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TANDEM MASS SPECTROMETRY: The Competitive Edge for Pharmacology

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INTRODUCTION

The notion of connecting two mass spectrometers to examine the chemistry of purified ion species was realized experimentally as early as 1954 (1), and ten years later instruments of this complexity had been constructed in five laboratories worldwide (2). The utility of collisions of fast-moving ions with neutral target atoms to promote fragmentation was introduced by Jennings in 1968 (3). The analytical value of collisional activation and tandem mass spectrometry for components of mixtures received attention through the next decade (e. g. 4–6), and was demonstrated with a variety of instrumental configurations. The commercial availability around 1980 of tandem mass spectrometers based on three-sector analyzers and on three quadrupole analyzers, and around 1985 of four-sector tandem instruments and of hybrids comprising two sectors and two quadrupole modules, rapidly increased the use of tandem (MSMS) techniques in analytical applications (7–14). Simply stated, these provide (*a*) a separation technique based on mass that removes chemical contamination (e. g. coelutants in HPLC peaks or matrix ions desorbed by fast atom bombardment) and separates components of mixtures, (*b*) reproducible and extensive fragmentation, and (*c*) increased selectivity through selected reaction monitoring. The selectivity and reliability provided by these powerful hyphenated instruments are compellingly relevant to problems in pharmacology. Applications of MSMS were included in about 100 papers in pharmacological journals in 1990.

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Table 1 Typical performance characteristics of some commercially available types of tandem mass spectrometers

	QQQ[a]	BEQQ[b]	EBEB[c] BEEB[b]
Range M/Z	1800	2000	4000
Resolution			
MS-1	unit	1:1000	1:3000
MS-2	unit	unit	1:1000
Limit of analysis	100 pmol	15 pmol	10 pmol
Collision energy	10–200 eV	10–200 eV	10–10000 eV

[a] See Ref. 17
[b] See Ref. 18
[c] See Ref. 14

This review illustrates the capabilities of tandem mass spectrometry with examples from the recent pharmacological literature, and discusses the more important experimental parameters for applications of this kind. The review focuses mainly on the capabilities of instrumentation that is currently available commercially, and that can provide unit resolution in both stages of ion analysis. The literature on tandem mass spectrometry techniques is extensive, and only selected references are included here.

INSTRUMENTATION

Viewed broadly, a tandem mass spectrometry experiment provides separation or analysis of two sets of ions, precursor and product ions. These analyses are usually separated spatially (1–14), although in several instruments currently under development they may be separated in time (15, 16). Most pharmacological applications to date have been carried out with instruments comprising two or more spatially distinct analyzers. Although this review does not provide technical descriptions of the various instrumental combinations, some references and general performance evaluations for commercially available instruments with tandem capability are offered in Table 1. The mass ranges and resolutions are those typically reported for tandem experiments. Resolution of double focussing measurements in either the hybrid or the four-sector instrument can be much higher. The implications of good mass resolution for pharmacologic applications are illustrated below. Sensitivities for tandem experiments reflect the ion optics and engineering skills of the manufacturers to some extent, and continued improvement can be expected. The use of array detectors, for example, improves sensitivity on four-sector tandem instru-

ments. The term "limit of analysis" used in the Table indicates the amount of sample needed to obtain an interpretable product ion spectrum.

Many new tandem configurations are under development (e. g. 19–23). Those that incorporate one or more time-of-flight analyzers are of particular interest because of their potential for high mass, high duty cycle, simplicity, and low cost. Similarly, the potential of quadrupole-based ion storage devices for providing temporally separated analysis of several generations of ions in a single compact instrument is intriguing.

Tandem mass spectrometry was used initially with electron impact, field ionization, and chemical ionization and both positive and negative ions were analysed. Early in the development of analytical applications, it was pointed out (4) that the optimal strategy uses an ionization technique that produces intense molecular ion species and minimal fragmentation. Molecular species with strong ion currents are then selected for extensive and reproducible fragmentation by collisional activation. Ionization of polar metabolites and biopolymers is usually accomplished with one of the desorption techniques, thermospray, or electrospray. These do not usually produce extensive fragmentation and are thus ideal for MSMS analyses. Fast atom or fast ion bombardment (FAB) has been the method of choice for many polar metabolites, and has been used extensively in combination with tandem mass spectrometry (10, 24). Thermospray, continuous flow FAB, and electrospray are also important ionization techniques for MSMS, not only because they favor molecular ion production, but because they permit interfacing high pressure liquid chromatography and capillary zone electrophoresis. Either cation or anion analysis may be the method of choice, depending on the analyte structure and the contaminating matrix. Sulfate analysis, for example, is more susceptible to anion mass spectrometry, in which a single [M-H]- molecular ion species is produced, while cation spectra of sulfates can contain a variety of molecular ion species carrying sodium and potassium cations in place of exchangeable protons.

