Electrospray Ionization Mass Spectrometry for Analysis of Low-Molecular-Weight Anticancer Drugs and Their Analogues

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In this study, several anticancer drugs and their analogues consisting of organic and organometallic compounds were analyzed by electrospray ionization mass spectrometry (ESI/MS) using a quadrupole mass spectrometer. Protonated molecular ions $[M + H]^+$ were observed for all of the compounds studied, and in the case of the two steroid sulfates, deprotonated molecular ions $[M - H]^-$ were obtained. Tandem mass spectrometry was performed on these quasimolecular ions, and the product ions formed provided useful fragmentation patterns that were characteristic for the compounds. This study provides evidence that ESI/MS is a sensitive technique for structure confirmation and identification of small organic and organometallic molecules. (*J Am Soc Mass Spectrom 1993, 4, 588–595*)

In 1968, Dole et al. [1] first introduced the idea of using electrospray as an ionization technique for mass spectrometry. Recently, Fenn and co-workers [2, 3] succeeded in interfacing the technique with modern mass spectrometers, and have generated a great deal of interest in this technique. Electrospray ionization mass spectrometry (ESI/MS) has made important contributions to biotechnology research because it is capable of accurately determining the molecular weight of proteins in excess of 200 kDa [4], and it has been extensively used for estimating the molecular weights of a wide variety of proteins (for review, see Smith et al. [5]).

The biotransformation and metabolism of drugs in the body play an important role in their pharmacologic actions. The parent drug and/or its metabolites are often present in the biological fluid at very low concentrations; hence reliable, sensitive, and specific analytical techniques are required for their detection. ESI/MS offers great potential in this area of research because it is a soft ionization technique that is capable of generating information about molecular weight. It has been successfully used to determine the molecular weight of small organic molecules at the femtomole level. Moreover, in conjunction with tandem mass spectrometry, it can provide fragmentation patterns of molecules, enabling structural confirmation. This study describes the analysis of four different classes of anticancer drugs by ESI/MS, in which the technique provides detailed information at high sensitivity.

Experimental

Reagents and Samples

The structures of the compounds discussed in this study are shown in Figure 1. Aminoglutethimide (AG) and 4-hydroxyandrostene-3,17-dione (4OHA) were provided by Ciba-Geigy Pharmaceuticals Ltd. (Horsham, UK). JM221 and JM216 were obtained from Johnson Matthey Technology Centre (Reading, UK). 5-Androsten-3,17-diol-17-sulfate was purchased from Sigma Chemical Co. (Poole, UK). Rogletimide (RG), tamoxifen, desmethyltamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, and 4-iodotamoxifen were synthesized by Dr. R. McCague in the CRC laboratory. Polyglutamated ICI D1694, CB3988, CB30-023, and 4OHA-sulfate were synthesized in our laboratory. All solvents used were high-performance liquid chromatography (HPLC) grade purchased from Romil Chemicals (Shepshed, UK).

Polyglutamated ICI D1694, CB3988, and CB30-023 were dissolved in methanol:water (50:50 v/v) containing 1% acetic acid. AG, RG, JM216, and JM221 were dissolved in methanol:water (50:50 v/v). 4OHA-sulfate and 5-androstene-3,17-diol-17-sulfate were dissolved in methanol:water (90:10 v/v). All chemicals were at a concentration of 10 ng/μL. Tamoxifen and its analogues were dissolved in methanol:water (50:50 v/v) at a concentration of 1 ng/μL. The solutions were further diluted where appropriate.

