Rapid On-line Profiling of Estrogen Receptor Binding Metabolites of Tamoxifen

Jeroen Kool,[†] Rawi Ramautar,[†] Sebastiaan M. van Liempd,[†] Joran Beckman,[†] Frans J. J. de Kanter,[†] John H. N. Meerman,[†] Tim Schenk,[‡] Hubertus Irth,[‡] Jan N. M. Commandeur,[†] and Nico P. E. Vermeulen[*,†]

Leiden Amsterdam Center for Drug Research (LACDR)/Division of Molecular Toxicology, Department of Chemistry and Pharmacochemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands, and Kiadis B.V., Zernikepark 6-8, 9747 AN Groningen, The Netherlands

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Here we present a high-resolution screening (HRS) methodology for postcolumn on-line profiling of metabolites with affinity for the estrogen receptor α (ERα). Tamoxifen, which is metabolized into multiple metabolites, was used as the model compound. Most of the 14 metabolites detected exhibited affinity for the ERα. The HRS methodology shows great potential for metabolite bio-affinity profiling and application in drug discovery and development.

Introduction

As biotransformation of drugs may lead to metabolites with different pharmacological and toxicological effects, profiling and screening for active metabolites are important in drug discovery and development to assess their contribution to the overall therapeutic and adverse effects on drugs.[1] While chemically reactive metabolites can also be named as active metabolites that might cause toxicological effects, in the context of this article active metabolites are primarily pharmacologically active metabolites. A major bottleneck in metabolic profiling remains in the detection and identification of active metabolites in complex metabolic mixtures.

Several years ago, a novel on-line high-resolution screening (HRS) (Figure 1) technology was developed, enabling screening of individual estrogenic compounds in mixtures.[2] HRS is based on separation technology, usually gradient HPLC, coupled on-line to a postcolumn biochemical assay. For on-line determination of estrogenic activity this assay is based on the interaction between the estrogen receptor (ER), more specifically the ERα, and the native fluorescent ligand coumestrol, which shows fluorescence enhancement when bound to the active site of the ERα. Estrogenic analytes injected into the HRS system bind to the ERα. Subsequently, the coumestrol added binds to the remaining free binding sites of the ERα, thereby showing a profound negative fluorescence enhancement.[2] This setup allows a sensitive way of detection,[3] while similar affinities are measured[2] as in real competitive assays, where receptor, radiotracer, and ligand are added simultaneously.

In the present study, this HRS-ERα technology was applied for screening and simultaneous identification of estrogenic metabolites of tamoxifen. Tamoxifen is a nonsteroidal antiestrogen that is used in the hormonal therapy of human breast cancer and it is widely used as a chemopreventive agent in women at risk for developing this disease.[4] Studies have shown that tamoxifen is metabolized by cytochrome P450s (Cyt P450s)[5] and Flavin-containing Monooxygenases (FMOs)[6] to multiple mono- and dihydroxylated and *N*-demethylated derivatives. In Table 1, the most important primary[5−13] and secondary metabolites[11,14−19] of tamoxifen are tabulated.

* Corresponding author. Tel.: +31 20 5987590. Fax: +31 20 5987610. E-mail: npe.vermeulen@few.vu.nl.
† Vrije Universiteit.
‡ Kiadis B.V.

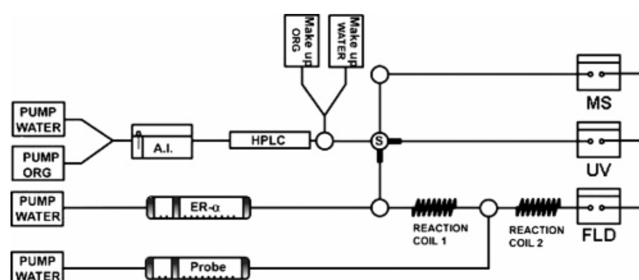

Figure 1. Schematic view of the HRS system: LC gradient with pump water and pump organic; makeup gradient with makeup pump water and pump organic; Flow split (S) to MS, UV detector, and ERα affinity assay; injection via an autoinjector (A.I.); ERα in superloop "ER-α" and probe ligand coumestrol in superloop "probe" are pumped into the ERα affinity assay with HPLC pumps. ERα affinity readout with a fluorescence detector (FLD).