Interfaced Chromatography

Some early advocates suggested that MSMS would replace gas chromatography mass spectrometry (GCMS). However, the highly complementary capabilities of the two quickly led to the interfacing of GCMSMS and LCMSMS instruments. The ability of gas or liquid chromatography to separate isomers with the same molecular weight, and to separate the analyte from much of the contamination in mixtures such as blood or tissue extracts, complements the ability of the tandem mass spectrometer to deconvolute coeluting components on the basis of their molecular weight and to provide structure characterization by collisionally induced fragmentation. Several empirical comparisons have shown that GCMSMS provides greater specific-

ity and lower limits of detection than GCMS or MSMS carried out on the same or similar instruments with the same ionization technique and sample preparation (25, 26). Senn reported a tenfold enhancement in the limit of detection of derivatized deacetylmetipranolol, the active metabolite of the beta-blocking drug metipranolol, compared to either GCMS or MSMS (27). Yost et al (28) found limits of detection were improved fourfold and eighty-fold for two halogenated analytes analyzed by GCMSMS with negative ion chemical ionization. Voyksner and colleagues (29) reported that both GCMSMS and GC-high resolution MS provided accuracy superior to MSMS, based on removing chemical inteference. In the general case limits of detection, identification, and quantitation can be improved by MSMS despite the loss of total ion current in collisional activation, because background or contaminating ions are eliminated and the signal-to-noise ratio is improved. It has also been demonstrated that these fairly complex instruments can be run with high throughput. Covey et al reported (30) the use of short columns in an HPLC interfaced to an atmospheric pressure chemical ionization source to screen 60 equine extracts an hour for phenylbutazone in the nanogram range by LCMSMS.

Figure 1A (30) shows a chromatogram reconstructed from the total ion current detected in an LCMS analysis of 3-hydroxy promazine for a standard sample injected at 0 min, an extract of control urine injected at 1 min, and an extract injected at 2 min from urine from a racehorse that had received promazine. These chromatograms may be compared with those from an LCMSMS experiment presented in Figure 1B. Chromatograms in Figure 1B characterize the same samples as the summed ion currents of product ions from collisionally activated mass 301 ions (protonated 3-hydroxypromazine). The increased selectivity and specificity are apparent.

Most of the interfaces developed for HPLC have been utilized with MSMS, e. g. thermospray (31, 32), continuous flow FAB (33, 34), electrospray (35, 36), and nebulizers (30). Both continuous flow FAB (37) and electrospray (36) have been used to interface tandem mass spectrometry and capillary zone electrophoresis. Mosely et al (37) report sensitivities in the femptomole range, with chromatographic separation efficiencies above 100,000 theoretical plates. There appears to be a consensus among scientists applying tandem mass spectrometry to problems in pharmacology that chemical extraction of blood, bile, and urine samples is required prior to MSMS or LCMSMS to assure specificity, reproducibility, and sensitivity, and to minimize instrumental downtime (e. g. 27, 30). Often derivatization, removal of salt, and other purification also contribute to an optimal analysis.

Collisional Activation

In most tandem mass spectrometry experiments precursor ions are selected in the first mass spectrometer, activated to promote decomposition by collisions

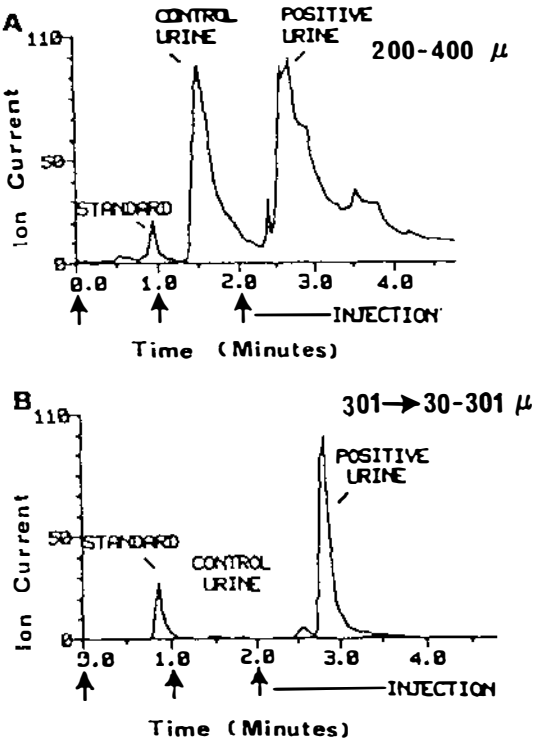

Figure 1 A: Chromatogram constructed by summing ions in the range 200–400 μ in an LCMS analysis of standard 3-hydroxy promazine, control urine, and urine from a horse administered promazine. B: Chromatogram in an LCMSMS analysis of the same samples constructed by summing intensities of product ions in the mass range 30 to 301 μ formed by collisional activation of mass 301 precursor ions. (Adapted with permission from Ref. 30.)

with atoms of an inert gas in a central collision chamber, and product ions are selected in the second mass spectrometer. Such activation greatly enhances fragmentation relative to regular unimolecular decomposition, and produces fragmentation less dependent on internal energy and consequently more reproducible. The nature of the fragmentation is altered somewhat, relative to unimolecular decomposition (e. g. 38, 39). For both scientific and commercial reasons, the effect of the energy of this collisional activation (determined experimentally by the kinetic energy of the projectile ions) has been thoroughly scrutinized. Fundamental considerations (7, 40–42) about energy deposition and fragmentation in impulsive collisions at various energies, and about the energy required for fragmentation, have been supplemented by experimental comparisons (18, 43–46) of the effectiveness of collisional activation at low energy (< 200 eV) and at high energy (> 2000 eV). There are clearly differences in the extent, nature, and reproducibility of fragmenta-

tion produced in these two energy regimes, as well as in overall transmission and sensitivity. In one direct comparison the production of sequence ions (fragment ions that provide information about amino acid sequences) from a tetradecapeptide by high energy collisions was found to require one sixth as much sample as production of sequence ions by low energy collisions (18). Such differences are widely acknowledged to become more pronounced as the molecular weight of the compound under analysis increases (18, 44–46). Activation by high energy collisions has been found to provide reproducible fragmentation patterns, from one laboratory to another and from one instrument to another, while spectra obtained using low energy collisional activation are reported to be highly sensitive to pressure and energy (e. g. 47, 48).

