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METABOLISM OF TOREMIFENE IN THE RAT

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Summary—Toremifene was labelled to a specific activity of about 20 μCi/mmol with tritium at positions 3 and 5 in the *para*-substituted phenyl ring. At these positions tritium is not eliminated within the metabolic pathways.

A mixture of unlabelled and labelled toremifene (5 or 10 mg/kg, 5 μCi/mg) was given i.v. or p.o. to Sprague–Dawley rats. The elimination of radioactivity was followed up by collecting urine and feces daily for 13 days. The elimination of toremifene which was similar after p.o. and i.v. administration took place mainly in the feces. About 70% of the total radioactivity was eliminated within 13 days, of this amount more than 90% in the feces. All applied radioactivity could be detected in three separate fractions according to the oxidative state of the side chain when counted by Berthold TLC Linear Analyzer. Each fraction was further separated into single metabolites by TLC or HPLC. Altogether 9 metabolites were identified and almost all methanol-extractable components were identified. The main metabolic pathways in the rat were 4-hydroxylation and *N*-demethylation. The side chain was further oxidized to alcohols and carboxylic acids. Small amounts of unchanged toremifene were found in the feces both after p.o. and i.v. administration indicating biliary secretion.

INTRODUCTION

Toremifene is a new compound and its metabolism has not been studied before. Toremifene structurally resembles tamoxifen and according to the chemical structure the same metabolic pathways can be expected. In the metabolic studies of tamoxifen ^{14}C-labelled tamoxifen was originally used [1]. The principal metabolic profile was thus obtained and several metabolites were identified by combining thin-layer chromatography and GLC–mass spectrometry. It appeared that tamoxifen was excreted mainly via feces and that total radioactivity had a long biological half-life in laboratory animals. However, only a small fraction of the total activity was identified. In later studies different methods were used: activation of tamoxifen and its metabolites by u.v. light and sensitive analysis of the resulting fluorescent phenanthrenes directly on TLC [2] or HPLC [3, 4]. The latter methods proved to be useful in analyzing the concentrations of tamoxifen and some of its metabolites in animal and human serum.

The aim of the present study was to clarify the metabolic profile of toremifene in the rat. To achieve this a new method was developed utilizing thin-layer chromatography and position-sensitive detection of radioactivity on the plate. A Berthold TLC Linear Analyzer was used and it appeared that all radioactivity could be localized on the plate. As TLC also purified the sample, identification of the metabolites by MS became possible. An almost complete metabolic profile of toremifene in the rat was thus obtained.

MATERIALS AND METHODS

Toremifene was synthetized in Farmos' chemical research laboratory (Oulu, Finland). It was used as a citrate salt and dissolved for dosing in solvent containing NaCl 8.65 g, PEG 3000 28.8 g, Tween 80 1.92 g, methyl-*p*-hydroxybenzoate 1.73 g and propyl-*p*-hydroxybenzoate 0.19 g in 1 l of distilled water. To avoid any precipitation of active compound only freshly prepared solutions were used. When dosing toremifene i.v. the solution was warmed (40°C) and mixed continuously with a magnetic stirrer.

Toremifene was labelled with tritium in positions 3 and 5 of the *para*-substituted phenyl ring. These sites are well suited for metabolic studies, because they do not participate in the metabolic reactions. All radioactivity can be recovered in different extraction and identification stages. The synthesis of tritiated toremifene was performed by tritiating the respective brominated derivative with Pd/C catalysis. The labile tritium atoms were washed and the stable product was purified by TLC. The specific activity of the final product was about 20 Ci/mmol and the radiochemical purity >95%.

Healthy female Sprague–Dawley rats (Alab Ab, Sollentuna, Sweden) weighing about 250 g were used in the studies. After dosing p.o. or i.v. (single dose or 1-week dosing p.o. to steady-state) the rats were immediately placed in single stainless steel metabolic

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Table 1. Excretion of total toremifene-derived radioactivity after p.o. and i.v. dosing

