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LIQUID CHROMATOGRAPHIC–ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRIC ANALYSIS OF TOREMIFENE METABOLITES IN HUMAN URINE

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SUMMARY

A liquid chromatographic–atmospheric pressure ionization mass spectrometric method has been developed for the analysis of toremifene metabolites in human urine after oral administration. This ionization source is a useful device for studying metabolites of toremifene because the total effluent from high-performance liquid chromatography is fed through an interface with a direct heating nebulizer and vaporizer at atmospheric pressure. To obtain good sensitivity the use of the right mobile phase is very important: ammonium acetate in methanol in the case of toremifene and its metabolites. Four unconjugated and three glucuronide-conjugated metabolites were detected in human urine. The majority of these were new and distinguishable from known metabolites.

INTRODUCTION

Toremifene, 2-{4-[(*Z*)-4-chloro-1,2-diphenyl-1-butenyl]phenoxy}-N,N-dimethylethylamine citrate, is a new triphenylethylene antiestrogenic substance developed by Farmos Research Laboratory in Finland (Farmos's compound Fc1157a). Toremifene has a potent antitumour activity similar to that of tamoxifen [1,2]. It has been shown to be especially effective in the treatment

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of advanced recurrent breast cancer. Side-effects are usually mild and transient, and toremifene is therefore well tolerated, even in long-term therapy [3].

The pharmacokinetics and biotransformation of toremifene in human serum after oral administration have been studied in our laboratory. In these studies, it was suggested that toremifene was mainly metabolized to N-demethyltoremifene, which has almost the same potency to bind to estrogen receptors as toremifene and occurs in human serum as a major metabolite. There was only a trace amount of 4-hydroxytoremifene.

In clinical studies, we are now investigating toremifene metabolites in serum and urine. Urinary metabolites may provide important information to explain the clinical efficacy of this substance. Especial interest is focused on the amine group metabolites because of their high antiestrogenic potency.

High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have been used to detect and quantify foreign compounds and their metabolites in biological samples. In TLC there are problems with the separation of all the metabolites. HPLC provides a useful tool for the separation of several kinds of metabolite. This approach has the advantage of requiring minimal clean-up of samples before analysis. It is, however, still limited by the non-specific nature of UV detection. Therefore these methods do not provide structural confirmation of the peak detected.

Development of a combined high-performance liquid chromatographic–mass spectrometric (HPLC–MS) method for toremifene and its metabolites would be of considerable assistance in the confirmation of an HPLC peak prior to quantitative studies and should assist in the observation and confirmation of new metabolic products. Liquid chromatography–atmospheric pressure ionization mass spectrometry (LC–API-MS) using an interface with a direct heating nebulizer and vaporizer has been developed in recent years and has the potential to provide a definitive method [4]. The API source is compatible with LC and offers a number of distinct advantages. It is not limited by the vacuum system constraints common in other types of system, therefore column effluents can be introduced directly into the API source. This technique appears to have the potential for producing the ions needed for a successful determination of toremifene metabolites.

We report here the results of our investigation into the development of a LC–API-MS method for the determination of toremifene and its metabolites in human urine.

EXPERIMENTAL

Reagents

Toremifene and its four analogues (Fig. 1) were generously provided by Farmos Research Laboratory (Turku, Finland). All chemicals were of analytical grade and were obtained from regular commercial suppliers.

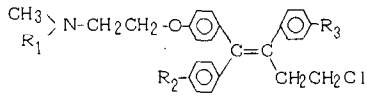

Fig. 1. Structures of toremifene ($R_1=CH_3$; $R_2=R_3=H$) and its four expected metabolites: N-demethyltoremifene ($R_1=R_2=R_3=H$), 4-hydroxytoremifene ($R_1=CH_3$; $R_2=OH$; $R_3=H$), N-demethyl-4-hydroxytoremifene ($R_1=R_3=H$; $R_2=OH$) and 4,4'-dihydroxytoremifene ($R_1=CH_3$; $R_2=R_3=OH$).

Administration

Toremifene was administered to a breast cancer patient at a dose of 480 mg once daily for five days. After the fifth administration, urine was collected over 48 h and kept at −20°C when not in use. Dosage and sample collection were carried out under the supervision of Professor Tsuneaki Senoo, Kawasaki Medical School (Okayama, Japan).