Instrumentation

Each of these solutions was infused at 1 μL/min with an infusion pump (Harvard Apparatus, Cambridge,
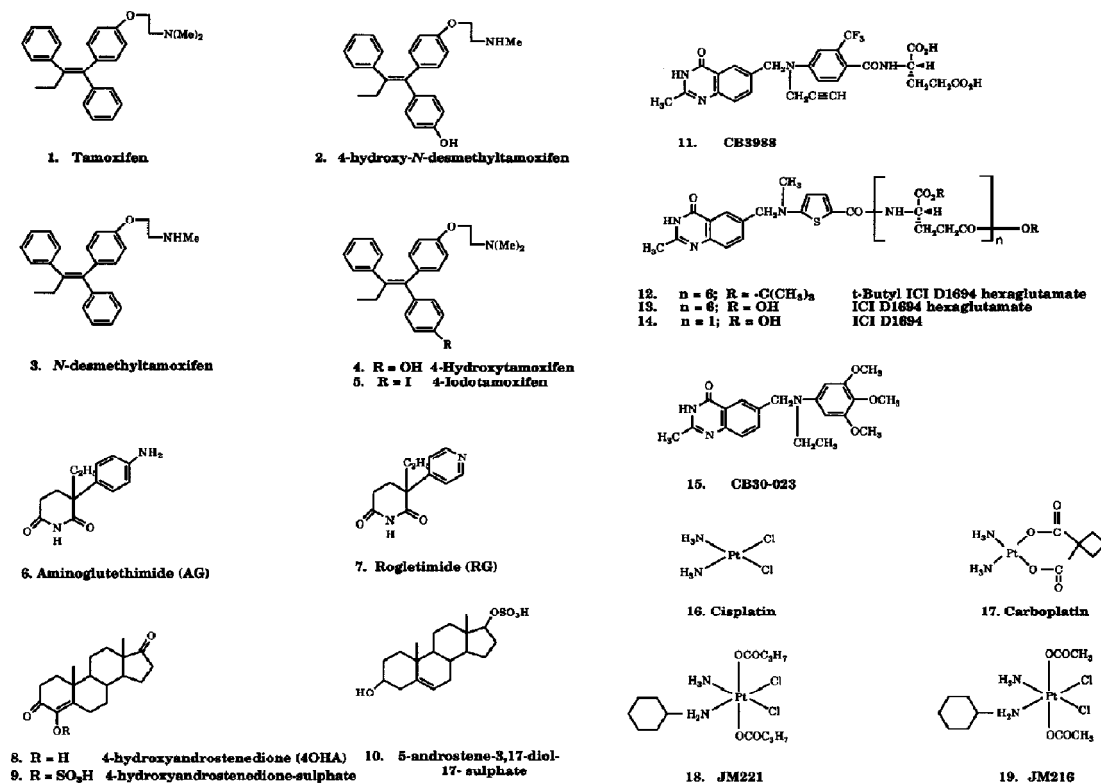

Figure 1. Structures of the compounds discussed in this study.

MA) into a TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ion source (Analytical, Branford, CT). A fused-silica uncoated capillary was used to connect the delivery syringe with the stainless steel spray needle. A potential difference of 3400–3800 V for the positive ions and 2900 V for the negative ions was applied between the grounded needle and the gold-plated ends of the glass capillary tube that transmit ions to the analyzer. Nitrogen heated to 60 °C was used as the drying gas.

Classical tandem mass spectra were obtained by opening the resolution in the first quadrupole (Q_1) so that the selected parent ion was transmitted to the collision cell in the second quadrupole (Q_2). Argon was used as the collision gas (0.3–0.5 mtorr), and the product ions were detected in the third quadrupole (Q_3). Alternatively, by increasing the voltage on the capillary, collision-activated dissociation (CAD) fragments were also produced in the high-pressure region between the glass capillary tube and skimmer within the electrospray source.

The DEC 2100 data system was controlled by Finnigan ICIS software (version 7). Full-scan spectra were acquired in centroid or profile mode. Profile data were averaged for 64 scans to improve the signal-to-noise ratio in the spectrum. Mass spectra in the positive ionization mode were obtained for most of the compounds. Negative ionization was used for the steroid conjugates. A scanning speed of 3 s per scan was used in all experiments.

Results and Discussion

Tamoxifen and Its Analogues

Tamoxifen (1) {z-1[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-1-butene} is a nonsteroidal antiestrogen compound. It has been widely used in the treatment of estrogen-dependent tumors [6]. Tamoxifen is extensively metabolized: 4-Hydroxy-N-desmethyltamoxifen (2) has been identified in human urine, and N-desmethyltamoxifen (3) and 4-hydroxytamoxifen (4) have been found in human plasma. Once formed, 4-hydroxytamoxifen is rapidly conjugated to the corresponding glucuronide and excreted [7, 8]. Hence 4-iodotamoxifen (5) was synthesized to block the formation of 4-hydroxytamoxifen in vivo [9]. Several methods are available for the analysis of tamoxifen and its metabolites in biological samples. The most commonly used procedure is HPLC separation followed by postcolumn derivatization by in situ irradiation of the column effluent and detection by fluorescence [10]. The limit of detection was less than

1 ng/mL [11]; however, this technique did not provide molecular ion or structural information until the appropriate fractions containing compounds of interest were eluted from the HPLC column and characterized by mass spectrometry [12]. Subsequently, the coupling of liquid chromatography with mass spectrometry (LC/MS) using a thermospray (TSP) ionization interface allowed on-line identification of the parent drug and its metabolite 4-hydroxy-N-desmethyltamoxifen (2) in urine samples by single-ion monitoring and further confirmation by gas chromatography/mass spectrometry (GC/MS) [7, 8]. GC/MS was demonstrated to be a specific and sensitive technique for analyzing tamoxifen and its metabolites, the only disadvantage being that it is necessary to derivatize the hydroxylated metabolites prior to analysis [13, 14].