Metabolite mixtures were generated by incubating tamoxifen with rat or pig liver microsomes. Pig liver microsomes were included because they were used for large-scale incubations to produce sufficient metabolites for additional NMR experiments. Metabolism of (*Z*)-4-hydroxytamoxifen, a potent antiestrogenic metabolite of tamoxifen, was also studied to identify secondary metabolites derived from this metabolite.

Results

On-line Estrogen Receptor α (ERα) Affinity Screening. Extracts (75 μL) from microsomal incubations were used and injected into the HRS system. Figure 2A shows the UV trace for tamoxifen and the metabolites formed after 90 min of incubation with rat liver microsomes. One major metabolite (peak 12) was observed at 18.2 min, whereas very small peaks (peaks 6, 8, and 14) were observed at 14.5, 15.0, and 23.5 min, respectively. The ERα affinity profile shown in Figure 2B, however, demonstrated that all four metabolites produced a strong ERα affinity signal. In addition, a significant ERα affinity signal was observed at 20.5 min (peak 13). The HRS analysis was repeated with a large volume injection (450 μL) to also enable detection of minor metabolites. The resulting UV chromatogram showed the appearance of at least three additional metabolites between 12 and 14 min (Figure 2C, peaks 1, 2, and 3). These three minor metabolites also showed ERα affinity responses (Figure 2D). Because of a complete occupation of the ERα upon injecting a large volume of the extract, the

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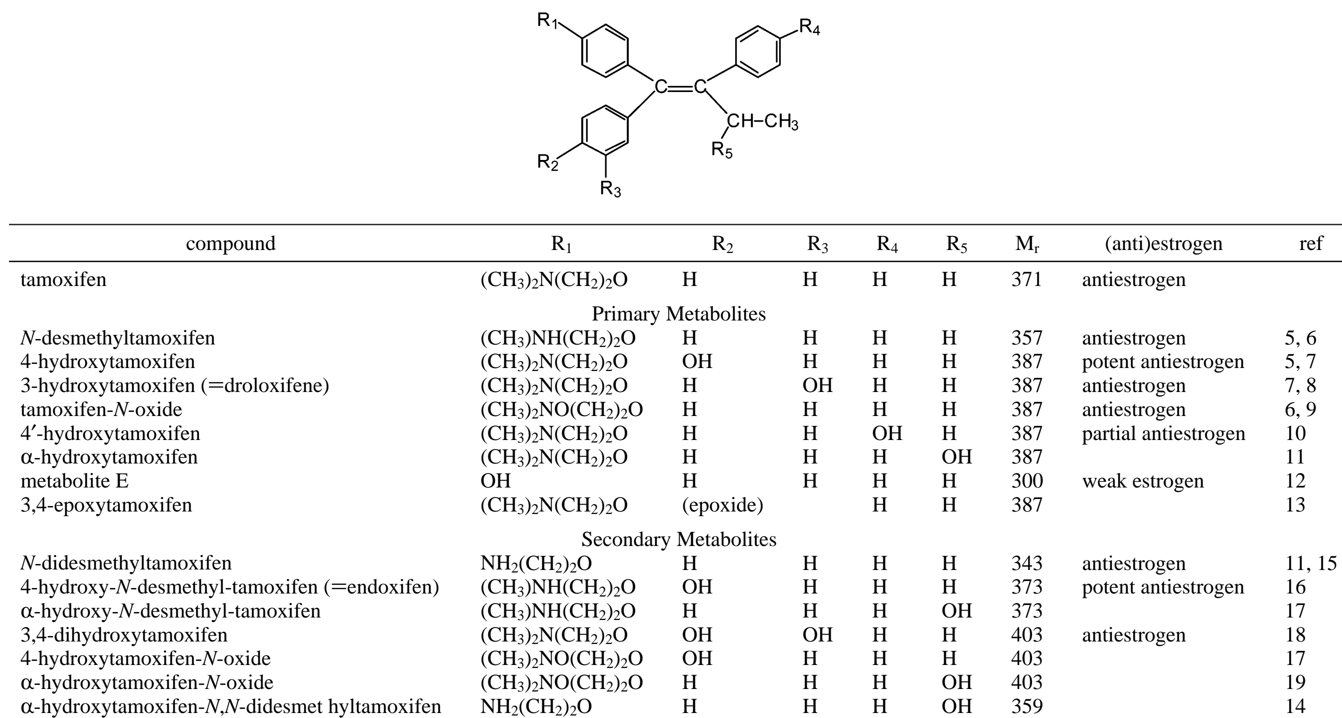
Table 1. Structures of Tamoxifen and Primary and Secondary Metabolites, As Derived from the Literature



