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METABOLIC HYDROXYLATION OF THE THIOPHENE RING : ISOLATION OF 5-HYDROXY-TIENILIC ACID AS THE MAJOR URINARY METABOLITE OF TIENILIC ACID IN MAN AND RAT

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Abstract—The metabolism of tienilic acid, a drug containing a thiophene ring, was reinvestigated in man, rat and dog. The major urinary metabolite in man and rat was isolated and completely characterized by comparison with a synthetic compound. This metabolite derives from the hydroxylation of the thiophene ring of tienilic acid in position 5. Its isomers, 3- and 4-hydroxy-tienilic acids, were synthetized but could be detected neither in man nor in rat urine.

Because of its particular behaviour toward electrophiles, 5-hydroxy-tienilic acid was found to react with diazomethane with the formation of a complex mixture of methylated products. This made difficult its measurement by a previously described GLC technique, after acidic extraction and methylation by diazomethane. A new very simple assay using HPLC and direct injection of urine is described in this paper. This assay led to a very precise and reproductible determination of tienilic acid and its hydroxylated metabolite in urine.

Up to 50% of tienilic acid is excreted in man or rat urine as 5-hydroxy-tienilic acid whereas this metabolite does not appear in dog urine. These data describe the first example of metabolic hydroxylation of the thiophene ring.

Metabolic activation of the benzene ring is now well documented, its hydroxylation by cytochrome P-450 dependent monooxygenases occuring very often with the intermediate formation of an arene oxide [1]. Several reports have also been devoted in the literature to metabolic activation of the furan ring [2–5]. However, very little is known on the metabolism of thiophene derivatives, only thiophene itself having been studied in that context [6]. The only metabolites of thiophene that have been so far identified are thienyl mercapturic acids which may be derived from reaction of glutathione with an intermediate thiophene 2,3-oxide [6].

We have been interested in the metabolism of tienilic acid, *1*, a drug containing a thiophene ring, which is the first natriuretic exhibiting at the same time uricosuric properties [7–8]. The metabolism of this drug had been studied in dog, mice, rat, pig [8–9] and man [10–13], and two metabolites had been identified in urine: the benzylic alcohol *2* coming from the reduction of the keto group and the diacid *3*. In dogs, metabolite *2* accounts for 85% of the administered dose whereas in rat, pig and man it only accounts for 5–30% of the dose. In rat and pig, a high proportion of the total urinary metabolites, respectively 70 and 60%, had not been identified. It was thus important to reconsider tienilic acid metabolism and to examine if the thiophene moiety is oxidized. Recently, Vinay *et al.* [10] have studied tienilic acid metabolism in man and shown that a metabolite different from *2* and *3* (Fig. 1) is formed. They proposed for it, on the basis of its mass spectrum, a structure where the keto group is reduced to the corresponding alcohol and where the thiophene group is hydroxylated. However, the site of hydroxylation could not be precised and no definite structural proof was given. We have thus undertaken a study of tienilic acid metabolism in order: (i) to isolate its major metabolite in human urine and to establish its structure unambiguously in order to know the major route of oxidative metabolism of the thiophene ring (ii) to determine the best assay for tienilic acid metabolites, based on their intrinsic properties, and (iii) to compare its metabolism in man and in various laboratory animals to find the best animal model for metabolic activation of tienilic acid in man.

MATERIALS AND METHODS

^{1}H NMR spectra were obtained on a Cameca 250 MHz spectrometer, and *mass spectra* on a Ribermag R10–10 quadrupole mass spectrometer with a field desorption direct introduction probe and either an electron impact mode or a chemical ionization (NH_3 or CH_4) mode. Gas chromatography (GC)-mass spectrometry (MS) analysis were performed with the same instrument coupled to a Girdel gas

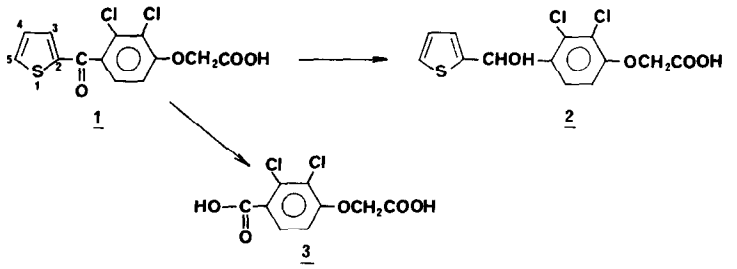

Fig. 1. Metabolites of tienilic acid previously found in human urine.

chromatograph by using a fused silica capillary column (Cpsil 5, 40 m × 0.32 mm, Chrompak).

