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Rapid drug metabolite profiling using fast liquid chromatography, automated multiple-stage mass spectrometry and receptor-binding[1]

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Abstract

Rapid drug metabolite profiling can be achieved using fast chromatographic separation and fast mass spectrometric scanning without compromising the separation efficiency. Fast chromatographic separations of drug and its metabolites can be achieved by eluting from a short narrow-bore guard cartridge column (20×2 mm I.D., 3 μm BDS Hypersil C_8) at flow-rate of 1.0 ml/min and with a gradient volume greater than 90 column volumes. The need for chromatographic separation is important for automated data dependent multiple-stage mass spectrometry (MS^n) experimentation. The total analysis time of 8 min permits profiling of metabolites in a 96-well plate in 13 h. The narrow chromatographic peaks resulting from the high flow-rate require the use of a mass spectrometer capable of fast scan speed due to the need to perform multiple MS experiments within the same chromatographic analysis.

A method has been developed for screening potentially biologically active in vitro microsomal metabolites by affinity binding with a receptor. After separation by centrifugal ultrafiltration, the bound ligands are released and characterized by LC–MS. In vitro microsomal metabolites of tamoxifen, raloxifene and adatanserin were screened for potential biological activity using this method. The in vitro metabolites of tamoxifen captured by the receptor include *N*-demethyltamoxifen and three species of hydroxytamoxifen; these data are consistent with those from a conventional binding study and bioassay. In addition, both hydroxyraloxifene and dihydroxyraloxifene are also recognized by the receptor. The specificity of the molecular recognition process is illustrated by the absence of binding with control microsomal incubate and with adatanserin and its metabolites. Therefore, active metabolites can be rapidly profiled by fast LC, automated MS^n, and receptor binding. This information can be obtained quickly and can add value to the drug discovery process.

Keywords: Multiple-stage mass spectrometry; Rapid metabolite profiling; Tamoxifen; Adatanserin; Raloxifene

1. Introduction

Pharmaceutical discovery has been transformed tremendously in the last decade by several new enabling technologies such as combinatorial chemistry, molecular biology and genomics. Such a revolution is brought about by the ability of combinatorial chemistry to synthesize a diverse and large library of compounds for high throughput screening against biomolecular targets which are made available through molecular biology and genomics [1–4]. The initial screens can produce many lead compounds, which can be further reduced to a few candidates

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using screening methods based on drug metabolism and pharmacokinetic principles [5]. Consequently, drug metabolism and pharmacokinetic studies are being conducted early in the drug discovery phase.

As a result, there has been an evolution in the paradigm for conducting metabolism and pharmacokinetic studies in drug discovery in the pharmaceutical industry. Several strategies have been explored to expedite preclinical pharmacokinetic studies and they include simultaneous dosing of a library of lead compounds at tolerable doses to an animal [n in 1 or cassette dosing] [6–8], plasma pooling from individual pharmacokinetic studies [9], in vitro permeability assessment of a library of lead compounds using Caco-2 cells [10] and plasma stability and protein binding of a library of lead compounds [11]. The main goal of these screens is to facilitate the selection of a few lead compounds with the most favorable pharmacokinetic parameters.

Increasingly, preclinical metabolism criteria are being used early in drug discovery to select lead compounds with better microsomal stability, minimal metabolic liability and absence of potential for drug–drug interactions. High throughput screening involving 96-well plate format is being implemented for investigation of microsomal stability, in vitro drug metabolism and drug metabolizing enzyme characterization [12,13]. These preclinical metabolism data together with the pharmacokinetic information can be used to further refine the chemical structures of lead candidates based on structure–activity relationships. The ultimate goal of these screens is to develop a drug that has a greater chance of success in the clinic.

To cope with the large number of lead candidates, metabolism and pharmacokinetic studies must also be conducted in high throughput mode. This has led to the development of automated 96-well plate sample preparation format and fast liquid chromatography–tandem mass spectrometric (LC–MS–MS) analysis. Often such fast LC–MS–MS method sacrifices the chromatographic separation efficiency for speed of analysis and relies extensively on mass separation for tracking of analytes. This type of experiments has been successful in high throughput preclinical pharmacokinetic studies. However, this analytical approach will encounter difficulty in the separation and identification of structurally similar metabolites often produced from in vitro microsomal incubations. This report describes the development of a fast liquid chromatographic separation method using a short narrow-bore guard cartridge column. This coupled with automated single-stage and data dependent multiple-stage mass analyses on an ion trap mass spectrometer (LCQ) was applied to rapidly profile metabolites. To provide even more structure activity information in the discovery paradigm, a receptor binding step can be incorporated to screen for potential biologically active metabolites. These structure–activity relationships from active metabolites can potentially contribute to the lead optimization process in drug discovery.

2. Experimental

2.1. Materials

Glucose-6-phosphate monosodium salt, 1 *M* magnesium chloride, 0.5 *M* ethylenediaminetetraacetic acid disodium salt, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate sodium salt, tamoxifen citrate salt, 4-hydroxytamoxifen, disodium phosphate heptahydrate were obtained from Sigma (St. Louis, MO, USA). Sodium chloride and 1 *M* sodium hydroxide were of Baker analyzed grade (J.T. Baker, Phillipsburg, NJ, USA). Ammonium acetate (BioChemika grade), 1 *M* triethylammonium acetate buffer pH 7 and formic acid (Chemika grade) were obtained from Fluka (Ronkonkoma, NY, USA). Monosodium phosphate monohydrate was purchased from Fisher Scientific (Atlanta, GA, USA). HPLC-grade water and acetonitrile, and potassium phosphate were from EM Science (Gibbstown, NJ, USA). Microcon microconcentrators with 30 000 M_r cut-off filters were purchased from Amicon (Beverly, MA, USA). Male Sprague–Dawley rat liver microsomes and human recombinant α-estrogen receptors were purchased from Xenotech (Kansas City, KS, USA) and Pan Vera (Madison, WI, USA), respectively. Adatanserin was obtained from Wyeth–Ayerst Research compound room (Princeton, NJ, USA).

2.2. In vitro rat liver microsomal incubations

All incubations were conducted with 1 ml final total volume in a screw-capped test tube and con-

sisted of 2 m*M* ethylenediaminetetraacetic acid, 10 m*M* magnesium chloride, 21–27 μM drug (free base), 2 mg microsomal protein, 3.5 m*M* glucose 6-phosphate, 0.4 unit glucose 6-phosphate dehydrogenase and 1.3 m*M* β-nicotinamide adenine dinucleotide phosphate. Potassium phosphate buffer pH 7.4 (0.1 M) was added to bring the volume to 1 ml. Incubation was carried out at 37°C for 1 h and the reaction was terminated by addition of 3 ml of cold acetonitrile. Following centrifugation at 790 *g* at 4°C for 10 min, the supernatant was transferred to another test tube and the acetonitrile was removed by evaporation under a gentle stream of nitrogen at 40°C prior to lyophilization to dryness. The residue was finally reconstituted in methanol–water (50:50) to give a concentration of 25 ng equivalent/μl of added drug.