Strategies are under development to supplement impulsive or "billiard ball" collisions with collisions that result in ion molecule reactions (49–54). In another approach, endothermic ion molecule reactions are designed to harness chemical energy to activate ions for fragmentation (55, 56). The effect of coulombic repulsion on collisional activation of multiply charged ions is also under intensive study (57–60). Surface-induced dissociation (61) and photon-induced dissociation (62) are also under development.

Scanning Strategies

Three general strategies may be realized in MSMS experiments. In the most widely used, precursor ions are selected for activation and the resulting product ions are recorded. This approach has been very effective for analysis of components of mixtures. In a second type of experiment a product ion may be selected and all precursor ions determined. This approach allows the investigator to define homologous families or classes of compounds, e. g. terpenes in oil shale (63), biogenic amines in wine (64), and sulfated azo dyes in regulated products (65). In the third strategy, the two mass spectrometers are scanned in parallel to permit detection of all decompositions that proceed with elimination of neutral fragments of a predetermined mass. This also allows interrogation of related compounds or related fragmentation pathways. These three kinds of measurements are commonly known as product ion scans, precursor ion scans, and constant neutral loss scans, respectively. All three are automated on hybrid and tandem quadrupole instruments, although the latter two have not yet been realized on tandem mass spectrometers based on four-sectors. Recently scan modes have been discussed for instruments that provide three (MS3) or more separate ion analyses (66).

APPLICATIONS IN PHARMACOLOGY

Applications of tandem mass spectrometry in pharmacology and in pharmaceutical sciences may be divided into four categories, structure elucida-

tion, detection of targeted analytes, isotope analysis, and quantitation. Drug discovery sometimes involves structure elucidation of natural products with biological activity, one of the most challenging uses of tandem mass spectrometry. In the pharmaceutical industry the drug discovery process also includes recognition and characterization of metabolites from candidate compounds administered to various animals. Tandem mass spectrometry can provide valuable support for these metabolism-screening studies. Once a candidate drug is identified, the specificity of tandem mass spectrometry can be interfaced with chromatographic techniques for quantitation of metabolites. Tandem mass spectrometry can also be used to support process development and quality control by providing rapid, specific identification and quantitation of impurities. These same capabilities for trace analysis and fingerprinting make tandem mass spectrometry valuable in supporting patents (27). The capability for stable isotope analysis is important in many strategies for quantitation, and is used to locate metabolites. In basic pharmacological research, the use of stable isotopes as tracers for mechanistic studies is best supported by high-resolution tandem mass spectrometry.

The place of mass spectrometry among the physico-chemical techniques used in structure elucidation of natural products is well established. Studies that use tandem mass spectrometry with collisional activation have tended to rely heavily on the fragmentation provided by the technique, and have required highly sophisticated interpretation. Examples relevant to this review include compounds with calcium binding (67), antineoplastic (68), and neurotoxic (69) activities, all from the series of natural product structures to which Gross and coworkers contributed measurements on their three-sector tandem mass spectrometer. A review of applications of FAB MSMS through 1988 contains other examples (70)

Drug Metabolism

Identification of structures of drug metabolites has always been facilitated by recognition in the metabolites of substructures or modules from the parent compound (27, 71). Thus ions that are characteristic of the drug family or of a class of metabolites (e. g. glucuronides (72)) have been profitably interpreted for many years in conventional mass spectra and more recently in product ion scans in tandem experiments. This feature of drug metabolites is also the basis of a strategy proposed for tandem mass spectrometry by Yost and colleagues (12, 73), in which precursors that fragment to selected product ions or characteristic neutral losses are scanned to screen extracts for molecular ion species of probable metabolites. Subsequently these molecular ions can be selected for activation to provide product ion spectra to characterize the nature of the metabolic modifications and their sites. (As always, limits are recog-

nized on the ability to discern stereoisomers and positional isomers in aromatic or fused-ring systems.)

These strategies have also been applied by Straub (74, 75) and others (26, 77) to screen extracts for glucuronides, sulfates, and glutathiones by recording precursor ions that decompose to class characteristic fragment ions or eliminate characteristic neutral species. Glucuronides can be recognized, for example, by the elimination of dehydroglucuronic acid, 176 atomic mass units (μ), while sulfate anions fragment to form SO_3- ions, 80μ, or HSO_4- ions, 97μ , depending on their aromaticity. Gaskell and coworkers have advocated glutathione screening by scanning constant loss of 129μ, loss of the gamma-glutamyl moiety (78), while Baillie and colleagues have proposed the loss of glycine methyl ester, 89μ , from derivatized glutathione conjugates (79). Analogous strategies have been extended to cysteine and mercapturate conjugates as well (79–83).