Synthesis of 3- and 5-hydroxy-tienilic acids. The aldehyde *7* (Fig. 2) was obtained from 2,3-dichloro-4-hydroxy benzoic acid, upon reduction by $LiAlH_4$ in tetrahydrofuran, selective alkylation by t-butyl-bromoacetate in CH_3CN of the phenol group and oxidation of the alcohol function by CrO_3. Reaction of *7* with the lithium derivative *8* or *9*, in ether at −78°, afforded respectively, after purification by chromatography (SiO_2), compounds *10* and *11* in 50 and 32% yield. Oxidation by MnO_2 gave *12* (yield: 75%), melting point (m.p.) of 103° and *13* (yield 85%, m.p. = 103°). Deprotection of their acidic and phenolic functions upon treatment by pure CF_3COOH, gave respectively *6* (yield 75%, m.p. = 209°) and *4* (yield 95%, m.p. = 220°). Both compounds gave satisfactory elemental analysis (C, H, N, Cl) and mass spectra (*6*: $m/z = 346\ M^{+\cdot}$ (8%), 311 (100%) other fragments at 247, 189, 127); *4*: m/z = 346 (1.5%), 162 (100%) other fragments at 311, 247, 206, 189, 126, 98, 73). Their ^1H NMR and u.v.-vis characteristics are indicated in Table 1.

Synthesis of 4-hydroxy-tienilic acid (Fig. 3). Reaction of the acid chloride *14* with 2,3-dichloroanisole afforded the ketone *16* (yield 85%), the ester function of which was saponified. The corresponding hydroxy-thiophene function was methylated upon treatment by CH_3I and the obtained dimethoxyketone demethylated by $AlCl_3$. The resulting compound *17* was then treated by t-butylbromoacetate in CH_3CN in the presence of K_2CO_3 giving a mixture of mono and dialkylated derivatives which were separated by silicagel chromatography. This led to the t-butyl ester of *5* (20% yield). Hydrolysis of its ester function with CF_3COOH gave *5* (yield 95%, m.p. = 222°C) which showed satisfactory elemental analysis (C, H, N, Cl) mass spectrum (*5* m/z = 346 (5.5%), 69 (100%), other fragments at 311, 247, 189, 127, 60) and ^1H NMR and u.v.-visible characteristics (Table 1).

Reaction of hydroxy-tienilic acids with diazomethane. Compounds *5* and *6* reacted with diazomethane (excess) in ether at 20° to give the corresponding derivatives *5a* and *6a* where the phenol and carboxylic acid functions are both methylated. Compounds *5a* and *6a* gave elemental analysis (C, H, N, Cl), mass and ^1H NMR spectra in complete agreement with the proposed structures: *5a*, ^1H NMR ($CDCl_3$, δin ppm vs $Si(CH_3)_4$) : 3.76 (3H, *s*, OCH_3), 3.78 (3H, *s*, $COOCH_3$), 4.75 (2H, *s*, CH_2), phenyl H : 6.76 and 7.25 (1H each, *d*, J = 8.5 Hz), thiophene H : 6.7 and 7.02 (1H each *d*, J = 1 Hz); mass spectrum [$m/z = 374\ M^{+\cdot}$ (34%), 261 (25%), 141 (100%), other fragments at 339, 273, 98].

6a, ^1H NMR = 3.8 (3H, *s*, OCH_3), 3.85 (3H, *s*, $COOCH_3$), 4.81 (2H, *s*, CH_2), phenyl H : 6.84 and 7.28 (1H each, *d*, J = 8.5 Hz), thiophene H : 6.89 and 7.69 (1H each, *d*, J = 5.3 Hz); mass spectrum [$m/z = 374\ M^{+\cdot}$ (11%), 339 (36%), 261 (8%), 141 (100%), other fragments at 127, 98].

Compound *4* reacted with diazomethane in identical conditions leading to a mixture of 3 isomers *18*, *19* and *20* (Fig. 5). Their structures were established by elemental analysis (C, H, N, Cl), mass spectrometry and ^1H NMR spectroscopy.

