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An Evaluation of Tandem Mass Spectrometry in Drug Metabolism Studies†

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The use of precursor ion and constant neutral loss scanning as a means of rapidly detecting drug metabolites is evaluated. Four clinically useful drugs, namely (i) cyclophosphamide, (ii) mifentidine, (iii) cimetropium bromide and (iv) haloperidol, were subjected to microsomal incubations to afford phase I metabolites. Aside from a minor clean-up procedure involving zinc sulfate precipitation of microsomal proteins and solid-phase extraction of metabolites using a Sep-pak C-18 cartridge, the mixtures were analysed directly by fast atom bombardment tandem mass spectrometry. It is demonstrated that such screening strategies are important in detecting novel metabolites. However, there are some problems associated with only using such methods, including (i) the possibility of not detecting metabolites that undergo unusual collision-induced dissociation fragmentation pathways, (ii) the non-detection of metabolites that have undergone metabolic change at unusual sites of reactivity, and (iii) production of artifacts derived from the parent drug by the primary ionization process. Examples are discussed that highlight both the strengths and weaknesses of such an approach.

INTRODUCTION

Contemporary methods for drug metabolite identification are usually based on the comparison of ultraviolet (UV) spectral data and high-performance liquid chromatography (HPLC) retention times of isolated 'unknown' metabolites with synthetic standards. Such methods of detecting drug metabolites and subsequently characterizing them can be a time-consuming process, as well as only affording very limited structural information.[1] Furthermore, phase I metabolism of a drug often results in only minor structural modification of the parent compound, e.g. oxidation, reduction, dehydration or hydrolysis.[2] These minor structural changes make it particularly difficult to determine suitable chromatographic conditions to effect HPLC separation of metabolites.

Mass spectrometry has contributed significantly to the structural determination and quantitation of drug metabolites.[3–6] More recently, tandem mass spectrometry (MS/MS)[7] has been increasingly used in both the characterization and quantitation of metabolites[8] found in complex biological matrices derived from both *in vitro* sources, e.g. microsomal incubates,[9,10] and *in vivo* sources, such as urine, blood, bile and feces.[11–15] In effect, MS/MS has been used to replace time-consuming chromatographic separation steps.

The use of MS/MS in the structural identification of drug metabolites was pioneered by Yost and coworkers,[16–18] and Straub and colleagues.[19–22] Perchalski *et al.*[16] first demonstrated the utility of MS/MS, using a triple-quadrupole instrument to rapidly identify a series of anti-epileptic drug metabolites found in urine. They also outline a protocol for the characterization of phase I metabolites. Subsequently, Straub went on to develop methods for the identification of phase II metabolites.[20,22] We and others have subsequently utilized precursor ion scanning[9,23,24] or constant neutral loss (CNL) scanning[10,25,26] in conjunction with product ion spectral acquisition to identify a wide range of metabolites.

In the present work, we describe some of the advantages and disadvantages associated with using the screening strategies of CNL and precursor ion scanning to detect the presence of drug metabolites in complex mixtures. In particular, we have investigated the *in vitro* metabolism of (i) the anti-cancer drug cyclophosphamide (1) and its interaction with DNA, (ii) the H_2-antagonist, mifentidine (2), (iii) the anti-muscarinic agent cimetropium bromide (3), and (iv) the neuroleptic compound haloperidol (4). The structures of the parent drugs are shown in Fig. 1.

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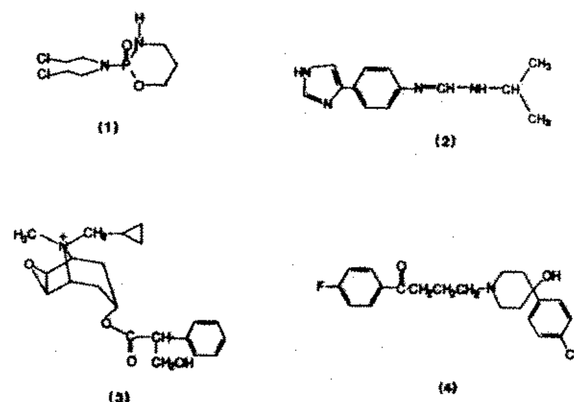


Figure 1. Structure of the parent drugs: cyclophosphamide (1); mifentidine (2); cimetropium bromide (3) (the anion Br⁻ is not shown); and haloperidol (4).