These powerful screening techniques (precursor scans and constant neutral loss scans) have not yet seen widespread use in pharmacology. In most published applications of tandem mass spectrometry to identification of xenobiotic metabolites, screening is carried out by radioisotope labeling or other means, and samples are purified chromatographically and analyzed by product ion scans to provide highly specific comparisons between metabolites from physiologic fluids and synthetic reference compounds. Illustrative examples include the N-oxide metabolite of monocrotaline isolated from rat urine (84), the major metabolite of a new inhibitor of angiotensin-converting enzyme fingerprinted with chemical ionization (85), and a phase I metabolite of the dihydrofolate reductase inhibitor trimetrexate analyzed by LCMSMS with thermospray ionization (86). Thermospray LCMSMS also provided differentiation between five isomeric metabolites of Temelastine and confirmation with synthetic standards (87).

Similarly, analysis of phase II metabolites with tandem mass spectrometry usually exploits the increased sensitivity and high specificity of product ion scans for comparison with synthetic reference compounds. Recent examples include the characterization of sulfate and glucuronide metabolites of fluphenazine by collisional activation on a hybrid mass spectrometer (88), urinary and biliary cysteine-containing metabolites of trichloropropane using a four-sector tandem instrument (89), stereo and regioisomeric mercapturate and cysteine conjugates formed when isomeric glutathione conjugates of napthalene oxide were administered intravenously to mice (81), the human urinary cysteine conjugate leukotriene E4 using continuous flow FAB on a triple quadrupole instrument (83), rat biliary glutathione conjugates of N-(l-methyl-3, 3-diphenylpropyl)formamide (90) and methyl isocyanate (91). The major metabolite of zidovudine (AZT, Retrovir) has been characterized from human urine by chromatography, NMR, and tandem mass spectrometry (92).

Glucuronic acid is conjugated to the 5'-hydroxyl group of the nucleoside analog. Collisional activation promoted the class characteristic loss of 176μ and also fragmentation characteristic of nucleosides. The site of conjugation was identified by examining fragment ions.

When reference compounds are not available, class-characteristic ions can usually be recognized directly in product ion spectra, revealing drug-relatedness or metabolite class. One example where this was a viable approach was the characterization of sulfinic and sulfonic acids as oxygenated metabolites of spironolactone (93). These structure assignments were initiated by the appearance of HSO_3- ions in the anion spectrum produced by collisionally activated dissociation (CAD) of the former and the loss of neutral SO_2 in the CAD anion spectrum of the latter.

Characterization (94) of the glucuronide conjugate that is the major human metabolite of etintidine was carried out without a reference compound, based in part on interpretation of its CAD spectrum. The spectrum was interpreted to reveal fragmentation characteristic of the parent drug, as well as the glucuronide, and to localize the point of conjugation outside the imidazole ring. In the absence of a reference glucuronide, NMR and enzymic hydrolysis were employed to confirm the assignment. A quaternary-linked glucuronide of lamotrigine has also been characterized by tandem mass spectrometry, NMR, and enzymic hydrolysis (95). Most interesting in this case, electrospray ionization was used for the MSMS experiment. Spectra from this study are reproduced in Figure 2. The conventional spectrum (Figure 2A) contains ions corresponding to molecular ion species formed by addition of either a proton or a sodium cation, and also comprising the three isotope peaks associated with two chloride atoms in the molecule. The product ion scan (Figure 2B) was recorded with collisional activation of monoisotopic cations of mass 432, and illustrates the significant improvement in signal-to-noise ratio that MSMS usually affords. Note also the class-characteristic loss of dehydroglucuronic acid, 176μ . The mass spectrometer used was a triple quadrupole.

Examples of glutathione conjugates characterized by tandem mass spectrometry, spectroscopy, chromatography, and NMR (without a reference compound) are the Gunn rat biliary metabolites of dimethylbilirubin (96). Isomeric metabolites were recognized as glutathione conjugates based on the characteristic elimination of 307μ. Collisionally induced cleavages characteristic of the tetrapyrrole allowed metabolic modifications to be regionally localized.

The high sensitivity of mass spectrometry often permits partial characterization of minor conjugated metabolites, including those that show up as contaminants of abundant conjugates. Selection of the molecular ion and characterization by collisional activation has provided a minor glucuronide derived from dihydroxylated fluphenazine without reference material