18 : ^1H NMR : 4.01 (3H, *s*, OCH_3), 3.84 (3H, *s*, $COOCH_3$), 4.8 (2H, *s*, CH_2), phenyl H : 6.82 and 7.30 (1H each, *d*, J = 8.5 Hz), thiophene H : 6.28 and 7.15 (1H each, *d*, J = 4.5 Hz); m/z = 374 ($M^{+\cdot}$, 60%), 141 (100%), other fragments at 339, 315, 299, 273, 261.

19 : ^1H NMR : 3.65 (3H, *s*, OCH_3), 3.89 (3H, *s*, $COOCH_3$), 4.86 (2H, *s*, CH_2), phenyl H : 6.9 and 7.28 (1H each, *d*, J = 8.5 Hz), thiophene H : 7.16 and 6.28 (1H each, *d*, J = 6 Hz).

20 : ^1H NMR : 3.64 (3H, *s*, OCH_3), 3.88 (3H, *s*, $COOCH_3$), 4.84 (2H, *s*, CH_2), phenyl H : 6.87 and 7.32 (1H each, *d*, J = 8.5 Hz) thiophene H : 8.18 and 6.18 (1H each, *d*, J = 6 Hz). Mass spectrum of a mixture of *19* and *20* : m/z = 374 ($M^{+\cdot}$, 70%), 339 (100%), 324, 266, 261, 251, 223, 142 and 127.

Human subjects. Studies were performed on 5 male and 5 female healthy volunteers aged between 24 and 40 years who were all non-smokers and did not refrain to smoke. Seven have taken no drug at least one month before the experiment, two declared to have taken no drug one week before and one woman was using contraceptive pills. The subjects were given one oral water load of 200 ml before taking tienilic acid and their urine was taken as a control. Then, they took a single oral dose of tienilic acid (250 mg) and 100 ml water every two hours to insure a correct hydration. Their urine was collected for given periods.

Treatment of animals. Male adult Sprague–Dawley rats (200 g) were given 30 or 100 mg/kg tienilic acid either orally or i.p. Urine was collected for 0–6, 6–24 and 24–48 hr. Some rats were pretreated with phenobarbital (80 mg/kg/day in saline for 4 days) or

with 3-methyl cholanthrene (20 mg/kg/day in corn oil for 3 days). After one day rest they were given 100 mg/kg tienilic acid i.p. and urine was collected.

Dogs were given 100 mg/kg tienilic acid orally as previously described [9].

GLC analysis of urinary metabolites of tienilic acid. The used GLC method was derived from that described by Desager *et al.* [13] and Hwang *et al.* [12], except that 5-chlorotienilic acid was used as standard.

A 5 ml urine sample was acidified (pH 2) with 1 M HCl and extracted twice with 3 ml ethylacetate. The organic phase was dried with Na_2SO_4 and reduced to 0.2 ml by solvent evaporation; the internal standard was added and then excess diazomethane (prepared from Diazald, Aldrich) in ether. After 10 min at 20°, an aliquot was silylated with 50 μl of bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethyl-silylchloride for 1 hr at 60°.

GLC analysis was either made on a glass packed column (2 m × 4 mm, SE 30 3% on chromosorb W) from 215 to 280° (5° per min) or on a fused silica capillary column (10 m × 0.32 mm, Cpsil 5 Chrompack) at 280° (helium, 25 cm/s). The method of Vinay *et al.* [10] (extraction with $CHCl_3$-ether 1 : 1, diazomethane treatment at 0°) was also used for comparison, but gave identical results.

HPLC analysis of urinary tienilic acid metabolites. HPLC analysis of the urine of rats treated by radioactive tienilic acid (^{14}C on the carbonyl group) custom synthesized by C.E.A. (France), by the technique previously described [11], gave three radioactive peaks corresponding to the solvent front and to compounds *1* and *2*. A modification of this procedure allowed the separation of 4 radioactive peaks corresponding to compounds *1*, *2*, *3* and *4*. The best separation was obtained with the following conditions : column : ultrasphere (150mm × 4.6 mm) with a precolumn, eluent: 0.1 M ammonium formate pH 4 and CH_3CN, either in gradient elution (0–40% in 10 min) or, for the quantitative determination of 5-hydroxy tienilic acid, with 24% CH_3CN (u.v. detection $\lambda = 380$ nm) and, for the quantitative determination of compounds *1* and *2*, with 43% CH_3CN (u.v. detector, $\lambda = 254$ nm). Direct injection of 20 μl of urine with a loop valve and integration with an electronic integrator (Hewlett–Packard) gave a very good precision and reproducibility of the determinations (less than 5% error).