compound	R_1	R_2	R_3	R_4	R_5	M_r	(anti)estrogen	ref
tamoxifen	$(CH_3)_2N(CH_2)_2O$	H	H	H	H	371	antiestrogen	
Primary Metabolites								
N-desmethyltamoxifen	$(CH_3)NH(CH_2)_2O$	H	H	H	H	357	antiestrogen	5, 6
4-hydroxytamoxifen	$(CH_3)_2N(CH_2)_2O$	OH	H	H	H	387	potent antiestrogen	5, 7
3-hydroxytamoxifen (=droloxifene)	$(CH_3)_2N(CH_2)_2O$	H	OH	H	H	387	antiestrogen	7, 8
tamoxifen-*N*-oxide	$(CH_3)_2NO(CH_2)_2O$	H	H	H	H	387	antiestrogen	6, 9
4′-hydroxytamoxifen	$(CH_3)_2N(CH_2)_2O$	H	H	OH	H	387	partial antiestrogen	10
α-hydroxytamoxifen	$(CH_3)_2N(CH_2)_2O$	H	H	H	OH	387		11
metabolite E	OH	H	H	H	H	300	weak estrogen	12
3,4-epoxytamoxifen	$(CH_3)_2N(CH_2)_2O$	(epoxide)		H	H	387		13
Secondary Metabolites								
N-didesmethyltamoxifen	$NH_2(CH_2)_2O$	H	H	H	H	343	antiestrogen	11, 15
4-hydroxy-*N*-desmethyl-tamoxifen (=endoxifen)	$(CH_3)NH(CH_2)_2O$	OH	H	H	H	373	potent antiestrogen	16
α-hydroxy-*N*-desmethyl-tamoxifen	$(CH_3)NH(CH_2)_2O$	H	H	H	OH	373		17
3,4-dihydroxytamoxifen	$(CH_3)_2N(CH_2)_2O$	OH	OH	H	H	403	antiestrogen	18
4-hydroxytamoxifen-*N*-oxide	$(CH_3)_2NO(CH_2)_2O$	OH	H	H	H	403		17
α-hydroxytamoxifen-*N*-oxide	$(CH_3)_2NO(CH_2)_2O$	H	H	H	OH	403		19
α-hydroxytamoxifen-*N*,*N*-didesmet hyltamoxifen	$NH_2(CH_2)_2O$	H	H	H	OH	359		14

resolution of the ERα affinity chromatogram was reduced profoundly due to tailing of the parent compound tamoxifen.

As shown in Figures 2E and 2F, all metabolites observed in incubations with rat liver microsomes, except for metabolite 1, were also observed after HRS analysis of pig liver microsomal incubations. The relative intensities of metabolites formed were significantly different, however. Most notably, the intensities of peaks 2 and 3 in pig liver microsomes were strongly increased when compared to those in the rat liver microsomes.

(*Z*)-4-Hydroxytamoxifen, previously considered as a metabolite of tamoxifen with the highest affinity toward the ERα,[10] was also incubated with rat and pig liver microsomes to identify possible secondary metabolites. Figures 3A and 3B show the UV trace and ERα affinity trace of an extract of a pig liver microsomal incubation. Three intense peaks, corresponding to metabolites with retention times of 13.1, 13.9, and 16.3 min, were observed with UV detection. These metabolites also showed a high response in the ERα affinity assay. In addition, a significant ERα affinity response was observed at 20.5 min, corresponding to metabolite 13, which was also observed in tamoxifen incubations (Figures 2B and 2F). The ERα affinity response observed at 16.3 min was caused by metabolites 10 and 11. The ERα affinity responses observed at 16.3 and 20.5 min were also seen with rat liver microsomes. The peaks at 13.1 and 13.9 min, however, were not observed in the rat liver microsomes (data not shown).