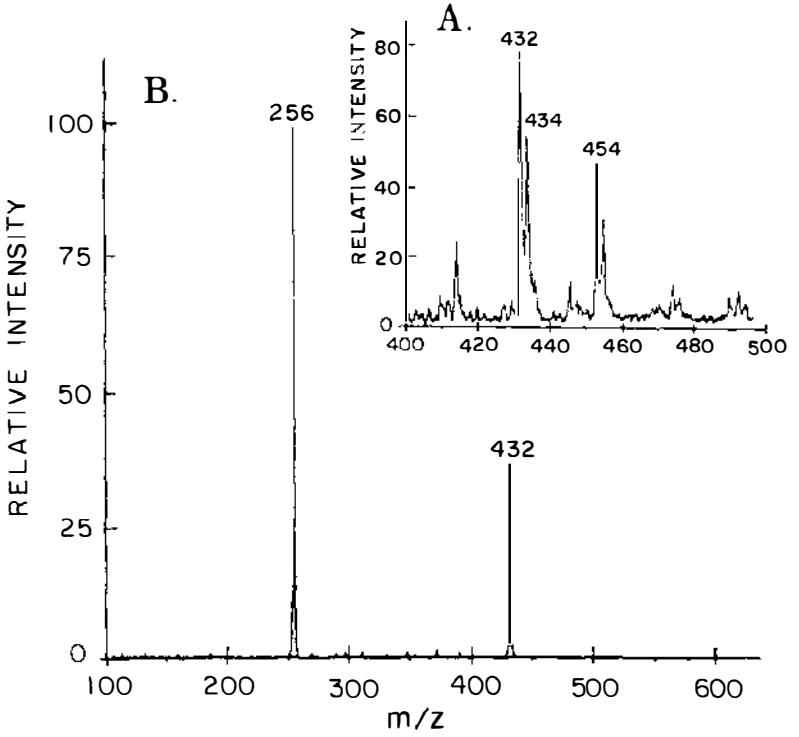

Figure 2 A: Partial mass spectrum obtained using electrospray for analysis of a quaternary ammonium-linked glucuronide of lamotrigine isolated from human urine. B: Product ion spectrum formed by collisional activation of the mass 432 ions in Figure 2A. (Adapted with permission from Ref. 95.)

(88), a minor dioxygenated metabolite of dimethylbilirubin glutathione (97), and a novel thiopyruvic acid adduct derived from the glutathione conjugate of naphthalene oxide (81).

Detection of Targeted Analytes

Two characteristics required for trace analysis and screening are sensitivity and specificity. Tandem mass spectrometry coupled with gas or liquid chromatography provides a very powerful combination of these two. It is widely viewed as the most definitive technique for regulatory issues, and in validating simpler but less specific screening methods.

For trace analysis tandem mass spectrometry is usually set up to transmit a precursor ion and a product ion of specified masses. Specificity is enhanced in this technique, often called selected reaction monitoring (SRM) by analogy to selected ion monitoring (71), when ions with heavier masses are monitored,

and sensitivity is optimized if ions of high intensity are selected (98). Various workers have pointed out that use of selective ionization techniques can further contribute to sensitivity, since the sensitivity of the instrument is not the limiting factor, but rather the signal-to-noise ratio.

For example, an MSMS procedure published (99) for confirmation of the antiparasitic drug ivermectin in animal tissue involves multistep extraction and cleanup, followed by selected reaction monitoring on a tandem quadrupole mass spectrometer, using chemical ionization with ammonia as reagent gas and collisional activation. Resolution in MS-I was lowered to transmit up to 4μ in order to increase sensitivity. A bovine liver sample spiked with ivermectin at 38 ppb and carried through the entire procedure produced a signal with 4520 ion counts, compared to a control liver's signal of 128 ion counts. The decomposition of the [M+NH4]+ molecular ion adduct to a relatively heavy fragment ion of mass 567 was determined to provide the least interference from other components of the biological matrix.

Chemical derivatization was employed to provide a rapid GCMSMS screen for metabolites of the anabolic steroid trenbolone acetate in human urine (100). Chemical ionization, collisional activation, and selected reaction monitoring were used, with detection limits at about 0.6 ng injected onto the column. Limits of identification were about tenfold higher. Interestingly, de Boer et al report that CAD provided only limited fragmentation, and thus did not increase the limit of detection relative to GCMS measurements. Nonetheless, the MSMS procedure did provide improved selectivity and improved limits of identification, which permitted characterization of trenbolone (hydrolyzed from its glucuronide conjugate) in large scale screening of the urines of athletes.

Further illustration of the flexibility of tandem mass spectrometry as a chromatographic detector is provided by Beattie & Blake (101), who program their triple quadrupole to monitor a series of precursor-product ion transformations that change as the chromatographic separation proceeds. They also concatenate several simultaneous SRM traces to produce chromatograms that reveal suites of isomeric metabolites or metabolite candidates in urine and plasma. This latter approach is reported to provide better selectivity/sensitivity (20 ng of standard injected on-column) than ion chromatographs reconstructed from product ion scans.

Quantitation

All the capabilities of tandem mass spectrometry should serve to improve pharmacokinetic assays: reduction of background interference, separation of components of mixtures with different molecular weights, controlled and reproducible fragmentation, and the ability to select both precursor and product ions in selected reaction monitoring. Selected reaction monitoring

strategies have been demonstrated, for example, for quantitation of leucine enkephalin (102), acylcarnitines (103), platelet activating factor (104), aldosterone (105), and several steroidal sulfates (106, 107). These assays are usually done with the addition of an internal standard, ideally a stable isotope-labeled analog (27, 71, 106, 108). However, structural homologs often prove satisfactory as well (97, 109).

Sensitivities are reported to be comparable to those of radioimmune assays (102, 105, 110), and the MSMS technique is recognized to provide much better specificity. Specificity is superior to high pressure liquid chromatography and to many bioassays.

As an example from pharmacology, an assay developed for low plasma levels of bromocriptine used in the treatment of Parkinson's disease incorporated a selective chemical ionization method as well as selected reaction monitoring (97). In the latter technique, MS-1 alternately passed precursor ions of a single mass from the analyte and precursor ions from the internal standard (ergotamine in this case) for collisional activation, whereas MS-2 alternately transmitted the corresponding product ions. (Selection of ions of higher mass and higher abundance provides greater selectivity and sensitivity to assays, as well as to screening discussed above.) The limit of detection for bromocriptine is reported to be about 1 pg/ml plasma, and calibration curves were found to be linear between 10 and 200 pg/ml. Precision was found to be ± 4% at the top of that range, and ± 13 % at the low end (n = 12). The assay was applied to follow the elimination phase in 30 human subjects, and the assay was reported to be selective, sensitive, accurate, rapid, and requiring relatively simple sample preparation.