For preparative isolation of the metabolites, the method was further modified to avoid the buffer salts. A Zorbax ODS column (250 × 9.6 mm) was used with a mixture H_2O : CH_3OH (92 : 8) as the eluent. Four hundred microliters of filtered urine were injected repetitively. From 8 ml of rat urine, 5 mg of metabolite *4* was thus obtained after lyophilisation of the solvent.

For the HPLC analysis of compounds *18*, *19* and *20*, the used system was : an Altex Ultrasphere silica column (250 × 4.6 mm), or, for preparative separation, a Whatman Partisil 5μ column (200 × 9.6 mm) with either CH_2Cl_2 : CH_3COOEt 97 : 3 or hexane : tetrahydrofuran 80 : 20 as eluents.

RESULTS

Study of tienilic acid metabolites in human urine by HPLC. Urine from human volunteers treated with tienilic acid was analyzed by high-pressure liquid chromatography (HPLC) using a modification of the technique of Randolph *et al.* [11]. By gradient elution with a proper buffer ionic strength four peaks containing the dichlorophenoxyacetic moiety of tienilic acid, as shown by mass spectrometry, were observed. The corresponding metabolites were isolated by semi-preparative HPLC allowing to assign the first (with the weakest retention time), third and fourth peaks respectively to compounds *3*, *2* and *1*. The second peak corresponded to an unknown metabolite the structure of which was determined by ^1H NMR spectroscopy and mass spectrometry. The molecular peak of its mass spectrum ($M^+ = 346$) was indicative of a monohydroxylated metabolite of tienilic acid and the analysis of the fragments suggested that the hydroxylation had occurred on the thiophene ring. Its ^1H NMR spectrum in d_6-dimethylsulfoxide showed two doublets ($J = 4$ Hz) for the thiophene protons, one of them being easily exchanged by D_2O (Table 1). Taking into account the relatively large coupling constant between the thiophene protons [14], two structures deriving from the hydroxylation of the thiophene ring of tienilic acid either in position 3 or in position 5 remained possible. Thus, we decided to synthetize by non ambiguous techniques the different isomers derived from tienilic acid by hydroxylation of the thiophene ring.

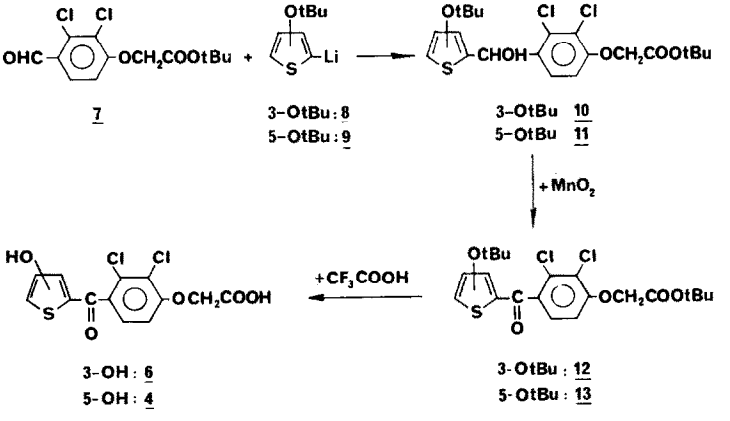

Fig. 2. Synthetic route to 3- and 5-hydroxy-tienilic acids.

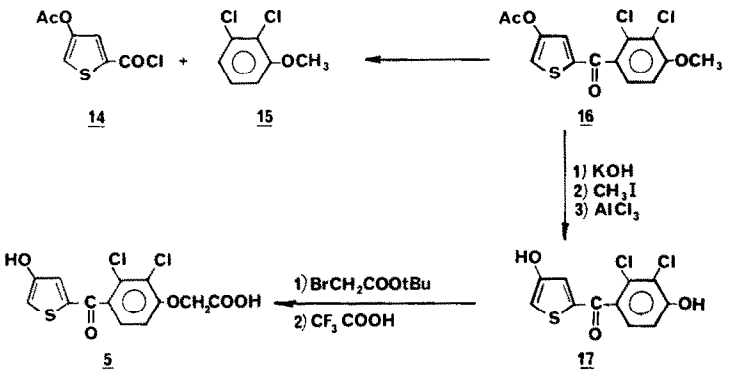

Fig. 3. Synthetic route to 4-hydroxy-tienilic acid.