2.3. Receptor-binding

The in vitro rat liver microsomal incubation was further diluted five-fold with phosphate−buffered saline (PBS) pH 7.4 prior to use in the binding study. All incubations, 200 μl final total volume in eppendorf tube, consisted of 106–135 pmol human recombinant α-estrogen receptors and in vitro rat liver microsomal incubate containing 106–135 pmol of added drug. PBS pH 7.4 was added to bring the volume to 200 μl. Incubation was carried out at 37°C for 2 h and the incubate was transferred to a Microcon microconcentrator (30 000 M_r cut-off) which had been passivated overnight by soaking in 1% (v/v) Aquasil (Pierce, Rockford, IL, USA) and rinsed twice with methanol–water (50:50). The microconcentrator was subjected to a final wash with PBS by centrifugation at 12 000 *g* for 20 min at 4°C. Separation of unbound drug and its metabolites was accomplished by centrifugation as before but for only 15 min. The retentate was washed twice with 200 μl of 1 *M* triethylammonium acetate buffer pH 7 which has previously been chilled at 4°C and then centrifuged at 12 000 *g* for 10 min at 4°C. The bound drug and its metabolites were released by addition of 200 μl of 0.5% (v/v) formic acid in acetonitrile–water (60:40) to the microconcentrator and stand at room temperature for 5 min prior to centrifugation as described above for 15 min. The filtrate was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 80 μl of methanol–water (50:50) prior to LC–MS analysis.

2.4. Chromatography

All chromatographic separations was carried out using a HP1090M liquid chromatograph (Hewlett–Packard, Palo Alto, CA, USA) which had been replumbed with 0.005 in. I.D. polyether ether ketone (PEEK) tubing to minimize peak broadening (1 in.= 2.54cm). An aliquot of sample (5 or 20 μl) was injected for chromatographic separation on a 2 mm I.D., 3 μm porous particles BDS Hypersil C_8 column (Keystone Scientific, Bellefonte, PA, USA) at oven temperature of 50°C. Separation efficiency was evaluated with 50, 20 or 10 mm length columns. Following injection, the column was washed with 10 m*M* ammonium acetate–acetonitrile (95:5) for 0.5 min prior to elution with a linear gradient up to 10 m*M* ammonium acetate–acetonitrile (5:95). The effects of a 14.5, 7, 6, 3, 2, or 1 min gradient were studied at various flow-rates. Flow-rates at 0.40, 0.80, 1.00 or 1.20 ml/min were examined.

2.5. Mass spectrometry

On-line LC–MS analyses were performed using the Finnigan MAT ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA, USA) operated in the positive ion electrospray ionization mode. The entire LC eluate was sprayed into the mass spectrometer at +4.5 kV and the mass spectrometer was optimized by tee-infusion with 10 ng/μl tamoxifen at the flow-rates (0.4, 0.8, 1.0 and 1.2 ml/min) used for chromatographic separation. Desolvation of the droplets was aided by the heated capillary set at either 230 or 240°C and by the auxillary and sheath gas set at 30 and 90, respectively. Ions were sampled into the mass spectrometer at an injection time of 150 ms with automatic gain control. Ultrahigh purity helium was used as the buffer gas. Each analytical scan from m/z 200 to 800 consisted of 3 μscan. Data dependent MS–MS was performed simultaneously at relative collision energy of 28% and with an isolation width of 2 daltons. Ejected ions were detected with electron multiplier set at gain of 4×10^5. Data acquisition and reduction was carried out with Navigator software version 1.2.

3. Results and discussion

3.1. LC–MS analysis

It is well recognized that LC method development for metabolite profiling can be time consuming and therefore often is the bottleneck in providing metabolite information by LC–MS for drug discovery. Standardization of LC and MS conditions is one reported approach to reduce development time [14]. Implementation of a standardized LC–MS method for rapid metabolite profiling requires considerable chromatographic resolution in the shortest possible time since commonly encountered isobaric metabolites are extremely difficult to differentiate by mass spectrometry. Such a desired chromatographic property can be achieved using a short column packed with small particles [15].

Currently, the column of our choice is a 3 μm porous particles narrow-bore column (50×2 mm I.D.). Reducing the column I.D. from the conventional 4.6 mm to 2 mm results in a five-fold gain in sensitivity [16] because of less dilution by the chromatographic process in the narrow-bore column. Less hydrophobic stationary phase such as C_8 is preferred due to its ability to handle diverse polarities of compounds without the strong retention of extremely hydrophobic compound. Also, this type of stationary phase required less washing to remove lipophilic materials from biological matrices. This is an important consideration when using an ion trap mass spectrometer with automatic gain control (AGC) to limit the total number of ions stored in the ion trap. Any strongly retained compound eluting in the second cycle of LC separation will increase the number of ions in the ion source. The LCQ operated under AGC will reduce the ion injection time to prevent space-charging in the ion trap. Consequently, this will affect its ability to detect minor components. On the other hand, if the compound under investigation is not adequately retained, it is likely that its polar metabolites will be difficult to separate because they will be less strongly retained on the column. Hence, a column with different stationary phase must be considered.

The selected mobile phase consists of water (A) and acetonitrile (B); each containing 0.1% (v/v) formic acid. After injection, the column was washed for 0.5 min with (A:B) (95:5) and then eluted with a 14.5 min linear gradient up to (A:B) (5:95) at oven temperature of 50°C. The flow-rate was at 0.4 ml/min and the total analysis time was 20 min. The choice of mobile phase is limited by the LC–MS interface to only volatile components that can be removed by the vacuum system following desolvation. Another consideration in the selection of mobile phase for LC–MS analysis is the mode of ionization. This mobile phase works optimally for positive ion electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), but it is not suitable for the generation of negative ions. An alternative mobile phase is 5 m*M* ammonium acetate and acetonitrile which can be used for detection of both positive and negative ions. The presence of ammonium cation is useful in analysis of neutral compounds by ionization through adduct formation.

The robustness of the analytical method is another important consideration for LC–MS analysis and can be achieved by diverting the first 1.5 min of the eluant from the mass spectrometer. The early eluant contains predominantly inorganics and polar nonvolatile species which can degrade the performance of the mass spectrometer. Currently, simultaneous acquisition of single MS and data dependent multiple MS (MS^n) data were performed within a single LC run. To expedite turnaround time, software has been developed to automatically print mass spectra and chromatograms of detected components. This standardized LC–MS procedure has been able to provide rapid metabolite profiling for drug discovery.

3.2. Fast LC–MS analysis

The need for high throughput in the new discovery metabolism paradigm necessitates the use of 96-well plate incubation format. The standardized LC–MS system described above will result in more than 24 h analysis time to profile the in vitro metabolites in one 96-well plate. Therefore, a shorter analysis time is necessary for high throughput analysis. Recently, a short guard cartridge column employed in the desalting mode prior to LC–MS analysis was used for rapid metabolite profiling. One drawback of such an approach is the lack of chromatographic separation of drug and metabolites which may suppress the ionization and detection of minor components. As a

result, there is considerable interest in the development of fast chromatographic separation.

Short narrow-bore column packed with 3 μm porous particles or 1.5 μm nonporous particles can provide fast gradient separation with good chromatographic resolution and sensitivity. The increased sensitivity is attributed to the small peak volumes obtained with these columns. Unless otherwise indicated, all chromatographic conditions used are as described in the experimental section. The HP1090M was replumbed with 0.005 in. I.D. PEEK tubing to give a total delay volume of about 0.5 ml to minimize peak broadening from extra column effects.