	Collection period of excretions (days after dosing)				
	0–1 (n = 20)	2–3 (n = 15)	4–6 (n = 10)	7–12 (n = 5)	13 (n = 5)
Dose i.v.					
Urine	2.1 ± 2.0	1.0 ± 0.5	0.1 ± 0.3	0.3 ± 0.3	ND
Feces	28.5 ± 8.7	27.4 ± 10.4	3.9 ± 2.2	1.4 ± 1.0	0.4 ± 0.5
Total	30.6	28.4	4.0	1.7	0.4
Cumulative	30.6	59.0	63.0	64.7	65.1
Dose p.o.					
Urine	1.5 ± 1.4	0.8 ± 0.4	0.3 ± 0.3	0.2 ± 0.2	ND
Feces	28.4 ± 11.9	24.3 ± 5.0	9.2 ± 6.8	7.2 ± 8.0	0.9 ± 0.3
Total	29.9	25.1	9.5	7.4	0.9
Cumulative	29.9	55.0	64.5	71.9	72.8

Dose of toremifene was 10 mg/kg and its radioactivity 5 μCi/mg. The values have been expressed as means ± SD of the total administered dose. ND = not detectable.

cages. Feces and urine were collected daily. The urine samples were collected in a tube in an ice bath. Feces samples were air dried, homogenized, weighed and stored at −20°C.

The total radioactivity of dried fecal homogenate was measured by burning an aliquot in a sample oxidizer (Junitek, Kaarina, Finland) and subsquent quantitation of the tritiated water by liquid scintillation counter (Model 81000, LKB-Wallac, Turku, Finland). For the metabolite analysis fecal homogenate was extracted 6 times with methanol and evaporated to dryness. After dissolving in 1 ml of methanol, the sample was applied onto a TLC plate (Silica Gel 60, Merck) as a line (Camag, Linomat II). The plates were developed using n-butanol–methanol (70:30) as eluent. Toremifene derived radioactivity was detected by a Berthold TLC Linear Analyzer. The radiogram showed typically three peaks (Fig. 5a), one of them consisted of amines, one of primary alcoholic and one of acidic compounds. Each metabolite group was scraped from the plate and extracted with methanol of identification of single metabolites. Amine metabolites were analyzed individually by second TLC (Silica Gel 60) with developing solvent chloroform:cyclohexane:triethylamine:ethanol (25:20:5:5). Primary alcohols were applied for identification to analytical RP-18 TLC plates and developed with acetonitrile:water (70:30) in pH 3 (HCl). Carboxylic acids were separated into individual metabolites on the similar plates as primary alcohols. Before direct injection to MS, the samples were purified by HPLC (Merck, LiChrosorb RP 18, acetonitrile 80%, water 20%, pH 3.0, formic acid). Before HPLC purification the carboxylic acids were esterified by HCl–methanol.

RESULTS

Excretion of total toremifene-derived radioactivity after i.v. and p.o. dosing (Table 1) shows that metabolism is similar after both dosing routes and indicates complete bioavailability of toremifene when given p.o.

The identification of toremifene metabolites was performed at two dose levels: 3 and 48 mg/kg. The dosing schedule was daily for 6 days p.o. According to the earlier calculated half-life of toremifene in the rat (14 h), this time was enough to reach the steady-state level in rat serum and feces. Total radioactivity of the feces daily (6 days) and 6 days after the dosing at the dose levels of 3 and 48 mg/kg is presented in Figs 1 and 2, respectively. The steady-state was reached within 6 days. The relative amounts of the three metabolic groups: amines, carboxylic acids and alcohols have been illustrated in Figs 3 and 4 at the dose levels of 3 and 48 mg/kg, respectively. The ratio of these three metabolic groups was similar at the

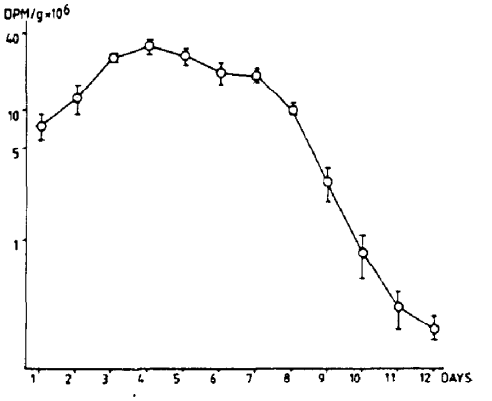

Fig. 1. Total radioactivity in feces vs time (dose 3 mg/kg per day). The 6th day is the last dosing day.