Sample preparation

To the urine (30 ml), 20 ml of 0.1 *M* phosphate buffer (pH 8.5) were added. The sample was extracted twice with 60 ml of *n*-hexane–diethyl ether (9:1, v/v). The organic layer was evaporated and the residue was dissolved in 0.2 ml of methanol.

To remove impurities, the urine sample was applied to HPLC, and the effluent from 5 to 20 min after injection was collected and evaporated. The residue was dissolved in 0.1 ml of methanol and aliquots were analysed by LC–API-MS.

The aqueous layer remaining after the organic extraction was adjusted to pH 5 with 10% phosphoric acid. β-Glucuronidase (Sigma, St. Louis, MO, U.S.A.), 100 000 U, was added, and the mixture was incubated at 37°C for 40 h to obtain the deconjugated metabolites. This was then adjusted to pH 8.5 with 0.1 *M* sodium hydroxide and extracted twice with 60 ml of *n*-hexane–diethyl ether (9:1, v/v). The sample was prepared as above and analysed by LC–API-MS.

HPLC conditions

A Shimadzu LC-6A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) with a loop injector was used. An SPD-2A detector (Shimadzu) was used at a wavelength of 237 nm equipped with a C-R3A data system. Toremifene and its metabolites were eluted isocratically using a mobile phase of 0.1 *M* ammonium acetate (pH 8) in 70% methanol on a 250 mm × 4.6 mm I.D. Zorbax CN (5 μm) column (Du Pont, Wilmington, DE, U.S.A.) at a flow-rate of 1.2 ml/min.

LC-API-MS

A Hitachi M-80B double-focusing mass spectrometer with an API source (M-8093 type) (Hitachi, Tokyo, Japan) and a Hitachi 0101 data-processing system was used for obtaining LC–API-MS data. The nebulizer and vaporizer temperatures were optimized at 350°C. The needle current was set to 15 μA.

The acceleration energy was determined to be 3 keV by the second electrode potential. Selection of optimal mobile phase and drift voltage during the flow injection analysis (FIA) was performed as reported previously [5].

RESULTS AND DISCUSSION

In the LC–API-MS studies, the HPLC mobile phase was optimized in order to obtain good separation and sensitivity using toremifene and its expected metabolites. The selected ion chromatograms of three substances, toremifene, N-demethyltoremifene and N-demethyl-4-hydroxytoremifene, using the $[M+H]^+$ ions at *m/z* 392, 406 and 408, respectively, as the monitoring ions are shown in Fig. 2. The most favourable intensity for all three substances was obtained using methanol–ammonium acetate (70:30, v/v) as the mobile phase. Diethylamine acetate and acetonitrile have high proton affinity, hence are unsuitable for the analysis of toremifene metabolites using LC–API-MS even if good separation is obtained. Furthermore, these compounds exhibited widely varying molar sensitivities. Toremifene was the most sensitive, followed by N-demethyltoremifene and N-demethyl-4-hydroxytoremifene. These sensitivity differences probably reflect differences in the gas phase proton affinities of the metabolites.

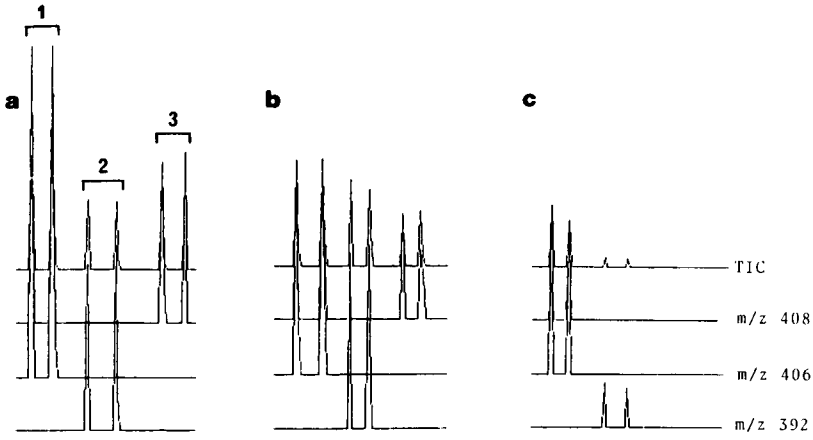

Fig. 2. Effect of the mobile phase on the $[M+H]^+$ ions of (1) toremifene, (2) N-demethyltoremifene and (3) N-demethyl-4-hydroxytoremifene. Mobile phases: a, methanol–ammonium acetate (7:3, v/v); b, methanol–water–acetonitrile–ammonium hydroxide (55:25:25:0.03, v/v); c, methanol–water–acetonitrile–diethylamine acetate (55:25:25:0.03, v/v).