Tamoxifen and adatanserin were chosen for this study because of differences in microsomal stability;adatanserin was more extensively metabolized. MICRA NPS 1.5 μm ODS column (33×3 mm I.D.) (Northbrook, IL, USA) was initially selected for fast LC–MS investigation because of its potential greater reduction in analysis time. However, there was inadequate separation of tamoxifen or adatanserin and its metabolites with a 6 min linear gradient at 0.45 ml/min (data not shown). This gradient of 11 column volumes should be adequate since it has been suggested that a gradient volume of 10 column volumes is a reasonable starting point in HPLC method development [17]. It appears that a gradient volume greater than 11 column volumes is required for adequate resolution of tamoxifen or adatanserin and its metabolites. This can be achieved by either retarding the gradient or increasing the flow-rate. However, retarding the gradient will extend analysis time and higher flow-rate will result in high back pressure which reduces the robustness of the method. Consequently, a compromised was made to use the larger 3 μm porous particles.

Fig. 1 (A and B) shows the chromatographic separation of rat liver microsomal incubates of tamoxifen and adatanserin, respectively, using a 10× 2 mm I.D., 3 μm BDS Hypersil C_8 column. Separation was carried out using a 1 min linear gradient at 0.40 ml/min which corresponded to 12.9 column volumes. The reconstructed ion chromatogram (RIC) of the base peaks from analysis of microsomal incubate of either tamoxifen (Fig. 1A) or adatanserin (Fig. 1B) shows two drug related peaks. Tamoxifen and adatanserin eluted at retention times of 2.96 (Fig. 1A) and 2.71 (Fig. 1B), respectively. Full scan ESI mass spectrum corresponding to the earlier eluting peak (2.84 min) in Fig. 1A is a composite mass spectrum of *N*-demethyltamoxifen (m/z 358), hydroxytamoxifen (m/z 388), dihydroxytamoxifen (m/z 404), and other endogenous compounds (Fig. 2A). Experimentally, the lack of separation is problematic for data dependent MS–MS data acquisition based on most abundant ion. As a result, only a product ion mass spectrum was acquired for hydroxytamoxifen from collision-activated dissociation of m/z 388 (Fig. 2B). This spectrum may represent a single metabolite to the untrained eye but is actually a composite of different isobaric metabolites, thus complicating structural assignment of a metabolic softspot. Such a conclusion from the product ion mass spectrum requires considerable time, familiarity with tamoxifen metabolism and mass spectra interpretation skills. Particularly, the extra time needed for interpretation is not compatible with providing fast metabolite profiling. Moreover, the commonly encountered regioisomeric metabolites are difficult to distinguish by MS–MS. Therefore, it is important to resolve different metabolites for the above reasons and because individual metabolites can have different activity as illustrated by the four isobaric hydroxytamoxifen metabolites. It has been reported that aromatic hydroxylation at the 3 and 4 positions promotes antiestrogenic activity [18] while α-hydroxylation of the ethyl group gives rise to an electrophile [19]. Similar composite mass spectra were also obtained for the earlier eluting peak at 2.55 min in Fig. 1B (data not shown).

To improve resolution, a column with twice the number of theoretical plates (20×2 mm I.D.) was employed. A slightly improved separation of the two samples was obtained with a 2 min linear gradient at flow-rate of 0.40 ml/min which was equivalent to 12.7 column volumes (Fig. 3A and B). Even under such condition, certain isobaric hydroxytamoxifen and hydroxyadatanserin components were still incompletely resolved. The total cycle time of about 8 min for these two columns suggested that metabolite profiling of 96-well plate could be accomplished within 13 h but with considerable sacrifice in separation efficiency. Hence, various gradients and flow-rates were evaluated on this 20×2 mm I.D. column in an attempt to improve separation efficiency.

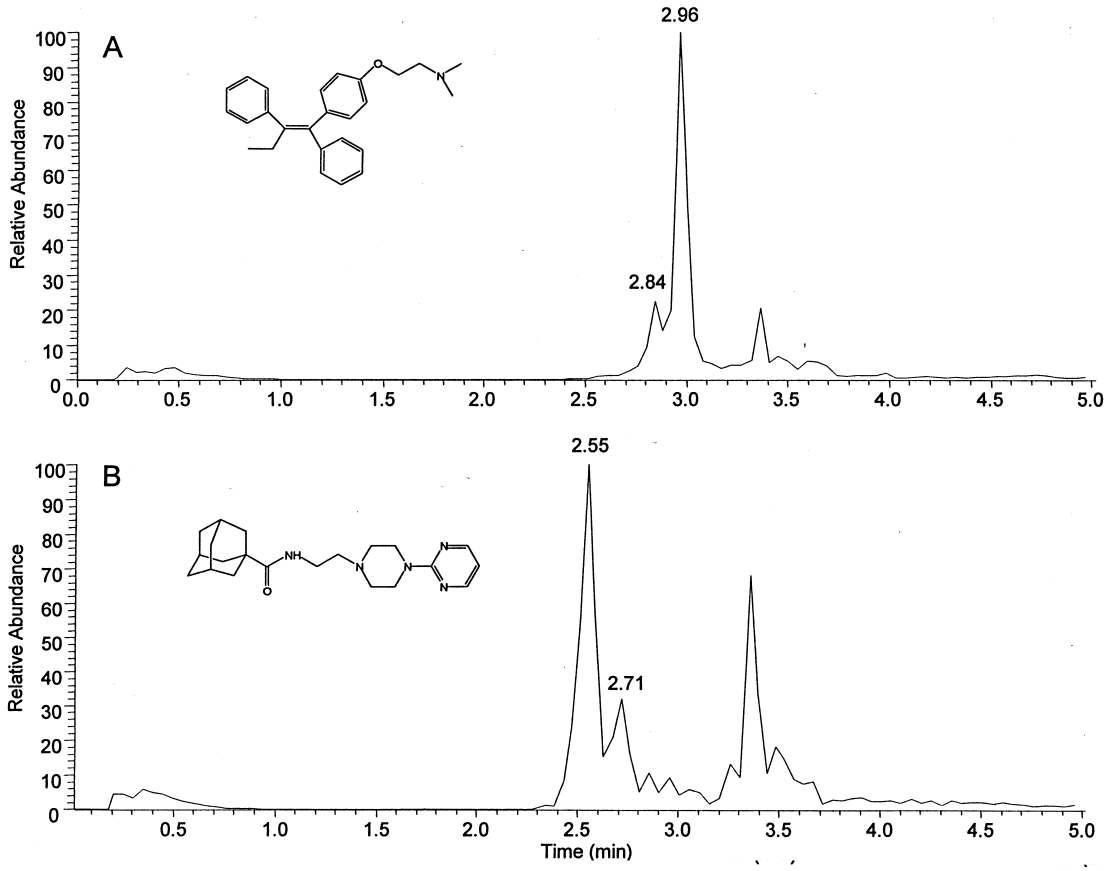

Fig. 1. Chromatographic separation efficiency by 10×2 mm I.D., 3 μm porous particles BDS Hypersil C_8 column using a 1 min linear gradient from 5 to 95% acetonitrile at 0.4 ml/min (12.9 column volumes). The reconstructed ion chromatograms corresponding to base peaks from LC–MS analyses of in vitro rat liver microsomal incubates of tamoxifen (A) and adatanserin (B).