Time Course of Metabolite Formation. With use of the HRS system, the time course of the formation of the various tamoxifen metabolites was also determined in order to distinguish between primary and secondary metabolites. As is shown in Figure 4A, metabolites 6, 8, 12, and 14 were formed soon after the start of the incubation of tamoxifen with rat liver microsomes, thus indicating that these metabolites are likely primary metabolites. Metabolite 13 was clearly formed at a later stage during the incubation. With pig liver microsomes (Figure 4B), metabolites 6 and 12 again were formed early and were predominant species. Metabolites 8 and 14 showed similar time response profiles in pig liver microsomes when compared to those in the rat liver microsomes. Metabolite 13 was formed to a lesser extent and appeared later on in the incubations. A similar metabolic pattern was seen for metabolite 3, which was only present in significant amounts after 90 min of incubation in the pig liver microsomes, thus indicating that metabolites 3 and 13 are most likely secondary metabolites. A major species difference in the formation was also observed for metabolite 2. While this metabolite was only formed in minor amounts in rat liver microsomes, it was one of the important metabolites contributing to the observed ERα affinity in pig liver microsomes.

Identification of Tamoxifen Metabolites. A simultaneously operated mass spectrometer (MS) allowed the parallel identification of the metabolites measured with the on-line ERα affinity assay. The ion traces of the expected pseudomolecular ions ($[M + H]^+$) of previously identified metabolites (Table 1) can be found in the Supporting Information. We have identified the metabolites 1−13 by MS, and ^1H NMR where appropriate. A summary of all metabolites identified, together with their retention time, pseudomolecular ion, and corresponding fragmentation pattern of their pseudomolecular ion are listed in Table 2. More detailed information regarding the metabolite identification can be found in the Supporting Information.

Discussion

The present HRS technology allowed on-line ERα affinity detection and identification of 14 individual metabolites in rat and pig liver metabolic mixtures. The relative abundances and ERα affinities of the individual metabolites could rapidly be detected in a single run. Affinity screening technologies based on dereplication processes do also allow receptor affinity screening of individual components in mixtures. However, they are usually not capable of detecting low-affinity compounds in the presence of high-affinity compounds due to tailing effects. Other screening technologies based on binding of active metabolites to, e.g., the estrogen receptor followed by off-line centrifugal ultrafiltration also allowed active metabolite characterization by LC-MS.[20,21] Those technologies, however, lack

