, which showed that this activity was cytochrome P-450-dependent, observed with this UV-visible technique were almost identical to those previously observed using an HPLC method [8]. The new method exhibits four advantages when compared to the HPLC technique. First it does not require the use of radioactive TA. Second, it is much more convenient and faster since it allows a rate measurement in less than 10 min without the necessity of any product separation. Third, it allows continuous monitoring of product formation giving a direct indication of rates of reaction. Finally, it would appear to be applicable to any 2-aroylthiophene. However, final proof of this will depend upon further experimentation.

Actually, 5-hydroxylation of 2-aroylthiophenes was demonstrated unequivocally with TA and SU and appears highly probable with CBT and MBT. Moreover, first data suggest that this hydroxylation also occurs on nine other 2-aroylthiophenes. Thus, this metabolic reaction appears general for this class of compounds.

This UV-visible assay is based on two particular physico-chemical properties of 5-hydroxy 2-aroylthiophenes, the existence of these compounds as anions at pH 7.4 and their strong absorbance at 390 nm. It should allow the determination of corresponding monooxygenase activities in various tissues or organs (with a detection limit around 0.02 nmol 5-OH product formed/nmol P-450/min). Finally it should be easily applied to various 2-aroylthiophenes of biological interest. For instance, we have applied it recently to a drug, nocodazole (Fig. 2), and obtained very preliminary results indicating that this thiophene-containing drug is hydroxylated at position 5 of the thiophene ring by rat liver microsomes.

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