The retention times from chromatographic separation of microsomal metabolites of tamoxifen and adatanserin at various gradients and flow-rates using a 20×2 mm I.D. BDS Hypersil C_8 column are tabulated in Tables 1 and 2, respectively. The retention times from a 20 mm long column clearly indicate comparable separation obtained with a longer column (50 mm) with 2.5 times higher number of theoretical plates. Clearly, there is only a slight difference (1.2-fold) in retention times for the two columns. The chromatographic separation fidelity has been preserved on the shorter column due to the 2.5 times gain in the gradient volume obtained at the same flow-rate and gradient. The data obtained implies that adequate separation of microsomal metabolites of either tamoxifen or adatanserin with the 20 mm long column will require a gradient volume of about 92 column volumes. This observation suggests that comparable separation efficiency is expected as long as the gradient volume is maintained with a change in flow-rate. In general, there is preservation of chromatographic separation efficiency with increasing flow-rates which is consistent with Van Deemter plots; this is clearly demonstrated by data obtained with metabolites of tamoxifen and adatanserin at flow-rates of 0.4, 0.8 and 1.0 ml/min (Tables 1 and 2). There was chromatographic separation for all compounds at increased flow-rates of 0.8 and 1.0 ml/min except for two dihydroxytamoxifen metabolites (TM2 and TM3) at 0.8 ml/min and two hydroxyadatanserin metabolites (AM4 and AM5) at 1.0 ml/min. This is noteworthy

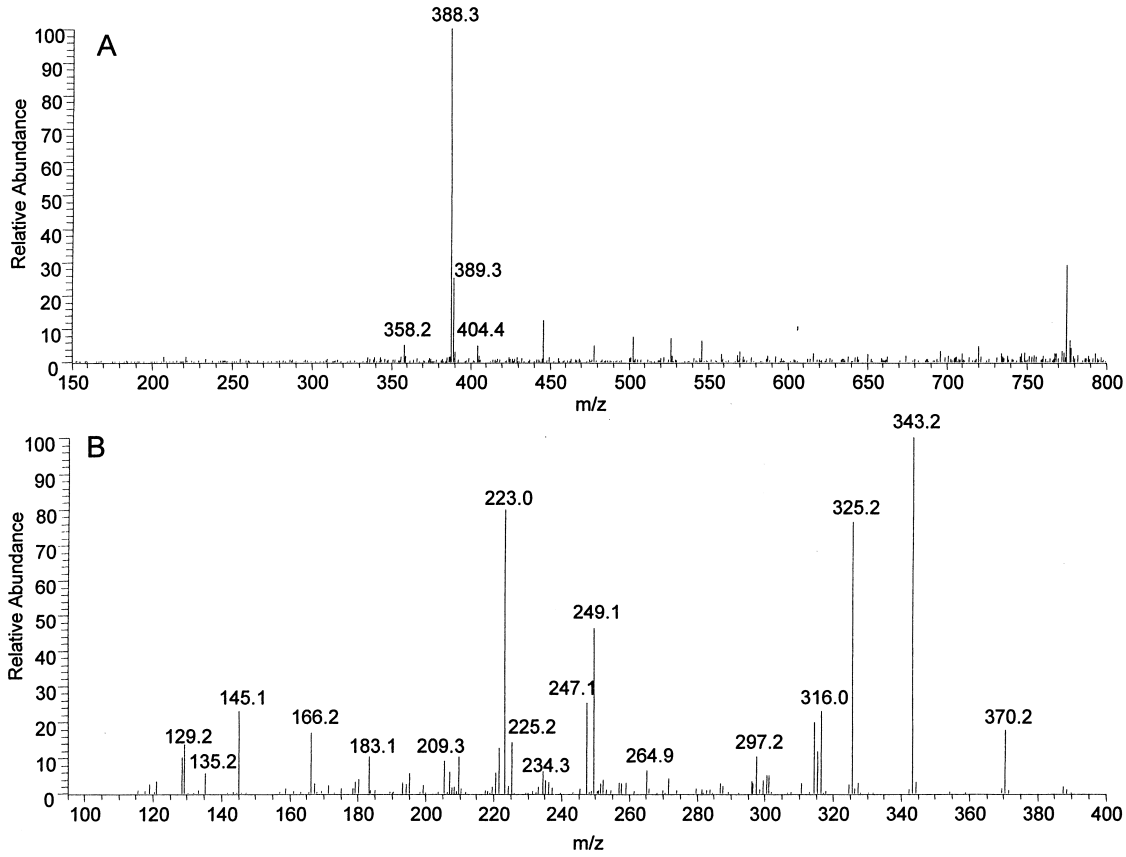

Fig. 2. The mass spectra of the peak at retention time of 2.84 min in Fig. 1A from silmultaneous automated full scan single-stage (A) and multiple-stage (B) mass spectrometric analysis of m/z 388 within a run.

since the drug and its metabolites are fairly similar in structure and many of the metabolites are isomers. This small drawback in separation efficiency is more than offset by the gain of 1.9 and 2.4 fold in analysis time at flow-rate of 0.8 and 1.0 ml/min, respectively. The backpressures of the column at 0.8 and 1.0 ml/min were 140 and 180 bar, respectively, which were acceptable for routine operation. The total analysis time including re-equilibration at 0.8 and 1.0 ml/min was 9.5 and 8 min, respectively. Attempt to further reduce analysis time was investigated using a 3 min linear gradient at 1.2 ml/min which resulted in 57.1 column volumes. The back pressure was relatively high at about 220 bar but it was still acceptable for analysis. Under this condition, two pairs of dihydroxytamoxifen metabolites (TM2 and TM3, and TM5 and TM6), all dihydroxyadatanserin, and three species of hydroxyadatanserin (AM3, AM4 and AM5) were not chromatographically resolved. The separation of tamoxifen and its metabolites into nine peaks instead of 11 and adatanserin and its metabolites into four instead of seven peaks at total analysis time of 5 min is still far superior than the desalting method. In this case, there was a three times gain in analysis time. Based on the above observations and balancing between separation efficiency, robustness and total analysis time, we recommend the use of 20×2 mm I.D. column packed with 3 μm particles and eluting analytes with a gradient volume greater than 90 column volumes at 1 ml/min for fast LC separation.

One consequence of fast chromatographic separation is smaller peak width with increased flow-rate. For example, the width of the chromatographic peak