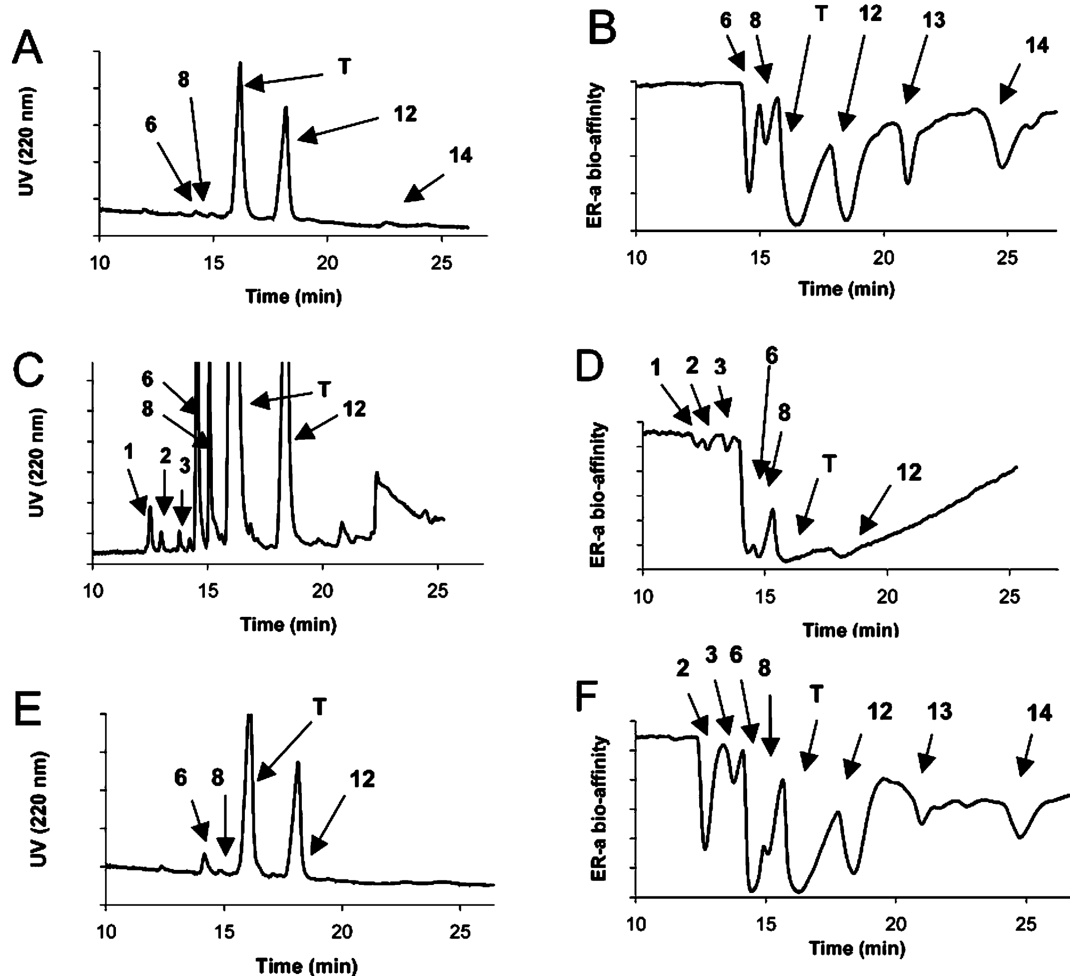


Figure 2. (A) HPLC chromatogram of tamoxifen incubated for 90 min with rat liver microsomes. Injection volume of 75 μL. (B) The corresponding ERα affinity trace. (C) HPLC chromatogram of tamoxifen incubated for 90 min with rat liver microsomes. Injection volume of 450 μL. (D) The corresponding ERα affinity trace. (E) HPLC chromatogram of tamoxifen incubated for 90 min with pig liver microsomes. Injection volume of 75 μL. (F) The corresponding ERα bio-affinity trace.

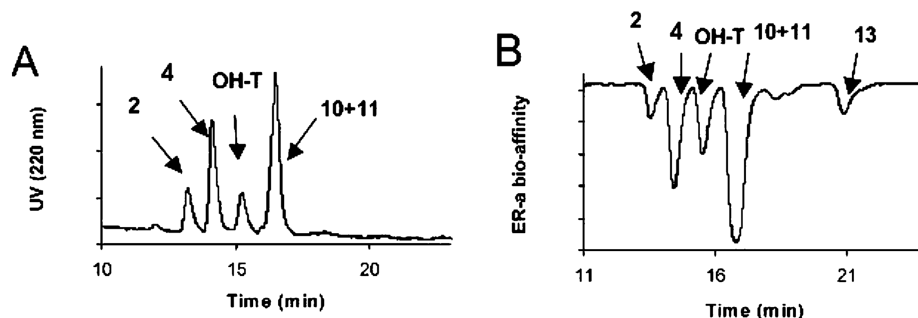


Figure 3. (A) HPLC chromatogram of (*Z*)-4-hydroxytamoxifen incubated for 180 min with pig liver microsomes. Injection volume of 75 μL. (B) The corresponding ERα affinity trace.

the ability to efficiently trap low-affinity ligands in the presence of high-affinity ligands or high concentrations of the parent compound.

The present results clearly showed that a wide range of tamoxifen metabolites contributed to the ERα affinity in microsomal liver fractions (Figure 2). Metabolite 13, identified as endoxifen, gave a significant contribution to the ERα affinity profile (Figures 2 and 3), despite the fact that it was present only in very low concentrations. The relevance of this metabolite in terms of ERα affinity was recently discussed by Stearns et al.[22] and Johnson et al,[16] who stressed the importance of this secondary metabolite for the antiestrogenic action of tamoxifen. Besides endoxifen (metabolite 13), we found that another metabolite (metabolite 14), present in low concentrations as well, contributed significantly to the ERα affinity. Further studies are needed to elucidate the structure and characteristics of this metabolite. Although the exact concentrations of the metabolites in the microsomal mixtures were unknown and although it is difficult to assess their relative abundance accurately based on the UV response, estimates about the relative ERα affinities based on abundance and corresponding affinity trace were made. Our results indicate that (*Z*)-4-hydroxytamoxifen and *N*-desmethyl-(*Z*)-4-hydroxytamoxifen are indeed metabolites with a high affinity compared to tamoxifen, which is widely described in the literature.[16] Besides these two metabolites, (*Z*)-4′-hydroxytamoxifen and metabolite 14 also show a high affinity