To determine the optimal drift voltage for each expected metabolite using a mobile phase of methanol–ammonium acetate (70:30, v/v), the drift voltage was varied from 125 to 220 V. The $[M+H]^+$ ions of each expected metabolite were used as the monitoring ions, and results are shown in Fig. 3. The drift voltage of each expected metabolite was optimized at 150–195 V. However, since the background in the low mass region interfered with the mass spectrum of each expected metabolite at drift voltages less than 170 V, the drift voltage was set to 195 V during measurement. No significant differences of optimal drift voltage between the expected metabolites were observed under these conditions. The averaged mass spectra of toremifene and its metabolites were obtained under these conditions. The injection volumes necessary to obtain mass spectra of toremifene and its expected metabolites were ca. 50 and 100 ng, respectively.

HPLC with UV detection of (a) standard toremifene and its expected metabolites, (b) unconjugated metabolites present in human urine and (c) the deconjugated metabolites are shown in Fig. 4. The glucuronide-conjugated metabolites were analysed as deconjugated metabolites after treatment with β-glucuronidase because it was difficult to extract and purify conjugated metabolites from human urine and to separate these metabolites by HPLC. The retention times of 4,4′-dihydroxytoremifene, N-demethyl-4-hydroxytoremifene, 4-hydroxytoremifene, N-demethyltoremifene and toremifene were 9.4, 11.7, 13.7, 16.0 and 19.5 min, respectively, which represents a good separation under these conditions. Since some metabolites of toremifene thought to be present in human urine were not detected as peaks and were interfered with by unknown compounds derived from human urine, it was difficult to estimate these metabolites by comparison with the retention times of expected metabolites in both HPLC profiles.

Fig. 5a shows the selected-ion chromatogram of N-demethyltoremifene, to-

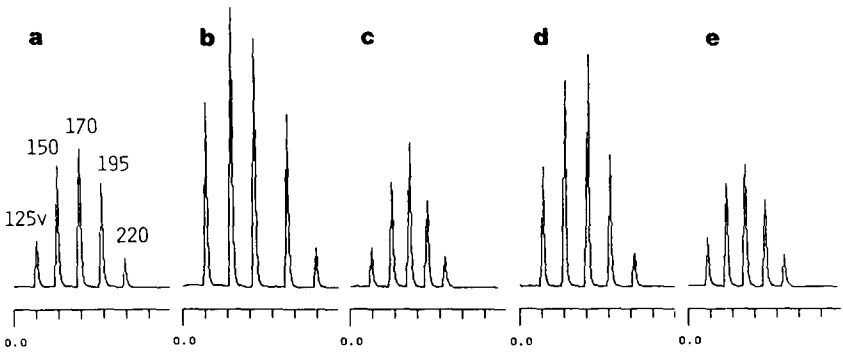

Fig. 3. Relative peak heights of toremifene and four expected metabolites at various drift voltages: (a) N-demethyltoremifene (*m/z* 392); (b) toremifene (*m/z* 406); (c) N-demethyl-4-hydroxytoremifene (*m/z* 408); (d) 4-hydroxytoremifene (*m/z* 422); (e) 4,4′-dihydroxytoremifene (*m/z* 438).

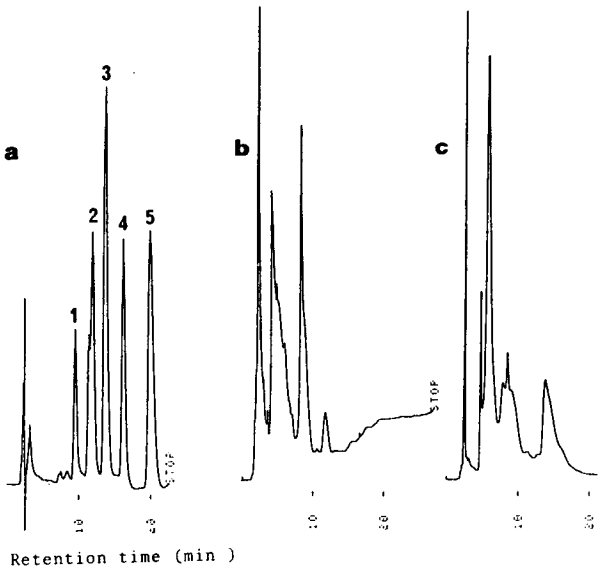

Fig. 4. HPLC with UV detection of (a) expected metabolites, (b) unconjugated metabolites and (c) deconjugated metabolites present in human urine after oral administration of toremifene. Peaks: 1 = 4,4′-dihydroxytoremifene; 2 = N-demethyl-4-hydroxytoremifene; 3 = 4-hydroxytoremifene; 4 = N-demethyltoremifene; 5 = toremifene.