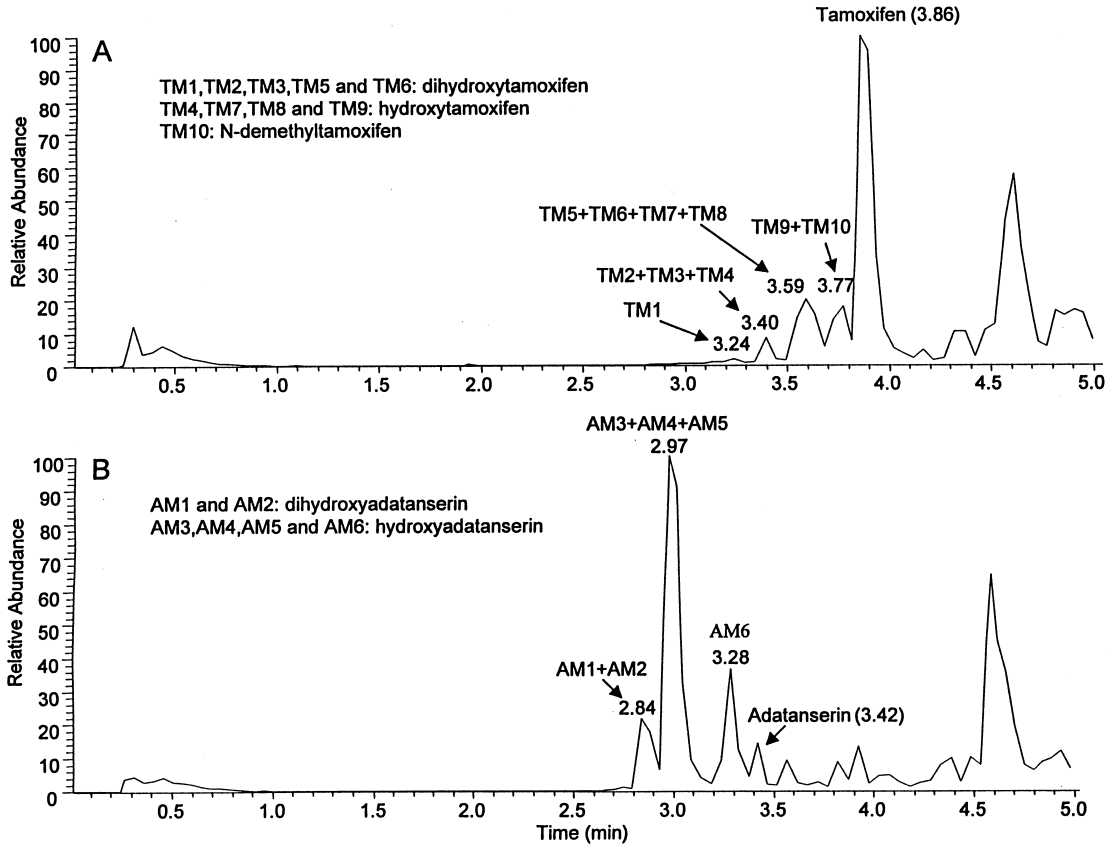

Fig. 3. Chromatographic separation efficiency by 20×2 mm I.D., 3 μm porous particles BDS Hypersil C_8 column using a 2 min linear gradient from 5 to 95% acetonitrile at 0.4 ml/min (12.7 column volumes). The reconstructed ion chromatograms corresponding to base peaks from LC–MS analyses of in vitro rat liver microsomal incubates of tamoxifen (A) and adatanserin (B). In each case, the labeled peaks correspond to the in vitro metabolites and several isomers are present in present peaks.

is about 20 s wide at 0.8 ml/min compared to about 8 s at 1.2 ml/min. Therefore, on-line LC–MS analysis at flow-rate of 1.2 ml/min requires operation of the mass spectrometer at a very fast scan speed. The reduced separation of tamoxifen and its metabolites at 1.2 ml/min may be attributed to an inadequate number of data points across the peak (Fig. 4A), because the ion trap mass spectrometer was operated at a total cycle time of about 2 s and hence only three to four data points were acquired across the peak. This accounts for the triangular mass chromatographic peaks in Fig. 4A; there is not sufficient number of data points taken across the peak to define the valley of separation of closely eluting components, as illustrated by the apparent poor separation for three components (TM9, TM10 and tamoxifen) indicated in Fig. 4A. By increasing the scan speed to 0.2 s per scan (Fig. 4B), 40 data points were acquired for an 8 s wide peak. The data clearly indicate that fast scanning is able to define better a narrow peak resulting in an apparent improvement in separation of the previous three components in Fig. 4B. The ion trap mass spectrometer is capable of faster scan speed for full scan mass analysis than triple quadrupole instruments and hence, would be suited for fast LC–MS analysis. Another instrument that is suited for fast LC–MS analysis is the time-of-flight (TOF) based mass spectrometer [20]. This is an important consideration because of the need to perform multiple MS experiments within a run to increase throughput. Furthermore, the narrow peak width combined with the

Table 1
The retention times of tamoxifen and its 10 metabolites from chromatographic separation on 50 and 20×2 mm I.D., 3 μm porous particles BDS Hypersil C_8 column at different gradient volumes obtained by changing gradients and flow-rates

Compound	Retention time (min)				
	50×2 mm I.D.,	20×2 mm I.D.			
	Gradient volume 36.9 ml[a]	Gradient volume 92.1 ml[b]	Gradient volume 88.9 ml[c]	Gradient volume 95.2 ml[d]	Gradient volume 57.1 ml[e]
TM1	7.91	6.67	3.59	2.85	2.32
TM2	8.62	7.49	4.03	3.19	2.51
TM3	8.81	7.58	4.03	3.19	2.51
TM4	9.22	7.77	4.16	3.28	2.56
TM5	9.69	8.52	4.50	3.59	2.75
TM6	9.97	8.71	4.64	3.68	2.75
TM7	10.53	8.98	4.73	3.77	2.79
TM8	10.93	9.29	4.90	3.86	2.88
TM9	11.38	9.79	5.17	4.12	2.97
TM10	11.88	10.05	5.29	4.17	3.05
Tamoxifen	12.45	10.52	5.50	4.39	3.14

TM1, TM2, TM3, TM5 and TM6: dihydroxytamoxifen.
TM4, TM7, TM8, and TM9: hydroxytamoxifen.
TM10: *N*-demethyltamoxifen.
Gradient volume is expressed in column volumes and calculated by multiplying gradient time from 5 to 95% acetonitrile with flow-rate and divided by the column volume of the column.
[a,b] 14.5 min gradient at 0.40 ml/min.
[c] 7 min gradient at 0.80 ml/min.
[d] 6 min gradient at 1.00 ml/min.
[e] 3 min gradient at 1.20 ml/min.

Table 2
The retention times of adatanserin and its six metabolites from chromatographic separation on 50 and 20×2 mm I.D., 3 μm porous particles BDS Hypersil C_8 column at different gradient volumes obtained by changing gradients and flow-rates

Compound	Retention time (min)				
	50×2 mm I.D.	20×2 mm I.D.			
	Gradient volume 36.9 ml[a]	Gradient volume 92.1 ml[b]	Gradient volume 88.9 ml[c]	Gradient volume 95.2 ml[d]	Gradient volume 57.1 ml[e]
AM1	5.69	4.72	2.65	2.07	1.90
AM2	5.95	4.97	2.79	2.15	1.90
AM3	6.46	5.29	2.95	2.31	2.02
AM4	6.65	5.50	3.05	2.39	2.02
AM5	6.90	5.73	3.16	2.39	2.02
AM6	8.44	7.04	3.82	3.00	2.40
Adatanserin	9.27	7.71	4.12	3.26	2.53

AM1-2: dihydroxyadatanserin.
AM3-6: hydroxyadatanserin.
Gradient volume is expressed in column volumes and calculated by multiplying gradient time from 5 to 95% acetonitrile with flow-rate and divided by the column volume of the column.
[a,b] 14.5 min gradient at 0.40 ml/min.
[c] 7 min gradient at 0.80 ml/min.
[d] 6 min gradient at 1.00 ml/min.
[e] 3 min gradient at 1.20 ml/min.