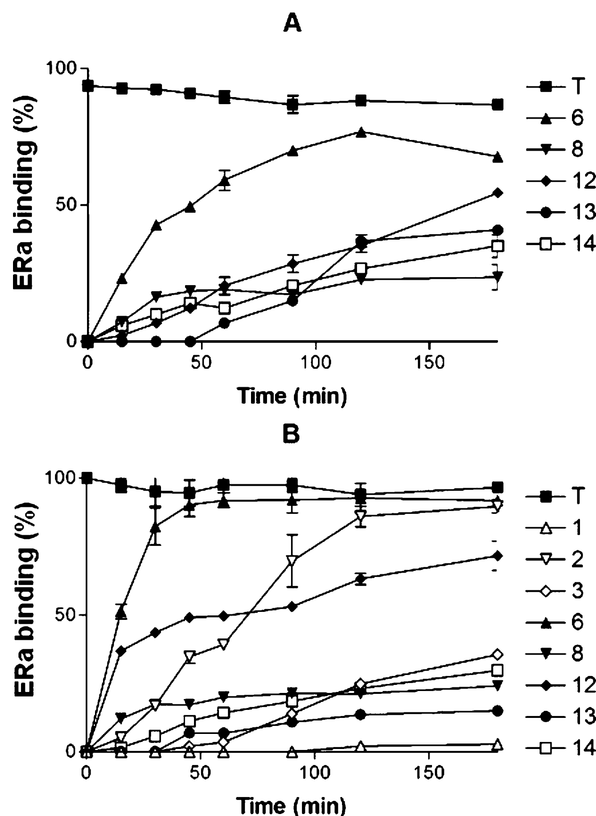


Figure 4. (A) Relative ERα binding of the metabolites formed in time, resulting from tamoxifen (T) with rat liver microsomes and (B) with pig liver microsomes.

for the ERα. The metabolites 2 and 3 and tamoxifen *N*-oxide show affinities similar to tamoxifen, while α-hydroxytamoxifen has a lower affinity. The di- and trioxygenated tamoxifen metabolites, which were formed from (*Z*)-4-hydroxytamoxifen, all show high affinities toward the ERα. These affinities are comparable to (*Z*)-4-hydroxytamoxifen. While affinities and/or (anti)estrogenicities for most metabolites have been described in the literature (Table 1), this study shows that also α-hydroxytamoxifen, at least three dioxygenated, two trioxygenated tamoxifen metabolites, and metabolite 14 show affinity for the ERα. Of these, only one dihydroxytamoxifen species (3,4-hydroxytamoxifen) with affinity for the ERα has been described in the literature to our knowledge.[23] The relative affinities of the other metabolites that were found in this study seem to correspond to those of the (anti)estrogenicities previously reported in the literature (Table 1).

Interestingly, the time dependency of metabolite formation in the microsomal mixtures could also be followed relatively easily and completely with the present HRS system (Figure 4). When rat and pig liver microsomal incubations were compared, similar metabolic profiles were found, although significant differences were seen in the formation rates of the metabolites. Generally speaking, pig liver microsomes produced more and faster metabolites contributing to the total ERα affinity.

Although this study only gives information regarding the ERα binding properties of tamoxifen and its microsomal metabolites, it can be envisioned that the metabolites formed also play a role in the complex selective estrogen receptor modulator (SERM) nature of tamoxifen.[24] Species differences in metabolic conversion[24] and tissue-specific metabolism[25] might also add to the complexity of explaining different effects seen with SERMs. Additional information in terms of functional activities of relevant metabolites binding toward the ERα (and ERβ) and species and tissue-specific metabolic profiles therefore would add to the explanation of the complex pharmacology of these SERMs.