remifene, N-demethyl-4-hydroxytoremifene, 4-hydroxytoremifene and 4,4′-dihydroxytoremifene using $[M+H]^+$ ions at *m/z* 392, 406, 408, 422 and 438, respectively, as monitoring ions, following the injection of 200 ng each of toremifene and its analogues. The separation of the expected metabolites in this selected-ion chromatogram was similar to that in the HPLC–UV profile. As shown in Fig. 5b, when a urine sample spiked with toremifene and expected metabolites (500 ng each) was analysed under the same conditions, a good separation was obtained without interferences, and the retention time was the same in Fig. 5a.

The mass spectra of the expected metabolites obtained by LC–API-MS are shown in Fig. 6. Each one revealed an $[M+H]^+$ ion accompanied by a chlorine isotope peak and a weak signal from an $[M-35]^+$ ion, probably due to the loss of HCl from the molecule.

The selected-ion chromatogram of unconjugated metabolites present in human urine using monitoring ions of *m/z* 408, 422, 438 and 440 is shown in Fig. 7a. The selected-ion chromatogram of deconjugated metabolites using monitoring ions of *m/z* 392, 406, 408, 422, 438 and 440 is shown in Fig. 7b. Five peaks, corresponding to the ions at *m/z* 408, 422, 438 and 440, were recognized in both chromatograms. Peak e had the same retention time as 4-hydroxytoremifene, but the other peaks (a–d) had slightly different retention times compared with those of the expected metabolites. Therefore peak e was thought to

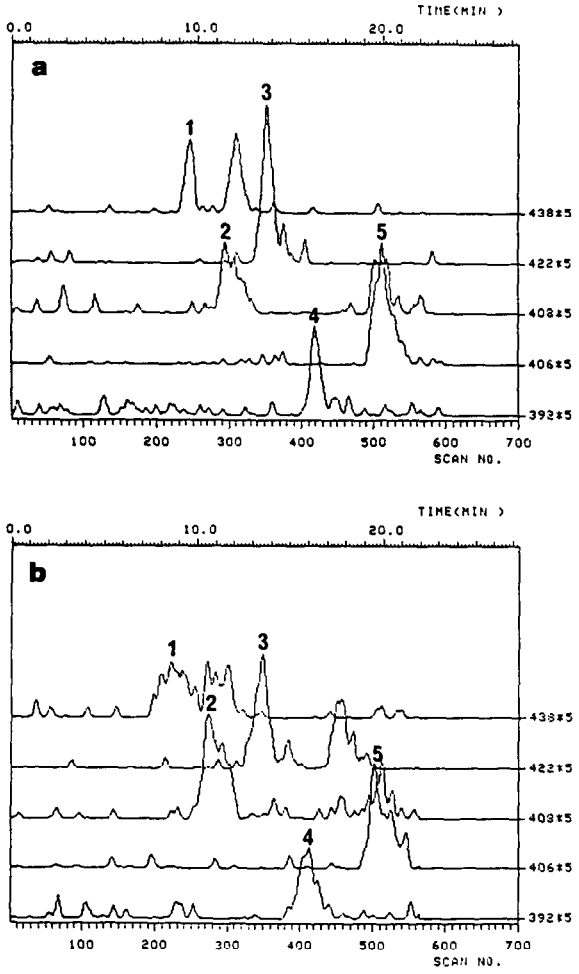

Fig. 5. Selected-ion chromatograms of (a) expected metabolites and (b) human urine spiked with expected metabolites on LC-API-MS. Peaks 1 = 4,4′-dihydroxytoremifene (*m/z* 438); 2 = N-demethyl-4-hydroxytoremifene (*m/z* 408); 3 = 4-hydroxytoremifene (*m/z* 422); 4 = N-demethyltoremifene (*m/z* 392); 5 = toremifene (*m/z* 406).

be 4-hydroxytoremifene, but peaks a–d were not identifiable from their retention times.