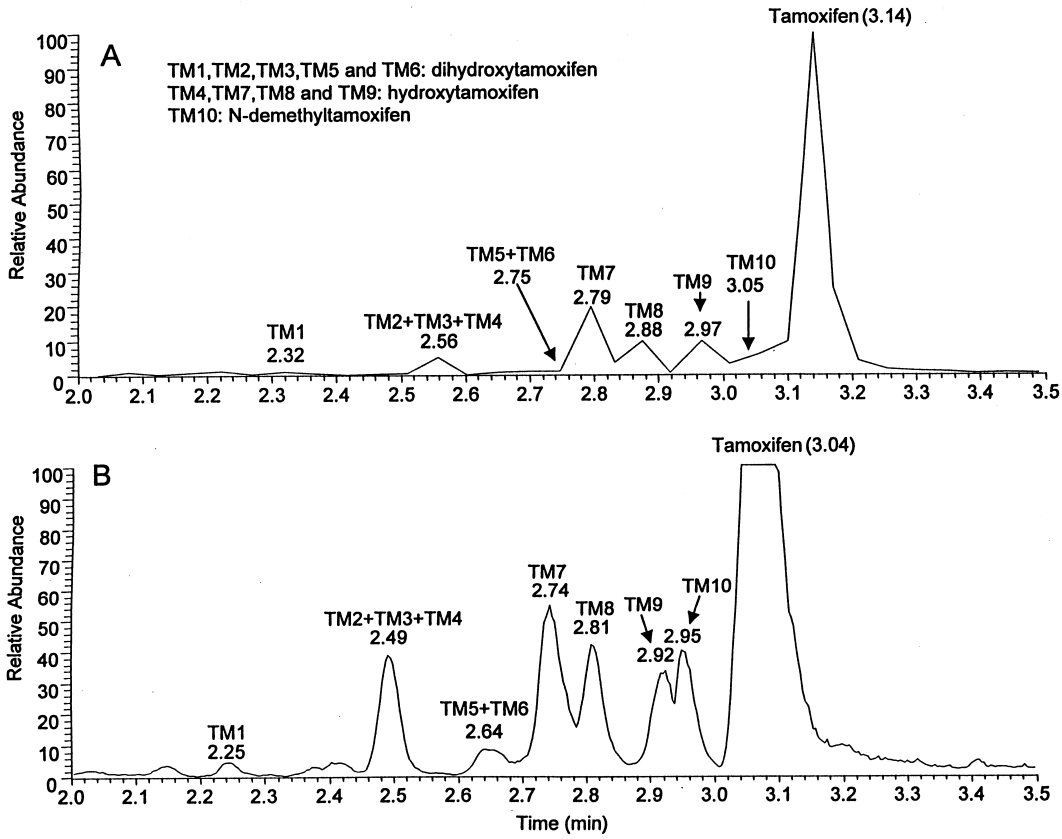

Fig. 4. The effect of scan speed of mass spectrometer on chromatographic separation of in vitro rat liver microsomal metabolites of tamoxifen using a 3 min linear gradient at 1.2 ml/min; other conditions are as described in Fig. 3. The reconstructed ion chromatograms from mass spectrometric analysis at total scan speed of about 2 s (A) and 0.2 s (B). The scan speed of 0.2 s is from scanning m/z 350-410 using a Finnigan MAT TSQ 700.

limited scan speed of mass spectrometers necessitate good chromatographic separation of drug and its metabolites for detection by data dependent MS^n data acquisition. The injection volume is also important because short narrow-bore columns have reduced loading capacity. Increasing the injection volume from 5 to 10 μl resulted in loss of resolution for several tamoxifen metabolites (data not shown). Finally, no post column split of the LC eluent was performed for the fast LC–MS experiments described here. However, the high flow-rate used to achieve fast chromatography may require to split the LC eluent prior to spraying into the mass spectrometer for development of a more robust metabolite profiling method. The post column split needed is a function of the atmospheric pressure ionization source design available on the instrument.

3.3. Biologically active metabolite screening by receptor binding

In vitro binding assays incorporating a biological target is routinely used in drug discovery to screen compounds. It is also recognized that the binding assay lacks the coupling and signal transduction systems of the functional bioassay which explains the potential false positive results. Nevertheless, binding assays can be used to discern active compounds. Furthermore, in vitro binding assay can be automated to operate in a high throughput mode.

A high throughput screen against receptors and in combination with LC–MS has been used in pharmaceutical industry for the identification of new chemical entity or to guide optimization of lead candidate using combinatorial chemistry [4,21]. Crucial to successful LC–MS identification of active leads is the need to separate the unbound ligands from the receptor-captured ligands. This physical separation can be accomplished by either on-line pulsed-ultrafiltration [22] or off-line using size-exclusion chromatography [21] or centrifugal ultrafiltration [23]. Subsequently, the bound ligands are released and characterized by LC–MS. Such an analytical method provides direct visualization of ligands bound to receptors. This is illustrated by the affinities of α-estrogen receptors toward estrogens but not progestins following centrifugal ultrafiltration prior to LC–MS analysis [24]. Except for its common application in selecting potentially active ligands, there have been few, if any, reports on the use of receptor-binding for identification of potentially active metabolites in drug metabolism study.

Biotransformation of a drug can lead to the formation of either biologically inactive or active metabolites. It is important to determine the biological activities of metabolites because the pharmacodynamic effect of the drug can be enhanced or antagonized by the active metabolite [25]. In addition, the oxidative pathways of drug metabolism may be affected by genetic polymorphisms and thus contribute to the variability of clinical responses [26]. Therefore, the early identification of active metabolites can expedite both the drug discovery and development processes. For example, the structures of the active metabolites will provide feedback for further refinement of the chemical structures to produce metabolically stable and, hence more active lead candidates. The following is an illustration using α-estrogen receptors to capture in vitro rat liver microsomal metabolites of tamoxifen, raloxifene and adatanserin for characterization by LC–MS.

Biologically active metabolite screening was carried out by adding α-estrogen receptors to an in vitro rat liver microsomal incubate of tamoxifen. The control was from incubation of NADPH fortified rat liver microsomes (without tamoxifen) with α-estrogen receptors. The RIC from analysis of the incubation of the rat liver microsomal incubate of tamoxifen with α-estrogen receptors is shown in Fig. 5A while Fig. 5B is the RIC corresponding to an in vitro rat liver microsomal incubate of tamoxifen at the same dilution used in the binding study. Comparison of the two RICs clearly indicates that the α-estrogen receptors captured tamoxifen, *N*-demethyltamoxifen (TM10), and hydroxytamoxifen (TM7-9); negligible binding was observed for the dihydroxytamoxifen metabolites (see inserts in Fig. 5A and B*).* TM7 was identified as 4-hydroxytamoxifen by comparison of retention time and product ion mass spectrum with reference standard (data not shown). Of particular interest is the finding that one hydroxytamoxifen isomer, TM4, did not bind to the receptor. This clearly suggests the specificity of the affinity binding process. However, there is an extra unlabelled peak in the RIC in Fig. 5A which has an identical M_r as tamoxifen (371 daltons) and is consistently detected in all samples including control microsomal incubate after incubation with the α-estrogen receptor. Also, no tamoxifen derived peak was detected from incubation of control rat liver microsomal incubate with the α-estrogen receptor (data not shown). This unlabelled peak may represent a compound in the microsomal preparation that is recognized by the α-estrogen receptor. However, it cannot be totally excluded that the unlabelled peak may be an artifact from nonspecific binding. This unidentified peak has very different MS–MS characteristic when compared with tamoxifen and its metabolites; therefore, this suggests that it is unrelated to the compound of interest. Similarly, microsomal metabolites of raloxifene were screened for activity by this receptor binding method (Fig. 6A and B). The bound ligands identified from the microsomal incubate were raloxifene, hydroxyraloxifene and dihydroxyraloxifene. Data from affinity binding with either a control microsomal incubate (data not shown), or with an in vitro microsomal incubate of adatanserin (Fig. 7A and B) showed no component bound to the α-estrogen receptor. Taken together, these data clearly demonstrated the binding specificity of tamoxifen and raloxifene metabolites to the α-estrogen receptor. In general, there was no dramatic difference in appearance of the RICs corresponding to the in vitro microsomal incubate of drug incubated with the receptor and without the receptor