Full-scan classical ESI/CAD mass spectra obtained from direct infusion of tamoxifen and 4-iodotamoxifen at 25 pmol/μL are shown in Figure 2a, b. For all compounds analyzed (1–3, 5), the major fragmentation occurred consistently at the dimethylaminoethane side chain, the pattern being similar to that found in spectra obtained under electron impact ionization (EI) mass spectrometry [14, 15]. In the case of compounds 2 and 3, the [M + H]$^+$ ions are seen at approximately 60% relative abundance, and they both give abundant fragment ions at m/z 58 (100%). This fragmentation is a

useful feature for characterization of the tamoxifen metabolites because their alkyl side chain is frequently altered. With 4-iodotamoxifen, cleavage of the iodophenyl ring was observed to give m/z 206.

Under ESI/MS, the minimum amount of sample consumed to acquire a single full-scan spectrum for tamoxifen was 2 fmol, and when acquired in the average profile mode, the signal-to-noise level was better than 5 to 1 (Figure 3a, b). These results suggest that ESI/MS can offer better sensitivity for analysis of this class of compounds than GC/MS. In view of this, on-line LC/ESI/MS was used to analyze plasma samples from patients receiving tamoxifen treatment (30-mg oral dose). The 3-h posttreatment plasma sample (2 mL) was extracted with hexane containing 2% butanol (3 × 2 mL). The extracts were combined and dried under nitrogen. The residue was dissolved in 10 μL acetonitrile, and 2 μL was analyzed by LC/MS. Separation of tamoxifen and its metabolite was achieved using a Suplex pKb-100 250 × 1-mm column (Supelco, Oakville, Ontario, Canada). The reconstructed ion chromatograms of N-desmethyltamoxifen and tamoxifen (m/z 358 and 372, respectively) are shown in Figure 4. Full-scan CAD spectra for these metabolites were identical to those of authentic reference materials. (Detailed results of this work will be described in a future communication.) These preliminary results illustrate that on-line LC/ESI/MS can be used directly to analyze plasma extracts with good sensitivity, and it avoids the need for derivatization required by GC/MS.

Aromatase Inhibitors

Aromatase is the cytochrome P_{450} enzyme responsible for converting the circulating androgens into estrogens in the final step of the steroid biosynthetic pathway. Inhibiting this enzyme provides a useful means of treating estrogen-dependent tumors, such as breast cancer. AG (6) is the first nonsteroidal aromatase inhibitor being routinely used for breast cancer treatment [16]. Recently, several new aromatase inhibitors have been developed. RG (7) is under Phase I clinical trials, and 4OHA (8) has just been licensed in the United Kingdom and New Zealand [17, 18].

HPLC is the most commonly used analytical method available for quantification of these drugs in biological fluids, with structural confirmation being carried out either by off-line [19] or on-line TSP/MS [20–22]. We evaluated the suitability of ESI/MS for the study of these compounds. AG and RG were infused of 500 pmol/μL into the mass spectrometer. Both compounds displayed their singly charged protonated molecules at m/z 233 and 219, respectively, and the corresponding dimers at m/z 465 and 437 respectively. Their CAD spectra (Figure 5a, b) resembled their EI spectra [19]. The principal fragments for both compounds involved the loss of [MH − C_2H_5]$^+$, to give m/z 204 and 190 respectively. For AG, other fragments are derived from ring cleavage and subse-
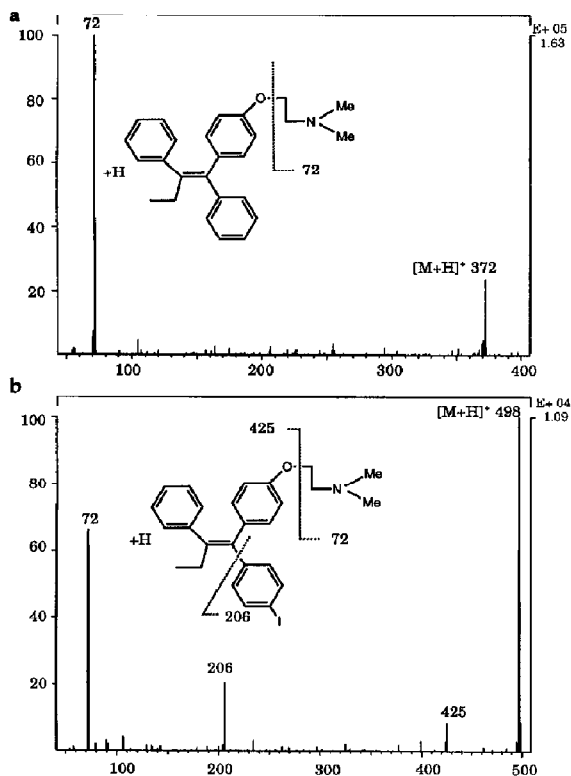