The mass spectra of peaks a–d are shown in Fig. 8. These peaks had an $[M+H]^+$ ion at *m/z* 440, 438, 408 and 422, respectively, accompanied by the chlorine isotope peak. In the mass spectrum of peak a, the $[M+H]^+$ ion did not correspond to any ion of the expected metabolites, but appeared 34 a.m.u. higher than that observed for toremifene. We therefore think that two hydroxy residues may be introduced on the ethylene bond of toremifene. In the mass

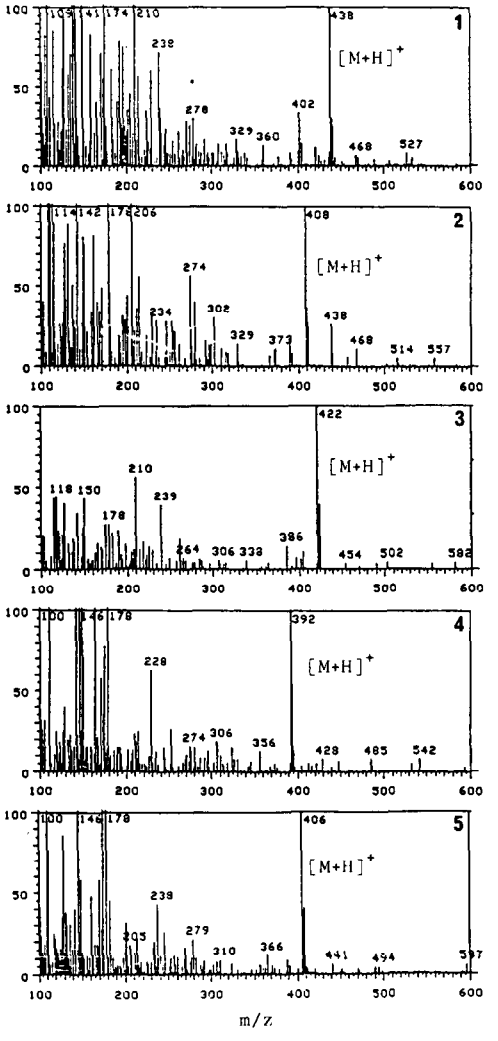

Fig. 6. Mass spectra of (1) 4,4′-dihydroxytoremifene, (2) N-demethyl-4-hydroxytoremifene, (3) 4-hydroxytoremifene, (4) N-demethyltoremifene and (5) toremifene on LC–API-MS.

spectrum of peak b, because it had the almost same retention time as peaks a and c, the ion at *m/z* 440 corresponding to the isotope peak of the ion at *m/z* 438 appeared with the same intensity as the $[M+H]^+$ ion at *m/z* 438. Furthermore, peak b gave an $[M+H]^+$ ion at *m/z* 438, 32 a.m.u. higher than that obtained for toremifene. We estimated that two hydroxy residues may be introduced into toremifene molecule but distinguished from 4,4′-dihydroxytoremifene. Peak c gave an $[M+H]^+$ ion at *m/z* 408, 16 a.m.u. higher than that obtained for N-demethyltoremifene. A hydroxy residue may be introduced into