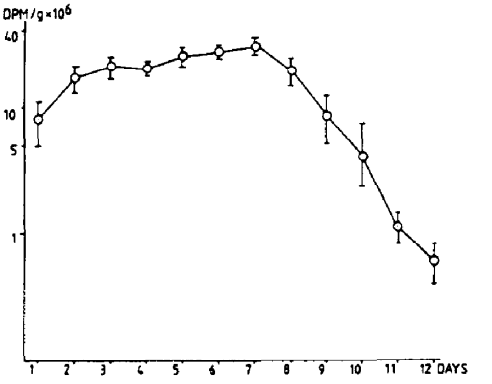

Fig. 2. Total radioactivity in feces vs time (dose 48 mg/kg per day). The 6th day is the last dosing day.

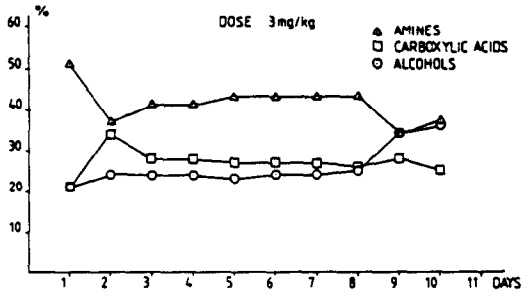

Fig. 3. Relative amounts of the three metabolic groups of toremifene during and after dosing (3 mg/kg per day). The 6th day is the last dosing day.

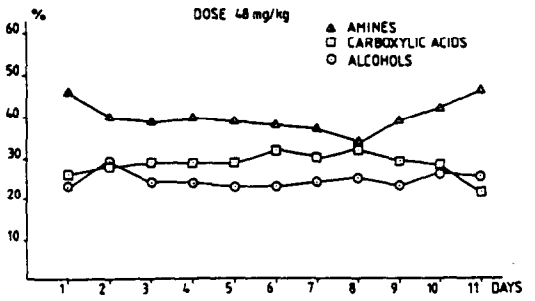

Fig. 4. Relative amounts of the three metabolic groups of toremifene during and after dosing (48 mg/kg per day). The 6th day is the last dosing day.

steady-state and after single dose with no significant difference between the dose levels of 3 and 48 mg/kg. Typical TLC chromatograms indicating the separation of metabolites have been shown in Fig. 5. The complete separation of the three main metabolic groups is shown in Fig. 5a. Each group was scraped from the plate and exposed to a second TLC. Figure 5b shows a typical TLC chromatogram of the amino metabolites. In the TLC chromatogram some radioactivity is regularly seen at the application site (peak 1) but the amount of the radioactivity is relatively small and the chemical structure of this radioactive peak is unknown. Figures 5c and d show typical TLC chromatograms of alcohols and acids, respectively. In the acid group there are two unknown major peaks. The structures have been identified as carboxylic acids by a mass spectrometer by indicating that they react with HCl–methanol producing the corresponding methyl esters. The structures have not been identified unambiguously by NMR analysis,