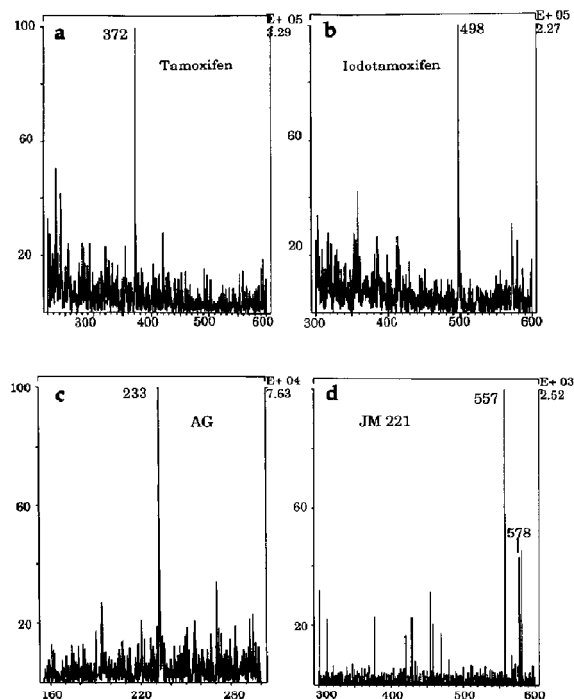**Figure 2.** Classical CAD mass spectra of (a) tamoxifen and (b) 4-iodotamoxifen.

Figure 3. Full-scan mass spectra of (a) 5 fmol of tamoxifen, (b) 5 fmol of iodotamoxifen, (c) 250 fmol of AG, and (d) 100 fmol of JM221.

quent loss of aniline at m/z 94, $[C_6H_8N]^+$. The fragment ion at m/z 133 (Figure 5b) is characteristic of RG. Metabolites derived from AG will normally produce this characteristic ion in CAD experiments. This feature has been used in metabolic studies by selecting and identifying the parent ions that fragment to give m/z 133 [23]. The detection limit for acquiring a full-scan spectrum for the parent drug AG is 5 pmol/μL, with a signal-to-noise ratio of 5 to 1 (Figure 3c). Although the sensitivity observed for ESI/MS is comparable to TSP/MS, ESI/MS has a distinct advantage in
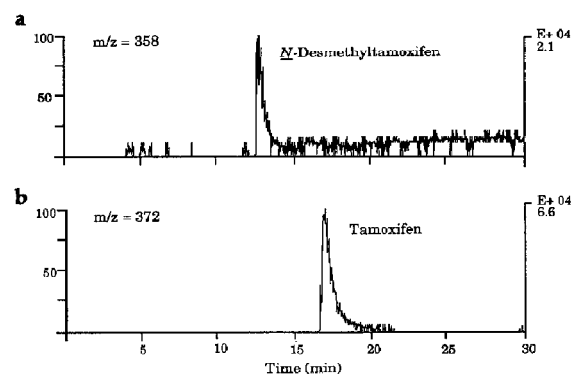

Figure 4. Reconstructed ion chromatogram of posttreatment plasma extracts from a patient who had received tamoxifen treatment: m/z 358 for N-desmethyltamoxifen and m/z 372 for tamoxifen.

that it is capable of detecting the conjugated metabolites as intact molecules [24]. Previous studies have shown that during LC/MS analysis, 4OHA-conjugated metabolites generally decompose under TSP conditions [21]. In contrast, the $[M - H]^-$ ion of 4OHA-sulfate (9) predominates when monitored by ESI/MS in the negative ionization mode. CAD was performed by increasing the voltage at the ESI source. Full-scan mass spectra of 4OHA-sulfate (9) and an analogue, 5-androstene-3,17-diol-17-sulfate (10), were obtained by direct infusion of a solution containing 3 pmol/μL of the compounds, equivalent to 150-fmol consumption of each sample (Figure 5c, d). Loss of the sulfate group (HSO_4^-) at m/z 97 was observed in both spectra. The ion at m/z 353, $[MH - 16]^+$, from 5-androstene-3,17-diol-sulfate results from the loss of a CH_4 from the methyl group at the C_{18} position, as suggested by Tomer and Gross [25]. For 4OHA-sulfate, the fragmentation pattern followed successive ring cleavage, as expected. Recently, with the help of ion spray ionization mass spectrometry, several sulfate- and glucuronide-conjugated metabolites of 4OHA have been identified for the first time from the urine samples of cancer patients [26].

Thymidylate Synthase Inhibitors

Thymidylate synthase (TS) is the enzyme responsible for catalyzing the methylation of deoxyuridine 5'-monophosphate to deoxythymidine 5'-monophosphate, which in turn becomes incorporated into the biosynthetic pathway leading to DNA. Quinazoline antifolates (QAs) are potent inhibitors of TS and work by mimicking the natural cofactor tetrahydrofolic acid. QAs are an important class of compounds for treating different cancers, including ovarian and colon cancer. Numerous QA analogues have been synthesized and analyzed successfully by ESI/MS.