Another published assay with well-validated accuracy and precision is the report by Straub & Lavandoski on the quantitation of the thermally labile N-oxide metabolite formed from a substituted benzazepine (111). Fast atom bombardment was used to provide precursor ions for selected reaction monitoring. In this case a pentadeuterio-analog of the analyte was used as the internal standard for assays in dog urine and plasma. Precision varied from 0.6 to 9.1% in urine spiked with 0.5 to 100 ug/ml of analyte. The authors reported that the lower limit was set by endogenous contributions to precursor ions of mass 212, and recommended the use of higher resolution in MS-1.

A third instructive example comes from Nelson & Foltz (110), who have developed a GCMSMS assay for lysergic acid (LSD) in blood and urine. The specificity of this assay is important in view of the false positive determinations produced by widely used radioimmune assays. Comparison of the GCMSMS technique with GCMS and direct probe MSMS procedures indicated that the more complex approach markedly reduces interference, e. g. from the isobaric diastereomer that is often a contaminant of LSD. A related compound was used as the internal standard, and multiple reaction

monitoring provided quantitation down to 50 pg/ml. The limit of detection was 20 pg/ml.

Polar conjugates have also been quantified by tandem mass spectrometry. LCMSMS assays for glucuronides were reported in 1982 (109) using selected reaction monitoring with paranitrophenol glucuronide as an internal standard. Scans for parents of HSO_3- ions have been demonstrated for quantitation of a steroid sulfate, using a dideutero-analog as internal standard (106).

Stable Isotope Analysis

The classical application of mass spectrometry for analysis of isotope abundances and positions is further enhanced by the use of tandem techniques (112, 113). One major use of stable isotopes has been discussed above, in isotope-labeled analogs used as internal standards for quantitation in pharmacokinetic studies. The introduction of two to five stable isotopes in non-exchangeable positions in the analyte of interest can provide an internal standard with nearly identical chemical and solubility properties, similar chromatographic retention, but molecular and (some) fragment ions clearly distinguishable by their mass. The ability of mass spectrometry to distinguish isotope-labeled drugs and metabolites has also been exploited historically to recognize metabolites in extracts (71, 114, 115). Isotope clusters can usually be recognized in a conventional mass spectrum, as for example, in an exemplary study by Wong and colleagues (86), who used LCMS to characterize metabolites formed by dogs that had received a 1:1 mixture of the dihydrofolate reductase inhibitor trimetrexate and a $^{13}C_2$, ^{15}N-labeled analog. Blake & Beattie (116) administered a 1:1 mixture of a ^{12}C- and ^{14}C-labeled experimental drug to rats and analyzed that isotope cluster by LCMS. The identification of isotope clusters by constant neutral loss in tandem experiments can provide heightened specificity and improved signal-to-noise ratios (78, 79).

Mechanistic studies is a third area in which stable isotope labels have traditionally been used in pharmacology. The isotope signatures of chloride, bromide, iron, etc, and distributions of labels in molecular ions can be assessed by conventional mass spectra, or by constant neutral loss or precursor ion scans. However, the ability of a high performance tandem mass spectrometer (97) to select labeled precursor ions with a monoisotopic composition can significantly enrich the isotope composition of the ions under study and clarify calculations of the distributions of labels in fragment ions. Of course, unit resolution of the product ions is also necessary for quantitative analysis of isotopes in fragment ions.

One example of the power of four-sector tandem mass spectrometers comes from a study of the mechanisms of both transferase-mediated and base-catalyzed conjugations between glutathione and phosphoramide mustard, the active metabolite of cyclophosphamide (117). Deuterium labels were in-

troduced at a position such that the intervention of a symmetrical cyclic aziridinium ion leads to the distribution of deuterium labels between two positions in the conjugated product. Direct displacement of chloride by glutathione would produce a product in which labels remain in the single original position. Although products of either reaction would have the same molecular weight, the isotope distribution could be determined from fragment ions in the product ion spectra following collisional activation. Adequate resolution and transmission in the tandem mass spectrometer permitted secondary isotope effects to be calculated for opening the aziridinium ring enzymatically or chemically. By contrast, an analogous labeling experiment demonstrated direct displacement in glutathione conjugation of cyclophosphamide.

Four-sector tandem mass spectrometry has also been used in another series of papers (96, 97, 118) to characterize metabolites of dimethylbilirubin excreted in Gunn rat bile. Conjugation with glutathione was postulated to proceed via an epoxide, and evidence for the involvement of P–450 monooxygenases was sought by incubation of microsomal preparations in an $^{18}O_2$ atmosphere. Partial spectra obtained by fast atom bombardment are shown in Figure 3 of (*a*) the metabolite formed in an $^{18}O_2$ atmosphere, and (*b*) the metabolite formed in an $^{16}O_2$ atmosphere. In the former case the protonated molecular ion selected for activation and analysis contains one atom of ^{18}O. Species even one mass unit lighter (e. g. all ^{16}O and one ^{13}C) or heavier are excluded with both MS-I and MS-2 run at 1000 resolution (10% valley definition). Some interpretation of the fragmentation is indicated in Figure 3. These two spectra illustrate the reproducibility of high energy collisional activation, and support the hypothesis that the labeled oxygen atom has been introduced into the vinyl side chain to which glutathione is also conjugated. This result is consistent with epoxidation as a Phase I transformation catalyzed by a P-450 monooxygenase.