3-Hydroxy-tienilic acid, *6*, and 5-hydroxy-tienilic acid, *4*, were prepared by condensation of the corresponding lithiated t-butyloxythiophene with the appropriate substituted benzaldehyde *7* [15], oxidation of the resulting benzylic alcohol by MnO_2 and deprotection of the phenol and acid functions by acidic treatment (Fig. 2).

4-Hydroxy-tienilic, *5*, was prepared by condensation of 2,3-dichloroanisole with the 4-acetoxy-thiophene-2-carboxylic acid chloride, *14*, liberation of the two phenol groups, reaction of the resulting diphenol *17* with $BrCH_2COOtBu$ and acid hydrolysis of the t-butylester of compound *5*.

Comparison of the ^1H NMR and u.v.-visible spectra (Table 1) and of the HPLC retention times (Fig. 4a) of the synthetic compounds *4*, *5* and *6* with the unknown metabolite of tienilic acid definitely showed that this metabolite is 5-hydroxy-tienilic acid *4*. Moreover, compounds *5* and *6* could not be detected by HPLC in urine of volunteers treated by tienilic acid, establishing that hydroxylation of the thiophene ring occurs mainly, if not exclusively, in position 5.

Study of tienilic acid metabolites in human urine by GLC. Tienilic acid and its metabolites had been previously studied by GLC [10, 12, 13] after (i) extraction of urine at pH 2 by an organic solvent, (ii) methylation of the acidic functions by diazomethane and (iii) further silylation of the alcohol functions by bis-trimethylsilyltrifluoroacetamide (BSTFA). By using this technique, Desager *et al.* reported a quantitative analysis of tienilic acid *1* and of its two metabolites *2* and *3* [13]. With a similar assay, Vinay *et al.* found another metabolite (Y) for which they proposed, on the basis of its mass spectrum, a structure derived from tienilic acid methyl ester by reduction of its ketone group and silylation of the corresponding alcohol and by hydroxylation of its thiophen ring and silylation of the corresponding phenol [10]. We repeated their experiments and actually found, by using their conditions, a new major peak different from those of compounds *1*, *2* and *3*. However, a GLC-MS analysis of this peak failed to give us the reported mass spectrum of *Y*. Moreover, we looked for the presence of *Y* in human

Table 1. Comparison of the ^1H NMR and u.v.-visible spectra of the main metabolite of tienilic acid and of compounds *4*, *5* and *6*

	^1H NMR*			u.v. - visible‡	
	OCH_2COOH	Phenyl H	Thiophene H	λ (nm)	(ε) (cm^{-1} mM^{-1})
4	4.92 (s)	7.14 7.46 (d,J = 8.5Hz)	7.22 6.15† (d,J = 4 Hz)	381 262 228	(27.0) (8.51) (14.0)
5	4.7 (s)	6.82 7.3 (d,J = 8.5Hz)	6.71 7 (d,J = 1.5Hz)	338 272	(5.68) (10.6)
6	4.92 (s)	7.08 7.36 (d,J = 8.5Hz)	6.73 7.86 (d,J = 5 Hz) 7.22 6.71 6.73 7.22	367 286	(9.74) (9.24)
Main metabolite	4.92 (s)	7.14 7.46 (d,J = 8.5Hz)	7.22 6.15† (d,J = 4 Hz)	380 262 228	(27.2) (8.6) (13.8)

* ^1H NMR in DMSO d6 at 30°, δ in ppm vs Si $(CH_3)_4$.
† Exchangeable by D_2O.
‡ u.v.-visible in buffer 0.1M ammonium formate pH 4.0.