Conclusions

The metabolites of tamoxifen formed upon incubation with rat or pig liver microsomes all showed ERα affinity. When (*Z*)-4-hydroxytamoxifen was incubated in rat and pig liver microsomes, five secondary metabolites with corresponding ERα affinities were detected.

It is concluded that the present on-line HRS technology allows the screening of metabolites in metabolic mixtures with ERα affinity in a very sensitive, selective, and rapid way. The HRS technology even allows to quickly screen active metabolite formation in time. This offers great perspectives for applications in drug discovery and development.

Experimental Section

Chemicals. β-Nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt was from Applichem (Lokeren, Belgium). Acetic acid (AcOH), methanol (MeOH), and acetonitrile (MeCN) were purchased from Baker (Deventer, The Netherlands). Both the MeOH and MeCN were of HPLC reagent grade. Tamoxifen and (*Z*)-4-hydroxytamoxifen were obtained from Sigma (Zwijndrecht, The Netherlands). Coumestrol was bought from Fluka (Zwijndrecht, The Netherlands). All other chemicals were of the highest grade commercially available.

Biological Materials. *Microsomes:* Rat and pig liver microsomes were prepared as described previously.[26] The protein concentration in the microsomes was 13 and 26 mg/mL, respectively. *Estrogen receptor*-α*:* The ligand binding domain (LBD) of the estrogen receptor-α (ERα; a kind gift of Dr. Marc Ruff, Laboratoire de Biologie et Génomique Structurales 1, IGBMC, Illkirch, France) was expressed in *E coli* according to Eiler et al,[27] but without estradiol in the medium. All subsequent steps were carried out at 4 °C. The cells were centrifuged at 4600 rpm for 60 min and the pellet was subsequently suspended in 50 mL of sodium phosphate buffer (10 mM; pH 7.4; adjusted with KOH) containing 150 mM NaCl. The cells were again pelleted at 4000 rpm for 15 min and the pellet was resuspended in 50 mL of the same buffer. This washing step was repeated twice. After the last washing step, the pelleted cells were suspended in 25 mL of the same buffer. Cells were disrupted by use of three French Press cycles (700 bar) followed by ultrasonic sound (microtip, 30% duty cycle output 7, 10 cycles). Finally, ultracentrifugation (100000*g*) for 1 h resulted in the soluble receptor in the supernatant. The ERα concentration (500 nM) was estimated by determining the B_{max} value (by titration with radio-labeled estradiol).[28] The B_{max} value was measured as the maximum amount of ligand binding extrapolated to a very high concentration of ligand. The soluble receptor stock solution was stored at −80 °C.

Microsomal Incubations. All liver microsomal incubations were performed according to Lim et al.[20] with slight modifications: 0.65 mM of NADPH was used instead of $NADP^+$. A potassium phosphate buffer (100 mM; pH 7.4) with 2 mM EDTA and 10 mM magnesium chloride was used. Incubations were performed with 200 μM tamoxifen. The microsomal protein concentration was 2.6 mg/mL both with the pig and rat liver microsomes. The incubation mixtures (20 mL) were extracted with 5 mL of ice-cold acetonitrile followed by two extractions with 10 mL of isopropyl ether each time. After evaporation, the residues were dissolved in 1 mL of 65% (v/v) aqueous ethanol and used directly for injection in the HRS system. A large-scale incubation (1 L) with pig liver microsomes (3 h) was done to obtain major metabolites for NMR identification. See Supporting Information for more details.

Chromatography. Unless stated otherwise, a 75 μL injection volume was used. The HPLC column was eluted at a flow rate of 250 μL/min with a mixture of H_2O:MeOH:AcOH (94.9:5:0.1) for 1 min. Subsequently, the MeOH concentration was increased to