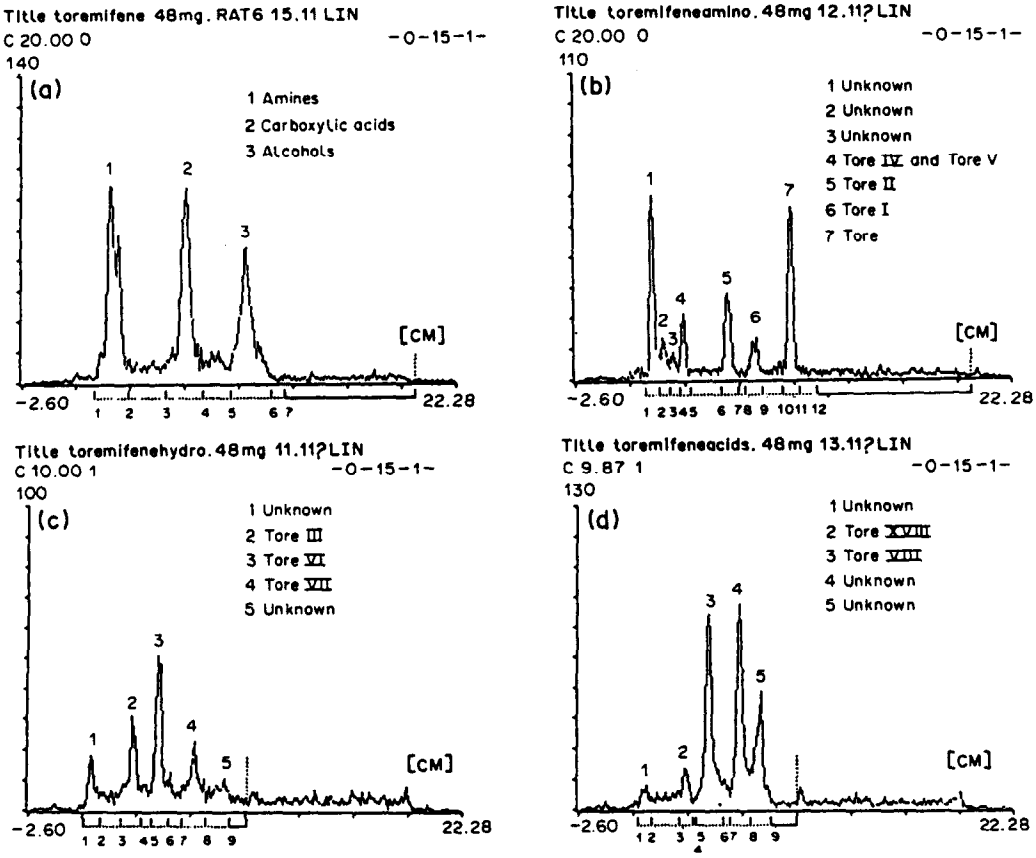

Fig. 5. Separation of toremifene and its metabolites by TLC. (a) A typical TLC-radiochromatogram of the methanol extract of feces where the three metabolic groups are well separated from each other. (b) A typical TLC-radiochromatogram of the separation of amines (peak 1 in Fig. 5a) to single metabolites. (c) A typical radiochromatogram of the separation of alcohols (peak 3 in Fig. 5a) to single metabolites. (d) A typical TLC-radiochromatogram of the separation of carboxylic acids (peak 2 in Fig. 5a) to single metabolites.