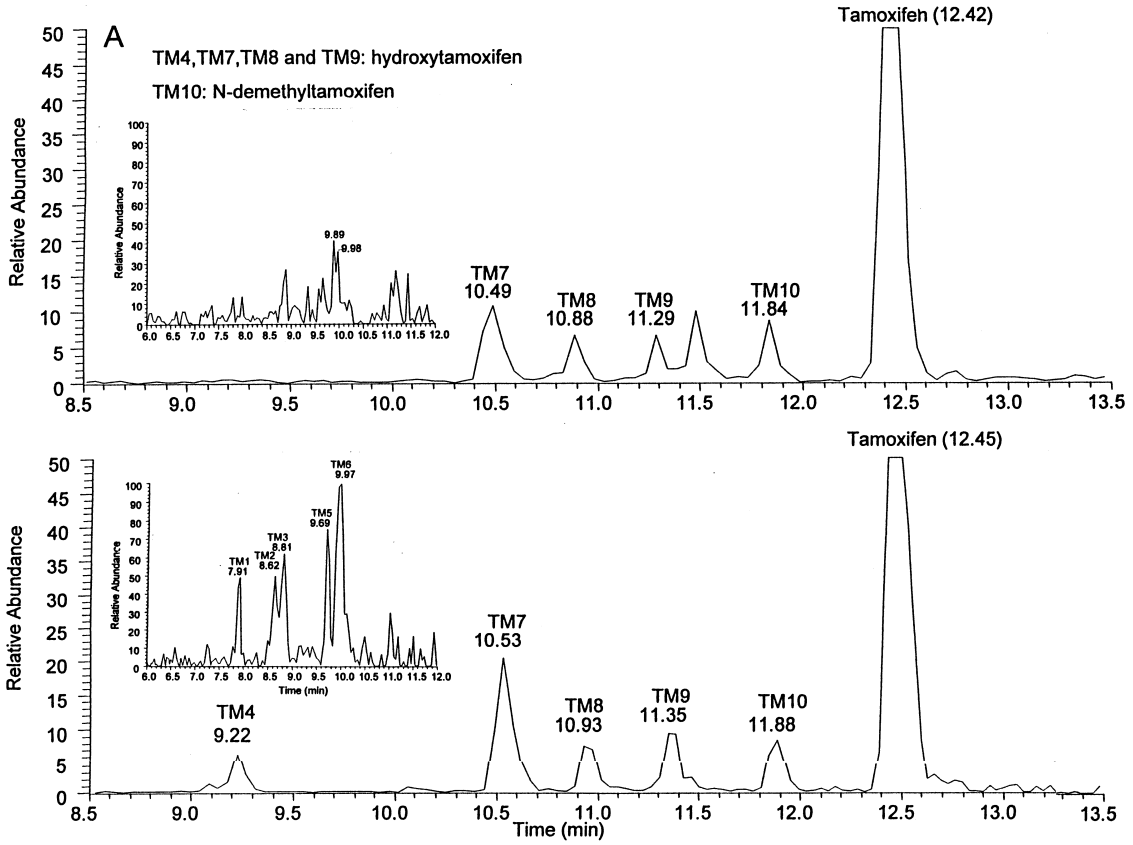

Fig. 5. Reconstructed ion chromatograms corresponding to summing m/z 358, 372 and 388 from LC–MS analysis of an in vitro rat liver microsomal incubate of tamoxifen after incubation with α-estrogen receptors (A). The lower reconstructed ion chromatogram (B) is from LC–MS analysis of an in vitro microsomal incubate of tamoxifen at the same dilution as that used in binding study. The inserts correspond to the RICs of the dihydroxytamoxifen metabolites. Binding study was carried out at equimolar of drug and α-estrogen receptor. Samples were analyzed using 50×2 mm I.D., 3 μm BDS Hypersil C_8 column with a 14.5 min linear gradient (5 to 95% acetonitrile) at 0.4 ml/min. Other conditions used are as described under chromatography section.

in the current investigation since the binding study was carried out at 1:1 stoichiometry. It is speculated that there will be more difference if the binding experiment is conducted with the stoichiometry of drug much greater than the receptor. In this case, the competition for receptor occupancy is determined by the affinity of the ligand and more of the greater affinity ligand will be captured by the receptor.

Thus, the removal of a methyl group or insertion of an oxygen atom (except TM4) did not disrupt the molecular recognition process by the α-estrogen receptor for these tamoxifen metabolites. Also, determination of the exact location of the oxygen insertion for TM4 can provide additional insight into the pharmacophore of tamoxifen. These binding data are consistent with the previous observed affinities of *N*-demethyltamoxifen and 4-hydroxytamoxifen by the estrogen receptor [27,28] and their antiestrogenic activities [18]. In fact, aromatic hydroxylation at position four of tamoxifen (TM7) resulted in a more active compound than tamoxifen [28]. This metabolite may be responsible for the majority of the in vivo antiestrogenic effects of the drug [18]. Other aromatic hydroxylated metabolite such as, 3-hydroxytamoxifen is also active [18] and may corresponds to the other two hydroxytamoxifen metabolites captured in our receptor binding experiment. Mono- and di-oxygenated metabolites of raloxifene