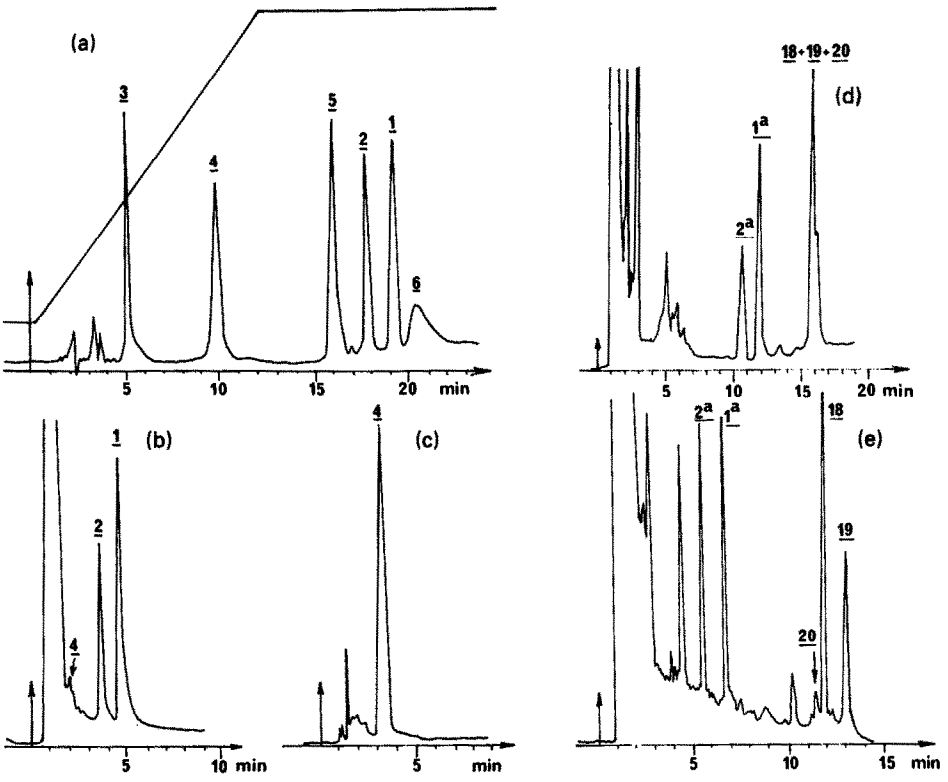

Fig. 4. HPLC and GC analysis of tienilic acid and its metabolites. (a) HPLC profile of a mixture of authentic samples of *1*, *2*, *3*, *4*, *5* and *6* (2 μg each). Conditions: Ultrasphere ODS (150 × 4.6 mm); Gradient elution (A:HCOONH$_4$ buffer 0.1 M; pH 4.0 + B:CH$_3$CN, from 17% B to 39% B in 12 min, flow 1 ml/min). U.v. detection at $\lambda = 254$ nm. (b) HPLC analysis of *1* + *2* in the urine of a volunteer. Injection of 20 μl filtered urine; conditions of (a) except for isochratic elution with 40% B. (c) HPLC analysis of *4* in the same urine. Similar conditions as in (b) except for isochratic elution with 24% B and u.v. detection at $\lambda = 380$nm. (d) GC analysis of an extract of human urine after methylation with diazomethane and silylation with BSTFA. Glass column SE 30 3% on Chromosorb W (2 m × 4 mm). Eluent N_2 at 30 ml/min. temperature gradient 215 to 280° at 5°/min. *1a* means here tienilic acid methylester. *2a* corresponds to the methyl ester and trimethylsilylether of *2*. (e) GC analysis of a similar extract with a capillary fused silica column Cp Sil 5 (10 m × 0.32 mm). Eluent He at 25 cm/sec at 280°. *1a* and *2a* as in (d).

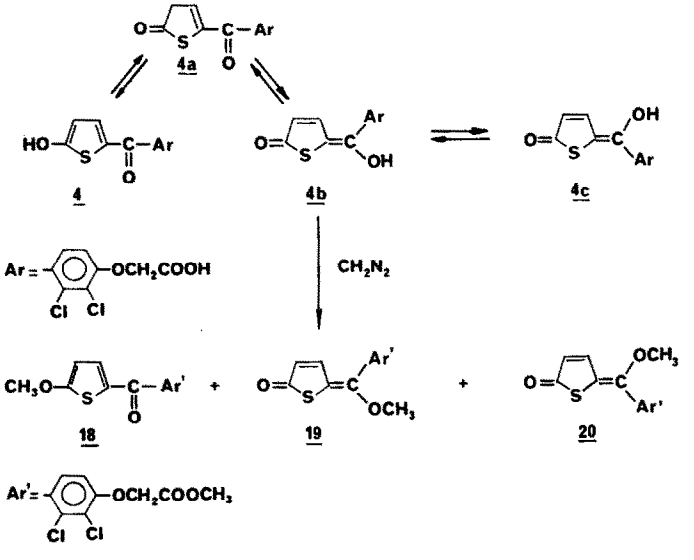

Fig. 5. Products resulting from treatment of *4* by diazomethane.