Biopolymers

Sequencing of peptides, carbohydrates, and nucleotides by tandem mass spectrometry has been extensively reviewed in recent years (119–121) and was addressed in at least five books that appeared in 1990 (122–126). Two classes of biopolymers may be of special interest to pharmacologists and toxicologists: biopolymers covalently altered by drugs, and biopolymers that are themselves therapeutically useful (127, 128). Although strategies for analysis of such macromolecules include mass spectrometry techniques other than MSMS, e. g. electrospray and laser desorption for molecular weight determinations, tandem mass spectrometry is the technique of choice for sequencing peptides, carbohydrates, or nucleotides covalently altered by drugs (e. g. 129–131).

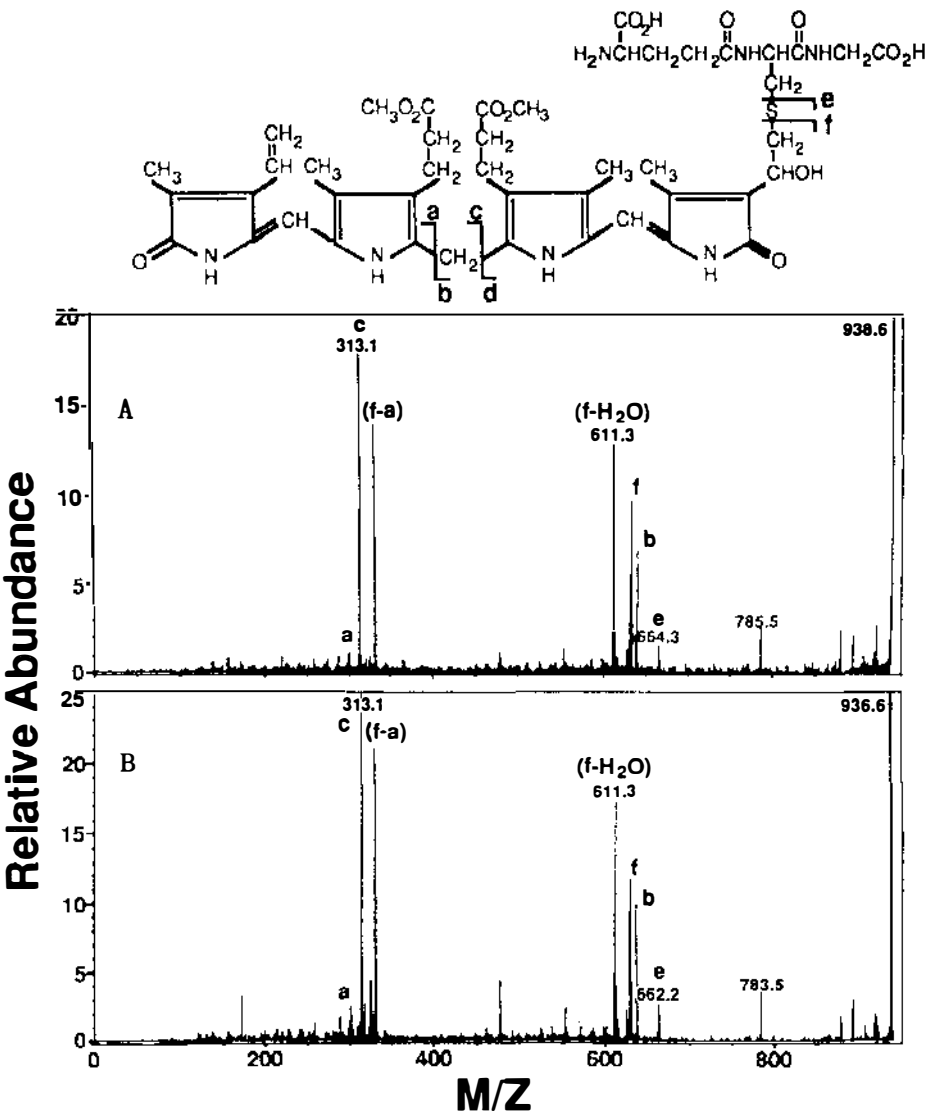

Figure 3 A: Spectrum obtained by collisional activation on a four-sector tandem mass spectrometer of a mono-oxygenated glutathione conjugate of dimethylbilirubin produced by incubation with Gunn rat microsomes in an $^{18}O_2$ atmosphere. B: Spectrum obtained by collisional activation on a four-sector tandem mass spectrometer of a mono-oxygenated glutathione conjugate of dimethylbilirubin produced by incubation with Gunn rat microsomes in an $^{16}O_2$ atmosphere. (Adapted with permission from Ref. 118.)

Enzyme activities have been found to be retained and half-lives significantly lengthened when proteins modified by attachment of polyethylene glycol (PEGation) are administered to animals or humans. Tandem mass spectrometry has been used along with other techniques to define the extent and sites of polyethylene glycol modification of superoxide dismutase (128). Figure 4 shows a product ion spectrum produced by high energy collisional activation of the tryptic peptide [114–126] derived from PEGated superoxide dismutase. The modification on lysine 120 is the succinimide linker left when polyethylene glycol is released by treatment with base (128). Sequence ions in the spectrum are designated following the system of Biemann (119).