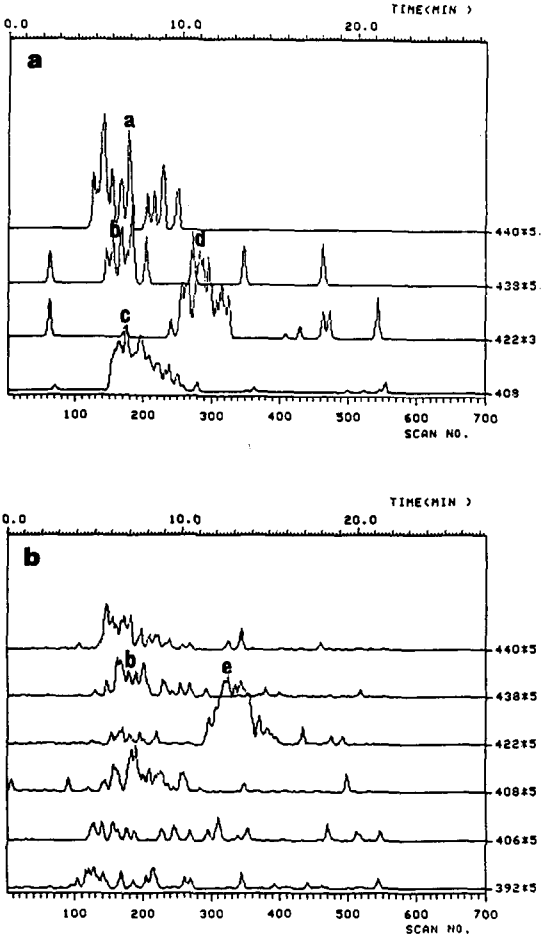

Fig. 7. Selected-ion chromatograms of (a) unconjugated and (b) deconjugated metabolites present in human urine on LC–API-MS.

N-demethyltoremifene molecule but distinguished from N-demethyl-4-hydroxytoremifene. The mass spectrum of peak d was almost the same as that of 4-hydroxytoremifene but there was a slight difference in retention time. A hydroxy residue may be introduced into a position other than the 4-position of the aromatic residue.

In the study of toremifene metabolites in human urine after treatment with β-glucuronidase, two peaks, b and c, were observed in the selected-ion chromatogram. Fig. 9 shows the mass spectra of peaks b and c. Peak b gave ions at m/z 438 and 468, which were taken to be the $[M+H]^+$ ion of the metabolites, accompanied by the chlorine isotope peak. Peak b possibly contained two metabolites: one of them may be dihydroxytoremifene and the other may be hy-

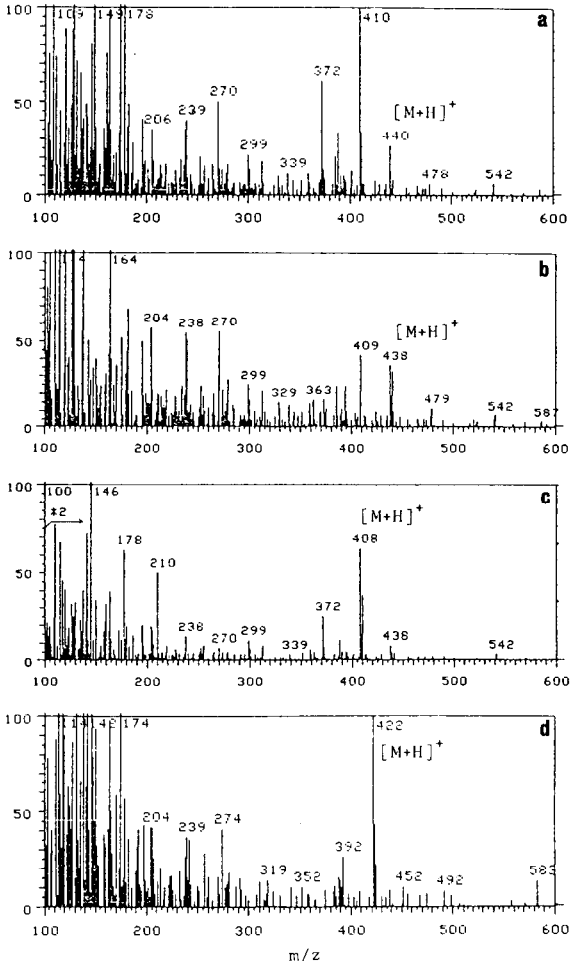

Fig. 8. Mass spectra of unconjugated metabolites present in human urine on LC–API-MS. (a) 1,2-Dihydroxytoremifene; (b) dihydroxytoremifene; (c) N-demethylmonohydroxytoremifene; (d) monohydroxytoremifene.

droxymethoxytoremifene. These two metabolites occur as glucuronide conjugates in human urine. The retention time of peak e was in good agreement with that of 4-hydroxytoremifene and gave the same spectrum as 4-hydroxytoremifene. This confirmed that peak e was 4-hydroxytoremifene, which also occurs as the glucuronide conjugate in human urine. The other peaks that appeared on the selected-ion chromatograms (Fig. 7a and b) may not be toremifene metabolites because their mass spectra did not have a chlorine isotope peak, which is characteristic of toremifene analogues.

Fig. 10 summarizes the toremifene metabolites expected to be present in human urine.