urine but could detect neither *Y* nor its main fragments even by mass fragmentometry. As the new peak of the GLC spectrum (Fig. 4d) was not well defined and badly tailing, we made the analysis on a fused silica capillary column and observed that this peak was resolved into three sharp peaks with an interconnective tailing plateau indicative of decomposition or isomerisation of a product on the column (Figs. 4d and e). GC-MS analyses give similar mass spectra all along this peak cluster. All these spectra were characterized by a molecular ion at $M^+ = 374$, corresponding to a methoxy tienilic acid methyl ester, by a peak at $m/e = 261$ corresponding to the dichlorophenoxy acetic acid methyl ester fragment, and by a peak at *m/e* : 141 or 142 corresponding to a methoxy-thenoyl fragment. These data indicated that all the compounds leading to the observed peaks cluster were isomers with a methoxy group on the thiophene moiety. Actually, the GLC analysis of an authentic sample of compound *4* after treatment by diazomethane in the same conditions led to an identical set of peaks (Fig. 4e). The compounds corresponding to the three main peaks were isolated by HPLC and studied by ^1H NMR and mass spectrometry. The major component was identified to the methyl ester of 5-methoxy-tienilic acid *18* and the minor components to compounds *19* and *20* (Fig. 5).

These results showed that treatment of compound *4* by diazomethane led to three products : the expected compound *18* derived from insertion of CH_2 into the O—H bond of the phenol group of *4* but also the compounds *19* and *20* derived from insertion of CH_2 into the O—H bond of the enol group of 4 tautomers (Fig. 5), *4b* and *4c*.

Moreover, we found that, while compound *4* added to control urine was stable for hours at room temperature, it was not very stable after extraction at pH 2 before diazomethane treatment. Actually, when this treatment was delayed, one found that about 50% of *4* is lost after 0.5 hr. This disappearance of compound *4* is presumably due to the involvement of the tautomeric form *4a* (Fig. 5) which has a methylene group very reactive, especially in acidic conditions [16], with compounds containing a ketone or aldehyde function that could exist in the assay medium such as tienilic acid itself, endogenous compounds from urine or the extraction solvent (ethylacetate). In fact, one found that compound *4* reacted with acetone at room temperature, *4* having completely disappeared after 2 hr.

Thus, the analysis of tienilic acid metabolites from urine by GLC after methylation by diazomethane exhibits several disadvantages : (i) the main metabolite *4* leads to several methylated products, (ii) some of these products, *19* and *20* are partly decomposed in the GLC used conditions explaining the complex pattern observed on Fig. 4(e), (iii) compound *4* is reactive in the acidic medium used for its extraction requiring that the diazomethane treatment is done immediately after extraction.

Whereas the GLC method seemed not suitable for correct quantitation of *4* in urine, the aforementioned HPLC method appeared very convenient since it allowed a direct quantitation of compounds *1*, *2*, *3* and *4* upon injection of urine itself and without any acidic treatment, solvent extraction and derivatization. This HPLC method was thus used (Fig. 4b, c) in the following studies and found to give very precise (error less than 5%) and reproducible quantitative determinations of tienilic acid and its metabolites in urine.

Urinary excretion of tienilic acid and its metabolites in man. By using the above described HPLC technique, we have determined the levels of tienilic acid and its metabolites in the urine of ten volunteers. All volunteers were found to eliminate tienilic acid rapidly, compounds *2* and *4* being the main metabolites. As shown in Table 2, 50–70% of the dose (250 mg of *1* administered orally) was eliminated as *1* + *2* + *4* after 6 hr and 60–88% after 24 hr. 5-Hydroxy-tienilic acid *4* accounted for more than 50% of the excreted products in urine, its proportion being as high as 70% in two volunteers. Anyway, it was always found as the main urinary metabolite.