The first example is C^2-desamino-C^2-methyl-N^{10}-propargyl-2'-trifluoromethyl-5,8-dideazafolic acid (CB3988) (11). CB3988 contains a trifluoromethyl group and is therefore amenable to study by ^{19}F-nuclear magnetic resonance (NMR) spectroscopy. The compound was designed and used as a probe for evaluating the utility of in vivo ^{19}F-NMR as a noninvasive method of determining the pharmacokinetics of this class of compounds [27]. Figure 6a is the spectrum obtained by directly infusing a solution of CB3988 into the mass spectrometer, whereas Figure 6b is the classical CAD spectrum, where only the 200-fmol sample is consumed to provide the spectrum. CAD spectra could be obtained either by increasing the voltage in the ESI source or by colliding the parent ion at Q_2 with argon. Identical spectra were obtained with both methods. The major fragment is at m/z 398, which was caused by cleavage of the peptide bond. The loss of m/z 173 could be attributed to the cleavage of the quinazoline ring or the loss of the —CO-glutamic acid moiety. The first proposal is the more likely explanation because
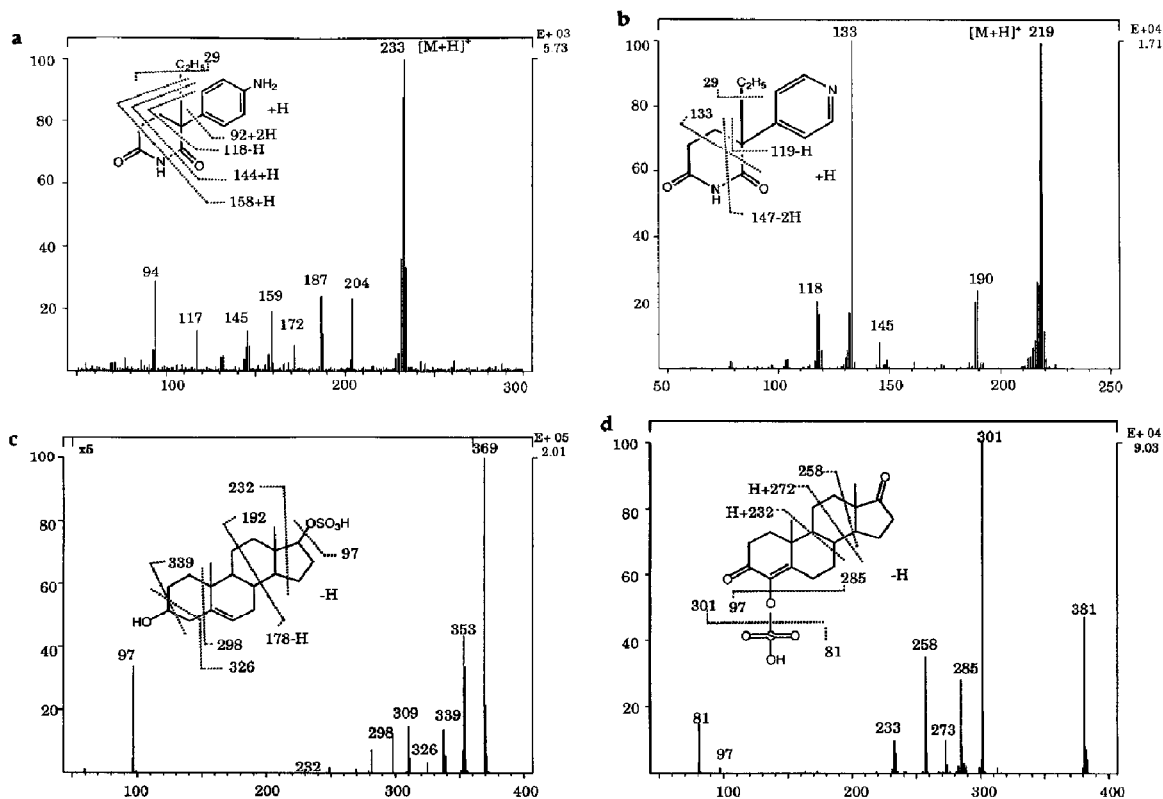
Figure 5. Classical CAD mass spectra of (a) AG and (b) RG; negative ion CAD mass spectra of (c) 4OHA-sulfate and (d) 5-androstene-3,17-diol-17-sulfate obtained by increasing the capillary voltage at the ESI source.

this fragmentation pattern is frequently observed in this class of compounds.