In the study of metabolism using LC–API-MS, it is important to get good

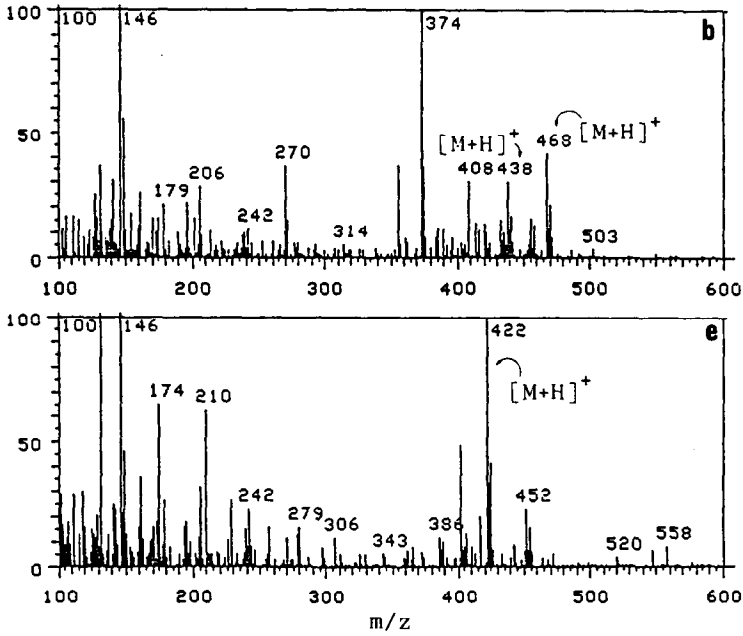

Fig. 9. Mass spectra of deconjugated metabolites present in human urine on LC-API-MS. (b) Dihydroxy- and hydroxymethoxytoremifene; (e) 4-hydroxytoremifene.

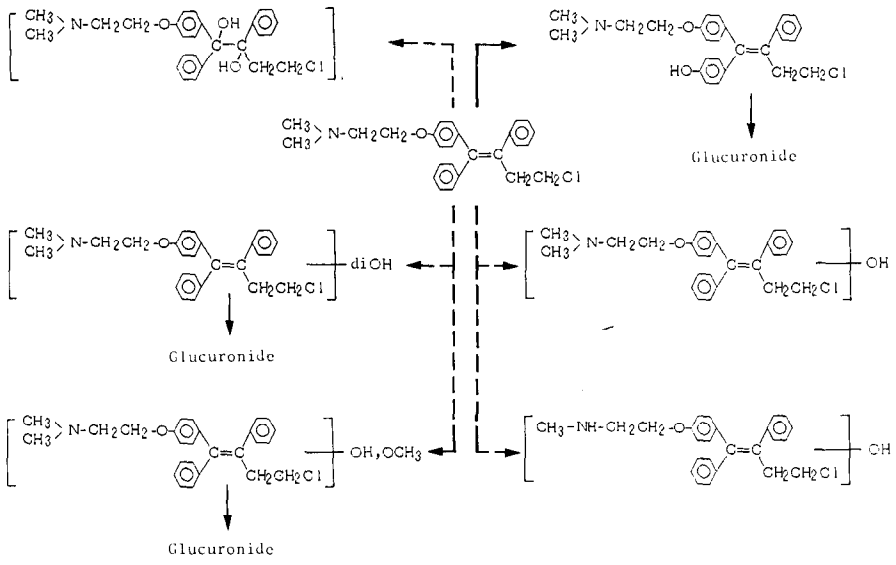

Fig. 10. Expected metabolites of toremifene present in human urine.

separation in the HPLC analysis since it is difficult to obtain the mass spectra of metabolites that are not separated from each other and from impurities present in biological samples. However, if each metabolite has a different molecular weight, it is possible to estimate them by the selected-ion method even if there is insufficient separation. Four unconjugated and three glucuronide-conjugated metabolites were observed in human urine after the oral administration of toremifene. Except for 4-hydroxytoremifene glucuronide, the structures of the other metabolites could not be confirmed. The structures of the unknown metabolites and their affinity for the estrogen receptor are under investigation.

CONCLUSION

LC–API-MS provides a useful method for the confirmation of toremifene and its metabolites in biological samples. The API source is a useful device for studying metabolites because the total effluent from HPLC is fed through an interface with a direct heating nebulizer and vaporizer at atmospheric pressure. In this case, the HPLC solvent must be carefully selected to obtain enough ion intensity. In our studies, ammonium acetate was required to obtain good results. Four unconjugated and three glucuronide-conjugated metabolites could be detected in human urine.

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