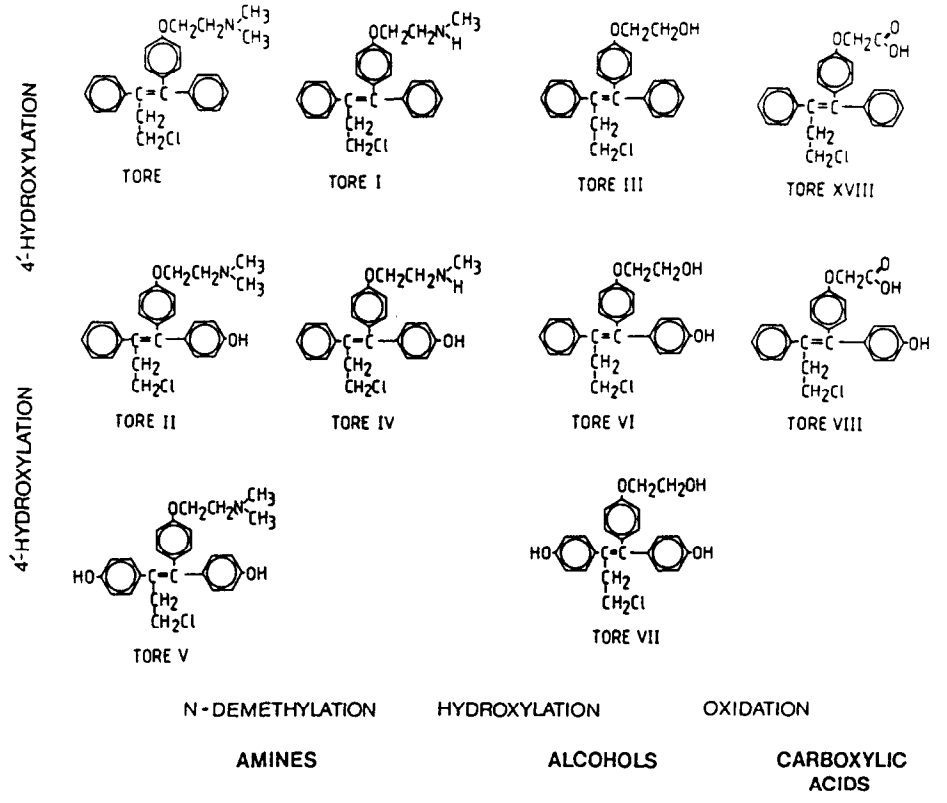

Fig. 6. The chemical structures of the identified toremifene metabolites in the rat feces. *Nomenclature:* TORE = toremifene; TORE I = *N*-demethyltoremifene; TORE II = 4-hydroxytoremifene; TORE III = (deaminohydroxy)toremifene; TORE IV = 4-hydroxy-*N*-demethyltoremifene; TORE V = 4,4'-dihydroxytoremifene; TORE VI = 4-hydroxy(deaminohydroxy)toremifene; TORE VII = 4,4'-dihydroxy (deaminohydroxy)toremifene; TORE VIII = 4-hydroxy(deaminocarboxy)toremifene; TORE SVIII = (deaminocarboxy)toremifene.

and the suspected reference compound has not been chemically synthetized yet. The radioactive samples have been used for quantitative determination of the metabolites and the MS and NMR analyses have been done from unlabelled steady-state samples. The metabolites were localized according to their fluorescence on TLC plates, scraped and reextracted with methanol. Finally their structure was confirmed with MS in comparison to synthetized compounds. Figure 6 shows the chemical structures of the identified metabolites of toremifene in the rat. The metabolic profile of toremifene was similar at the dose levels of 3 and 48 mg/kg.

The chlorine atom of toremifene was found in all analyzed material. This shows that chlorine does not participate in the metabolic pathways. One can conclude that toremifene and tamoxifen therefore have no identical metabolites.

DISCUSSION

The metabolic reactions of toremifene have been elucidated. This was achieved by using tritiated toremifene. In this case the tritium label seems to be very stable and is not decomposed or metabolized. With the developed all methanol extractable radioactivity could be localized on the TLC plates. By TLC the metabolites could first be divided to three groups: amines, alcohols and carboxylic acids. Each group could be further separated to individual metabolites. All the most important metabolites have now been characterized.

About 70% of the total radioactivity could be extracted with methanol (6 times) from the feces. The rest of the radioactivity may consist of the same metabolites that were found in methanol extract. The feces are porous material and perhaps absorb lipophilic compounds, so that it is probably impossible to extract 100% of toremifene-derived radioactivity from this kind of material. The treatment of feces with β-glucuronidase/arylsulphatase did not increase the extraction efficiency of the fecal extracts. Instead, bile contains conjugates of the metabolites because the extractability of radioactivity increased from 30 to 60% during β-glucuronidase/arylsulphatase treatment in preliminary studies. At present, however, the importance of conjugation in the extraction of toremifene metabolites has not been completely elucidated. Conjugates are probably hydrolyzed in the intestinal tract to unconjugated

metabolites which are reabsorbed and reconjugated. Fromson *et al.*[1] reported data for tamoxifen showing extensive amounts of conjugates in the feces. It seems that those data contain some artefacts for example carboxylic acid metabolites which are one major metabolite group for toremifene are not reported for tamoxifen. Similar types of acidic metabolites, one assumes, must also occur with tamoxifen.

The amount of toremifene-derived radioactivity in the urine was small when compared to feces (Table 1). This is in good agreement with tamoxifen studies [1]. We did not try to analyze conjugated and non-conjugated metabolites separately, because of the low amount of activity in the urine.

The metabolites of toremifene in human feces and urine are qualitatively similar to the rat metabolites, but there are quantitative differences in single metabolite profile. The result of human metabolism and the biological activity of the metabolites will be published elsewhere. The chlorine atom in the toremifene molecule is stable in metabolic reactions. Therefore tamoxifen and toremifene have no identical metabolites. The exact biological role of the chlorine atom in toremifene and in its metabolites cannot be defined at present. On the other hand, tamoxifen has carcinogenic potential in 12-month studies in the rat liver, but toremifene has not [5]. This difference is due to the chlorine atom: one may propose that it inhibits formation of biologically reactive metabolites.

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