Table 2. Tamoxifen and (*Z*)-4-Hydroxytamoxifen Metabolites Found upon Incubation of the Respective Parent Compounds in Rat and/or Pig Liver Microsomal Incubations[a]

metabolite no.	metabolite name	ret. time UV	$[M + H]^+$	MS/MS
1	α-hydroxytamoxifen	12.6	388	370, 325, 247
2	dioxygenated tamoxifen	13.1	404	359, 332, 315, 265, 239, 223, 166, 145
3	dihydroxy tamoxifen	13.8	404	359, 332, 249, 239, 161
4	trioxygenated tamoxifen	13.9	420	N.D.
5	monohydroxy tamoxifen	14.2	388	343, 316, 249, 223, 145, 129
6	(*Z*)-4-hydroxy tamoxifen	14.5	388	343, 316, 249, 223, 166, 145, 129
7	*N*-desmethyl-oxygenated tamoxifen	14.7	374	N.D.
8	4′-hydroxy tamoxifen	15.0	388	343, 316, 249, 223, 129
9	desmethyltamoxifen	16.1	358	N.D.
10	dioxygenated tamoxifen	16.3	404	N.D.
11	trioxygenated tamoxifen	16.3	420	N.D.
12	tamoxifen *N*-oxide	18.2	388	371, 343, 327, 300, 129
13	*N*-desmethyl (*Z*)-4-hydroxytamoxifen	20.5	374	279, 371
14	N.D.	23.5	N.D.	N.D.
T	tamoxifen	16.1	372	327, 209

[a] Retention times and mass spectral data are given. N.D. = not determined.

95% in 18 min via a linear gradient. A postcolumn gradient of 5 min at H_2O:MeOH:AcOH (4.9:95:0.1) was subsequently applied. The organic modifier percentage in the HPLC effluent was diluted continuously to bioassay compatible levels. For this purpose a counteracting MeOH−H_2O makeup gradient at a flow rate of 750 μL/min was applied postcolumn to the HPLC eluate as reported previously.[29] The total flow rate after adding the makeup gradient was 1000 μL/ min. The MeOH percentage directly after the makeup gradient was constant at 24%. This flow was then split into three directions at flow rates of respectively 750, 150, and 100 μL/min. The 750 μL/min flow was split toward an UV detector, the 150 μL/min flow was split toward the MS detector, and the 100 μL/ min was introduced into the ERα affinity assay. The final MeOH concentration in the reaction coils of the bioassay after mixing in ERα and ligand (coumestrol) solutions was 9.6%.

HRS Apparatus. A schematic view of the HRS apparatus is shown in Figure 1. Agilent 1100 (Waldbronn, Germany) HPLC pumps, fluorescence detector (λ_{ex} 340 nm; λ_{em} 410 nm), and UV detector (220 nm) were used. All hardware was controlled by Kiadis B.V. software (Groningen, The Netherlands). A Phenomenex Prodigy C18 (100 × 3.2) 5 μm analytical column (Torrance, USA) with a Phenomenex Security Guard column (C18-ODS, 4 mm length × 2 mm i.d.) AJO-4286 was used. The ERα and coumestrol solutions were housed in 150 mL of Pharmacia superloops (Uppsala, Sweden) at 0 °C. Detailed information can be found in the Supporting Information.

On-line Estrogen Receptor α Affinity Assay. A modified version of the on-line ERβ affinity assay previously described by Oosterkamp et al.[2] was used. ERα (10 nM; 100 μL/min) was incubated with HPLC effluent in a Tefzel reaction coil for 10 s. Then, coumestrol was added (373 nM; 50 μL/min), allowed to incubate for 15 s, and subsequently directed to a fluorescence detector. The ERα and coumestrol solutions were prepared in sodium phosphate buffer (10 mM; pH 7.4; adjusted with KOH) containing 150 mM NaCl. More information can be found in the Supporting Information.

Mass Spectrometry. Mass spectrometry (MS) was performed using a Thermo Finnigan LCQ Deca MS detector (San Jose, CA) in positive ion electrospray ionization mode in a similar way as described by Schobel et al.[29] All scans were recorded in the data-dependent scan mode which allowed normal MS data, recorded in the 50−600 *m*/*z* range, and MS/MS fingerprints to be obtained in an alternating manner. The MS/MS scan was set in such a way as to obtain MS/MS data of the first five most intense ions recorded in the previous MS scan. Detailed information can be found in the Supporting Information.

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Supporting Information Available: A figure with ion traces of LC-MS measurements, detailed description of the identification of tamoxifen metabolites, and detailed information of the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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