The second example is the tert-butyl protected ICI D1694 hexaglutamate (12), a synthetic precursor to ICI D1694 hexaglutamate (13) [28]. ICI D1694 hexagluta-mate is an active metabolite of ICI D1694 (14), a drug that is currently undergoing Phase I clinical trials. Studies have shown that the polyglutamated forms of ICI D1694 are approximately 100-fold better inhibitors of TS compared with the parent drug and are also not readily effluxed from the cell, indicating that these metabolites are in fact the active species [29]. The mass spectrum (Figure 6c) of the tert-butyl protected precur-sor (12) exhibits a strong protonated molecule at m/z 1496 and a doubly charged ion at m/z 749. Then a successive loss of tert-butyl groups from both the singly and doubly charged ions were detected. ESI/MS worked equally well in confirming the structure of deprotected antifolate polyglutamates, such as ICI D1694 hexaglutamate (13).

The third example is CB30-023 (15). This compound was designed as a possible lipophilic inhibitor of TS [30], and 5 ng of material was consumed to give the CAD spectrum (Figure 6d). Two intense fragment ions

are detected at m/z 210 and 172, which again sug-gested the loss of the quinazoline moiety.

Platinum Anticancer Drugs

Cisplatin (cis-dichlorodiammineplatinum II) (16) and its less toxic analogue carboplatin (cis-diammine cy-clobutane-1,1-dicarboxylato-platinum) (17) are organo-platinum complexes that are extensively used for the treatment of a wide variety of solid tumors, including ovarian and testicular cancers [31]. These two drugs have a similar spectrum of clinical activity, and devel-opment of resistance often compromises their overall efficacy. Hence, development of analogues capable of overcoming resistance to these drugs is highly desir-able. At the Institute of Cancer Research, a program is currently underway to develop such compounds along with platinum complexes capable of being adminis-tered orally. Critical to the success of these programs is the understanding of the pharmacokinetic behavior of novel platinum compounds, such as the platinum (IV) dicarboxylate complexes JM221 [ammine-bis(butyl-ato)dichloro(cyclohexylamine)-platinum(IV)] (18) and
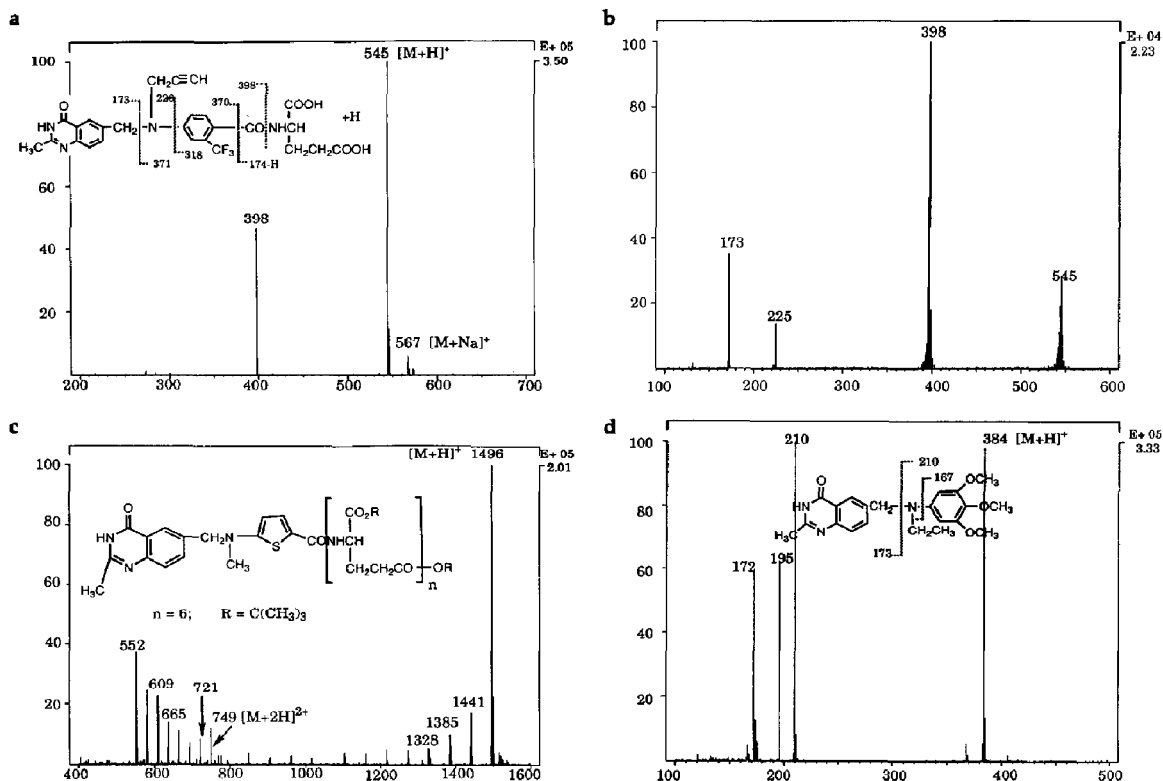
Figure 6. ESI mass spectrum of (a) CB3988 and classical CAD mass spectrum of (b) CB3988, (c) tert-butyl protected ICI D1694, and (d) CB30-023.