Treatment of urine with glucuronidase and sulfatase before the HPLC analysis did not change significantly the observed amounts of compounds *1*, *2* and *4*, indicating that glucuronides or sulfates of *2* and *4* are not present in urine. Some minor very polar metabolites (< 5%) have not yet been identified. In agreement with previous results [9, 10], a GLC analysis showed that metabolite *3* accounted for less than 1% of the dose after 24 hr.

Comparison of urinary excretion of tienilic acid metabolites in humans, dogs and rats. Compound *4* was also found as the main urinary metabolite of

Table 2. Measurement (HPLC) of tienilic acid and its metabolites in urine from humans, rats and dogs

	% of the dose excreted within 24 hr			
	Total = *1*+*2*+*4*	*1*	*2*	*4*
Humans*	78§ (58–88)‖	22 (15–36)	11 (6–18)	45 (32–56)
Rats†	42 (40–45)	8 (4–16)	2 (0.5–3)	32 (20–40)
Dogs‡	43	5.5	36	<1

* 250 mg of *1* orally; 10 subjects.
† 100 mg/kg of *1* i.p.; 10 rats.
‡ 100 mg/kg of *1* orally; pooled urine from 3 dogs.
§ Mean value
‖ Extreme values.

tienilic acid in rat. Upon administration of 100 mg/kg of *1* (i.p.), 42% of the dose was recovered in urine after 24 hr under the form of metabolite *4* (32%), metabolite *2* (2%) and tienilic acid (8%) (Table 2). When a lower dose of *1* (30 mg/kg) was administered orally, the amount of metabolite *4* excreted within 24 hr increases from 32% to about 44%. It is noteworthy that pretreatment of the rats with 3-methylcholanthrene or phenobarbital did not significantly modify the excretion pattern of tienilic acid and its metabolites (data not shown).

Very different results were obtained in dog. Traces of compound *18* could be detected in the urine of dogs treated by tienilic acid (100 mg/kg) only by GLC-MS fragmentometry. 5-Hydroxy-tienilic acid *4* was under the limit of detection of the HPLC technique. As previously reported [9, 17], compound *2* is the major urinary metabolite of *1* in dog (Table 2).

DISCUSSION

The aforementioned results report the first example of metabolic hydroxylation of a thiophene ring, and show that the hydroxylation of this ring in position 5 of tienilic acid is a major route of biotransformation of this compound in man and rat. In that respect, it is noteworthy that, in certain volunteers, up to 56% of administered tienilic acid was found in urine under the form of 5-hydroxy-tienilic acid. The other compounds, *5* and *6*, which could have been derived from the hydroxylation of the thiophene ring of tienilic acid, have been synthetized, like *4*, by unambiguous techniques, but have not been detected in the urine of man or rat.

5-Hydroxy-tienilic acid *4* is in tautomeric equilibrium with different conjugated keto derivatives (Fig. 5). The existence of tautomer *4a* was shown by the fast exchange of the proton in position 4 of metabolite *4* with D_2O (Table 1). These keto compounds have a nucleophilic character and can react with mild electrophiles either by the oxygen atoms of their phenol or enol function or by the carbon in position 4 of the thiophene ring [16]. This explains the complex mixture obtained upon treatment of *4* with diazomethane (Fig. 5) which makes difficult the analysis of this compound by GLC after methylation. This also explains the slow disappearance of *4* after extraction of urine by solvents at pH 2, presumably because of reaction of the active methylene group of *4a*, in acidic conditions, with electrophilic compounds such as aldehydes or *1* itself or other ketones. Because of these intrinsic properties of *4*, its measurement in urine by HPLC after direct injection was found by far superior to its measurement by GLC after acidification, solvent extraction and methylation by diazomethane.

The above described results on urinary excretion of tienilic acid and its metabolites in man, dog and rat are consistent with those previously described in the literature [9, 12, 13, 17] if one takes into account that the previously unknown tienilic acid metabolite is 5-hydroxy-tienilic acid. As previously indicated [9] compound *2* is the major urinary metabolite of *1* in dog contrary to man and rat where *4* is the main metabolite.

Our results clearly show the importance of the hydroxylation of the thiophene ring of tienilic acid in man, suggesting that such a metabolic pathway should be considered for other drugs containing a thiophene ring. They also lead to a rational method of determination of 5-hydroxy-tienilic acid in urine, allowing to avoid artefacts due to the particular reactivity of this compound. Finally, they show that tienilic acid metabolism is similar in man and rat at least when one takes into account the products eliminated in urine.

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