FUTURE DIRECTIONS

Instrumentation for mass spectrometry is evolving at a rapid pace, currently emphasizing analysis of larger molecules. Simpler and cheaper analyzers are under development to provide MSMS experiments, including time-of-flight and quadrupole ion traps. Tandem mass spectrometers based on time-on-flight instruments will readily incorporate ionization by laser desorption. Quadrupole based ion storage devices, and Fourier transform ion cyclotron resonance mass spectrometers, which provide product ion spectra from precursor ions selectively retained in storage cells, will offer possibilities to examine older, cooler precursor ions and to incorporate kinetic considerations into analytical strategies. It is clear that tandem mass spectrometers will continue to become simpler to operate, under computer control.

The feasibility of MSMSMS, MS^4 and MS^5 have been demonstrated with hybrid (132), quadrupole (133), four-sector (134, 135), and ion trap (22) instruments. Strategies incorporating these additional structural filters are under development for analytical applications.

Ion activation is also an area receiving attention, with efforts underway to develop more efficient fragmentation, some control of the type of fragmentation that takes place, and more reproducible low energy activation. Collisional activation of metal ion adducts, for example, can provide very different fragmentation than protonated molecules. Collisions leading to ion molecule reactions have been suggested, as well as thermodynamically tailored collisions leading to reactive complexes.

The possibility of directing ion molecule interactions in a tandem mass spectrometer has led to exploration in several laboratories of methods to distinguish diastereoisomers (136–140). Differences are reported in product ion spectra of fused ring lactams and steroids (137), diastereomeric hexoses adducted with sodium cations (136), and glycosidic components in glyco conjugates (27, 136, 138, 139)). With high energy collisions, reproducibility is reported to be ± 5% (137), although reference compounds are required to

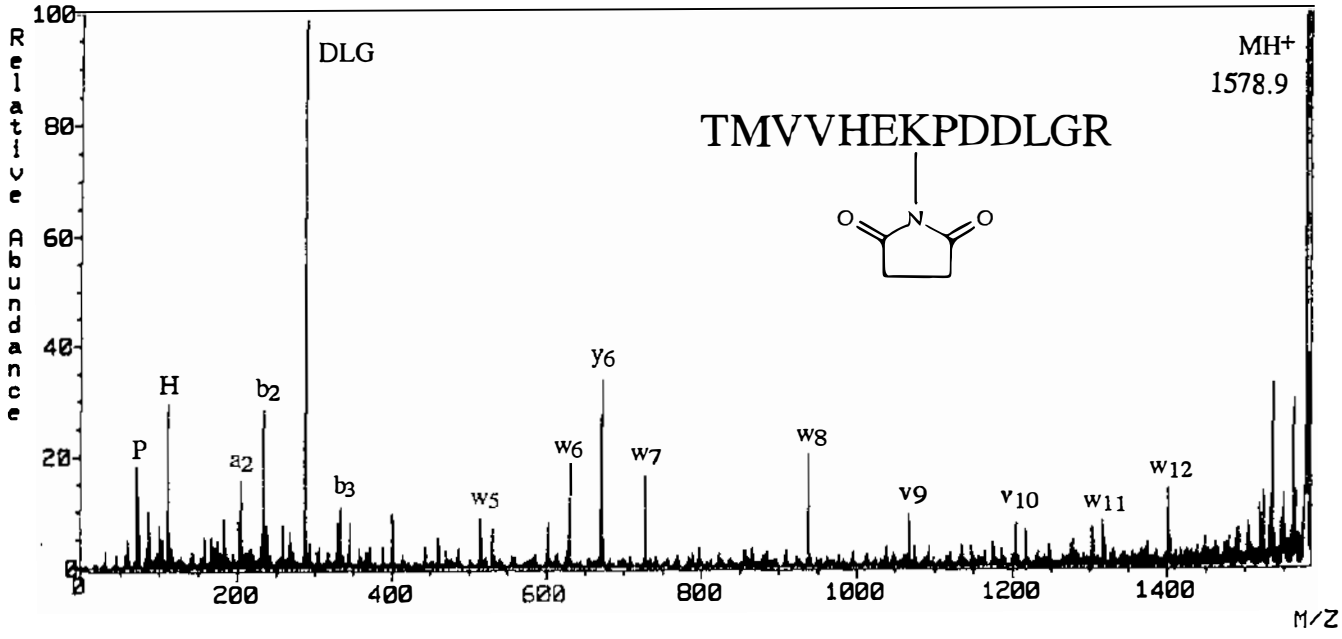

Figure 4 Spectrum of product ions formed by high energy collisional activation of tryptic peptide [114–126] from PEGated superoxide dismutase after treatment with base to release polyethylene glycol. (Adapted with permission from Ref. 128.)

assign the isomers. Chiral target gases and chiral adducting agents offer possibilities as well.

Lastly, the rapid pace of development of tandem mass spectrometry for analysis of biopolymers suggests that it will provide much more support in the future for the development of enzymes, receptors, and growth factors as therapeutic agents, as well as precise studies of the interactions of biopolymers with drugs and other xenobiotic substances.

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