JM216 [ammine-bis(acetylato)dichloro(cyclohexyla-mine)-platinum(IV)] (19). JM216 is currently undergoing Phase I clinical trial as an orally administrable drug [32].

The analytical techniques currently available for studying platinum complexes in biological samples are limited and mainly involve HPLC using UV [33] and/or off-line atomic absorption spectrometry detection [34]. To date, mass spectrometry [35] and NMR spectroscopy [36] have made a limited contribution because of their poor sensitivity for studying these compounds; however, our recent studies on cisplatin and its second-generation analogues have demonstrated that ESI/MS may have adequate sensitivity for identification of metabolites of these compounds [37]. In the present report this study is extended to the analysis of novel third-generation platinum complexes.

The mass spectra obtained by direct infusion of a solution containing JM221 and JM216 (20 pmol/μL) are shown in Figure 7a, c. Both spectra showed strong sodium and potassium adduct ions. The intensity of the protonated molecule $[M + H]^+$ can be enhanced by addition of a low concentration of acetic acid (1%) to the mixture; however, addition of higher concentration of acetic acid (5%) caused substantial fragmentation, resulting in an abundant signal at m/z 292,

$[Pt—C_6H_{11}NH_2]^+$, for JM216 and m/z 469, $[M — OCOC_3H_7]^+$, for JM221 (data not shown). Fragmentation of these coordination complexes often occurs without increasing the voltage at the ESI source, and increasing this voltage results in successive loss of ligands from the molecule, as shown in the CAD spectrum of JM216 (Figure 7c). The fragment ions include m/z 523, $[M + Na]^+$, m/z 501, $[M + H]^+$, m/z 441, $[M — CH_3OCOH]^+$, and m/z 308, $[M — 2 \times (OCOCH_3) — 2 \times HCl]^+$. Classical CAD spectra from both compounds provided an extra strong signal at m/z 98, suggesting a loss of the cyclohexylamine ligand (see, for example, Figure 7b). Major fragment ions from JM221 include m/z 469, $[M — OCOC_3H_7]^+$, m/z 380, $[M — 2 \times (OCOC_3H_7)]^+$, m/z 344, $[M — 2 \times (OCOC_3H_7) — HCl]^+$, and m/z 308, $[M — 2 \times (OCOC_3H_7) — 2 \times HCl]^+$ (Figure 7b). Another unusual observation from this type of compound is the strong tendency for the formation of dimers $[2M + H]^+$, $[2M + Na]^+$, and $[2M + K]^+$ at m/z 1001, 1023, 1039, respectively, in the case of JM216 (Figure 7d). Meng and Fenn [38] observed multiple ion cluster formation when they studied arginine by ESI/MS, but the mechanism of their formation is unknown.

An aliquot (5 μL) of human plasma ultrafiltrate obtained from plasma incubated with 120 μM JM216

for 1 h at 37 °C was analyzed by LC/MS. LC/MS separation was achieved using a 250 × 1-mm PLRP-S reversed-phase column (Polymer Laboratories, Shropshire, UK). The mobile phase consisted of acetonitrile and water. The gradient elution profile was 0–20 min; 10–19% acetonitrile; 20–25 min; 19–90% acetonitrile; 25–35 min; 90% acetonitrile. The parent drug has a retention time of 33 min, as shown in Figure 7e, and the mass spectrum is shown in Figure 7f. These data,

together with our recent report [37], suggest that ESI/MS has adequate sensitivity for elucidating structures of nonvolatile platinum complexes in biological samples.