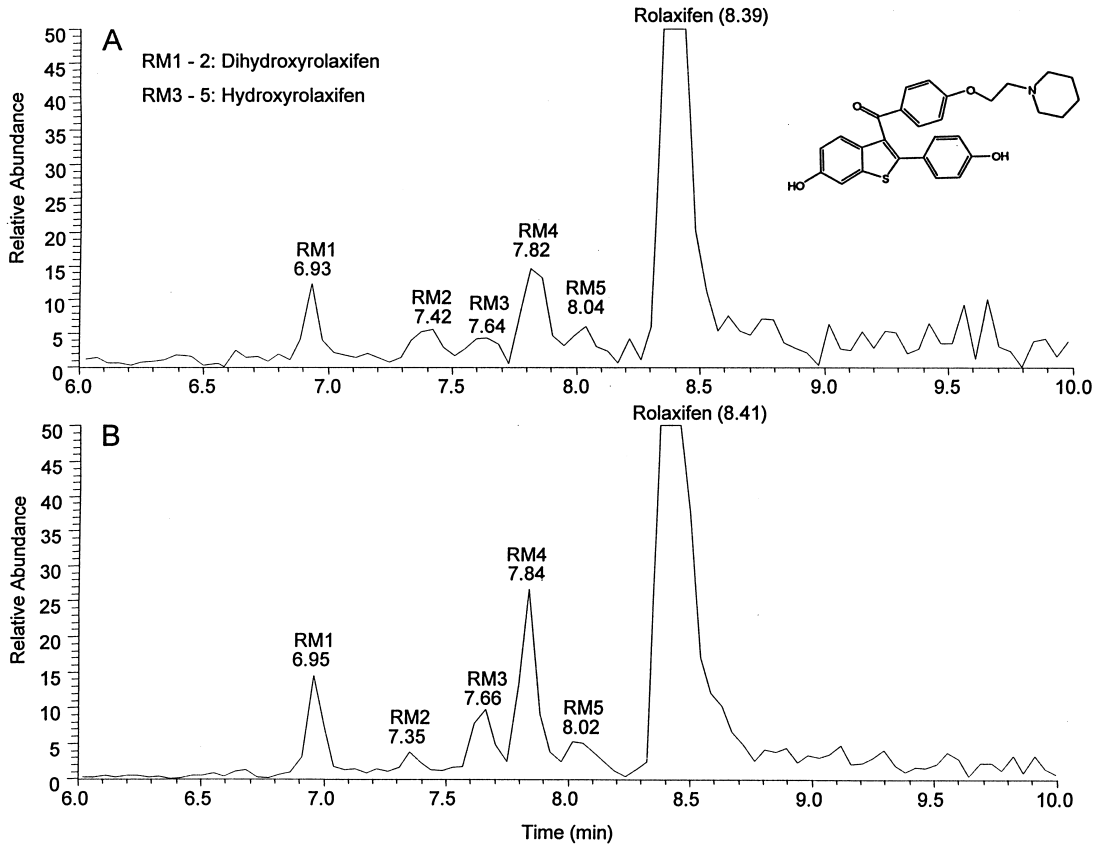

Fig. 6. Reconstructed ion chromatograms corresponding to summing m/z 474, 490 and 506 from LC–MS analyses of an in vitro microsomal incubate of raloxifene after incubation with α-estrogen receptor (A). The lower reconstructed ion chromatogram (B) is from LC/MS analysis of an in vitro microsomal incubate of raloxifene at the same dilution as that used in binding study. Other conditions as described in Fig. 5.

are also recognized by the estrogen receptor. This is not surprising since insertion of two oxygen atoms to the same aromatic ring such as 3,4-dihydroxytamoxifen resulted in higher affinity for the receptor than tamoxifen [29]. Adatanserin and its metabolites did not bind because they lack the estrogen receptor pharmacophore.

The structures of the aromatic hydroxylated metabolites of tamoxifen and raloxifene have hydroxyl group(s) separated by a hydrophobic spacer which resembled the postulated structure of the pharmacophore for the estrogen receptor [30]. The knowledge of these structure–activity relationships can help the refinement of the structure of lead compound to generate better quality leads or second generation drugs. For example, droloxifene (3-hydroxytamoxifen) and idoxifene (4-iodopyrrolidininotamoxifen) are both antiestrogenic compounds generated by modifying the three and four position of tamoxifen. These data clearly shows the value of screening for active metabolites by receptor binding early in drug discovery, which permits further lead optimization to yield a more potent compound or a candidate with a more predictable clinical response.

4. Conclusions

A paradigm for rapid metabolite profiling can be accomplished by coupling fast LC and fast scanning MS. In vitro oxidative metabolic pathways often produced many isomeric metabolites where insuffi-

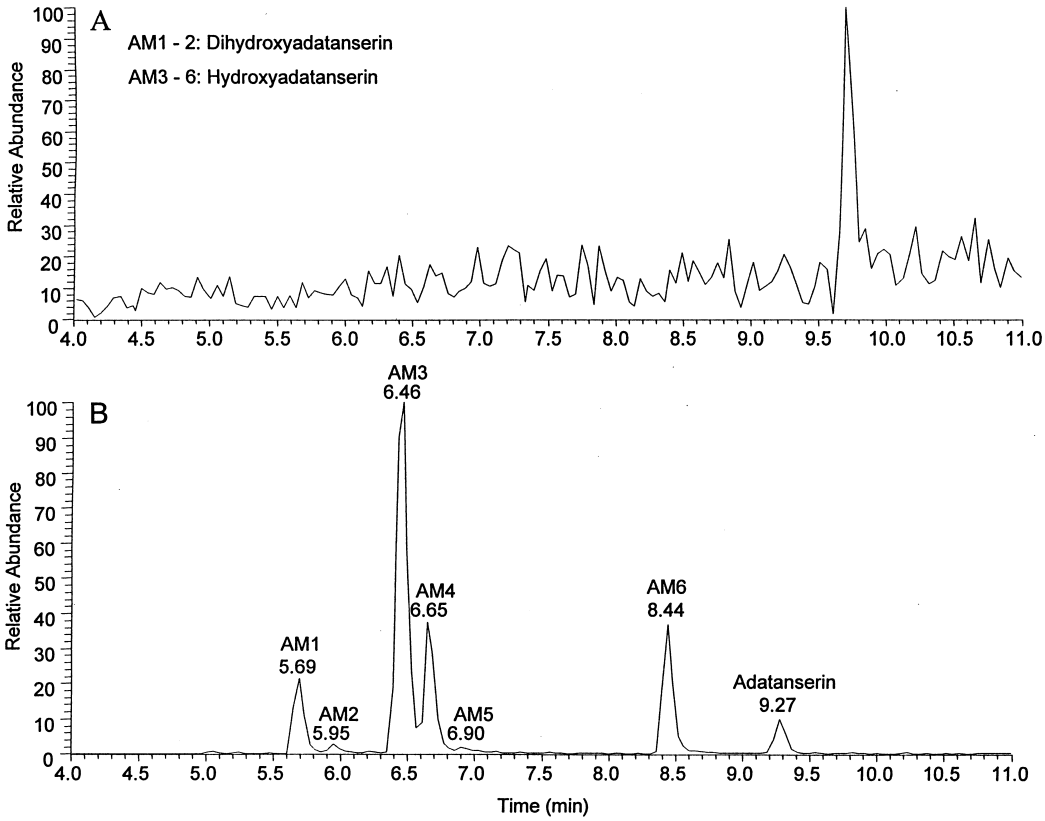

Fig. 7. Reconstructed ion chromatograms corresponding to summing m/z 370, 386 and 402 from LC–MS analyses of in vitro microsomal incubate of adatanserin after incubation with α-estrogen receptor (A). The lower reconstructed ion chromatogram (B) is from LC–MS analysis of an in vitro microsomal incubate of adatanserin at the same dilution as that used in binding study. Other conditions as described in Fig. 5.

cient chromatographic separation will result in poor quality mass spectra which can compromised structural assignment of metabolites. Fast chromatography with sufficient resolution of isomeric metabolites can be achieved by using short (20 mm long) narrow-bore (2 mm I.D.) guard cartridge column packed with small (3 μm) C_8 porous particles and high flow-rates (up to 1 ml/min). This fast LC method provided excellent resolution of in vitro metabolites of tamoxifen and adatanserin in 8 min, suggesting that the method can profile metabolites in a 96-well plate in 13 h. The resulting narrow chromatographic peaks necessitate the use of fast scanning mass spectrometers (such as ion trap and TOF) to obtain quality mass spectra for structural elucidation.

In vitro metabolites can be screened for potential biological activity by incorporating a receptor binding experiment in the paradigm. Results from the receptor binding experiments and structural informations from the LC–MS experiments can provide structure–activity relationships so that the chemical structures of lead drug candidates can be further optimized. This proof of concept may be illustrated by examples where structures of active metabolites have led to the development of second generation drugs such as draloxifene and idoxifene which closely resembled tamoxifen metabolites. Thus, rapid metabolite profiling to generate metabolite structure information coupled with a screen for potential activity by receptor binding can make significant contribution to the discovery of new drug candidate.

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