Conclusion

The direct introduction of samples for ESI/MS analysis provides a fast, simple, and sensitive technique for
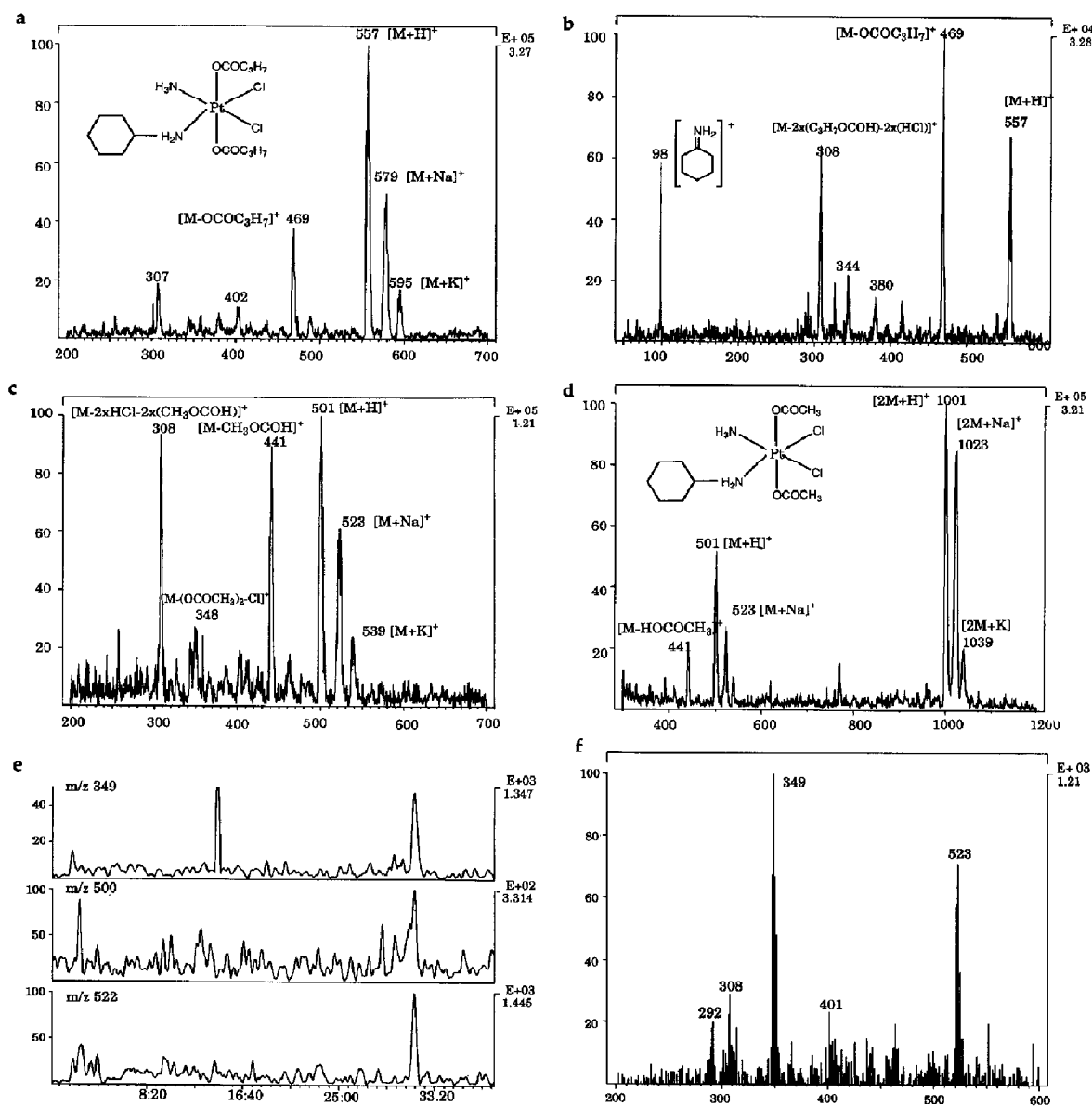

Figure 7. (a) ESI mass spectrum of JM221 obtained by direct infusion of the sample; (b) mass spectrum of JM221 obtained by CAD of m/z 557; (c) ESI mass spectrum of JM216 obtained by direct infusion of the sample; (d) mass spectrum of JM216 showing the dimer at m/z 1001; (e) reconstructed ion chromatograms of an LC/MS analysis of a human plasma ultrafiltrate after incubation with JM216; (f) mass spectrum of JM216 observed in the sample.

structure confirmation and identification of small organic and organometallic compounds. It is also feasible to utilize on-line LC/ESI/MS for identifying polar metabolites of these components at low concentrations in biological samples. In comparison to fast-atom bombardment (FAB) ionization, ESI/MS has better sensitivity, and the resulting spectra are free from matrix signal interference. For example, the limits of detection to obtain a full-scan mass spectrum of JM221 by static FAB/MS, continuous flow FAB/MS, and ESI/MS are 200 and 10 pmol and 100 fmol, respectively. In addition, a further advantage of ESI/MS over FAB/MS as an on-line technique is that no matrix needs to be incorporated in the LC mobile phase. Because ESI/MS is relatively easy to operate and accepts low flow rates, LC/ESI/MS may be automated; however, because of the low flow rate, a splitter or a microbore column is required to comply with this condition. On the other hand, in some situations where only small absolute amounts of sample are available, the microscale analysis using a microbore column may be an advantage. The simple technique of obtaining a CAD spectrum by increasing the voltage of the ESI source is especially useful if pure compounds are used. A complex mixture might well lead to contributions from several compounds to the CAD product ions. ESI/MS has undoubtedly made a significant contribution toward drug development and drug metabolism, as illustrated by the results presented here.

Acknowledgments

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