EXPERIMENTAL

Materials

Cyclophosphamide (1) was a gift from P. E. Mirkes, University of Washington, USA. Mifentidine (2) and the formamide (10) and *N*-hydroxylamine (13) products were synthesized and supplied by the Instituto De Angeli (Milan), Italy, as was the parent drug, cimetropium bromide (3). Haloperidol (4) was obtained from Sigma Chemical Co. (St. Louis, USA).

Mass spectrometry

All mass spectra were obtained on a VG 70-SEQ instrument of EBQ_1Q_2 configuration, where E is an electrostatic analyser, B is the magnet, Q_1 is an r.f.-only quadrupole collision cell and Q_2 is a mass filter quadrupole. EB and Q_2 correspond to mass spectrometer one (MS_1) and two (MS_2), respectively. All synthetic standards and microsomal incubation extracts were ionized by positive ion fast atom bombardment (FAB) mass spectrometry. Xenon atoms from a model B11N saddle-field fast-atom gun (Ion Tech, Teddington, UK) were used at 8.5 keV as the primary ion beam. The secondary ions produced by the fast xenon atoms are accelerated out of the source region to an energy of 8 keV.

Product ion spectra. Molecular ions (either protonated MH^+ or M^+) were selected with a resolution of ~1000 using MS_1 and subjected to collision-associated dissociation (CAD) using either argon or air as the collision gas. Collision cell conditions were varied between 20 and 220 eV collision energies and 10^{-5} to 10^{-8} mbar of pressure. The resulting product ions were mass analyzed in Q_2 and a product ion spectrum was acquired by scanning Q_2 over the appropriate mass range, with 10–15 scans being obtained in the multi-channel analysis (MCA) mode.

Precursor ion spectra. Q_2 was set to transmit the product ion(s) of interest and MS_1 was scanned at 5–30 s per decade. Collision cell conditions were as described above, and spectral acquisition was via the MCA mode.

Constant neutral loss spectra. MS_1 and MS_2 were scanned simultaneously with a mass difference of 54 Da, as described previously.[10]

***In vitro* incubation procedure.** Various animal hepatic microsomal incubations with parent drugs as well as control incubations, in which either substrate or cofactors were omitted or heat-inactivated microsomes were used, were carried out as described previously.[10]

RESULTS AND DISCUSSION

Yost and coworkers[16,18] suggested, in their original work, a strategy for screening mixtures of drug metabolites without having to resort to complex clean-up and purification procedures. They suggested that a product ion spectrum of the parent drug should be acquired, and any major neutral losses or abundant fragment ions noted. Subsequently, both CNL and precursor ion spectra should be obtained on the drug metabolite mixture. Finally, product ion spectra on those ions detected in the screening scans (CNL or precursor ion) should be acquired and compared with spectral data obtained on available pure synthetic standards.

In general, we have adopted a similar approach in our investigation of the *in vitro* microsomal metabolism of a number of clinically interesting drugs. However, we have recently demonstrated that a simple clean-up procedure involving zinc sulfate precipitation of microsomal proteins followed by solid-phase extraction of drug metabolites using a Sep-pak C-18 cartridge dramatically improves the signal-to-noise ratio to both FAB mass spectra and also CNL spectra of drug metabolite mixtures.[27] Therefore, we have routinely used this preliminary clean-up step prior to mass spectral analyses.

It is apparent that such methods are extremely useful in the detection of both expected and novel metabolites. However, the routine use of this strategy to detect the presence of metabolites in complex biological mixtures has highlighted the limitations of using only this approach.[28] We discuss, in the examples presented below, the advantages and disadvantages of using the screening techniques of precursor ion and CNL scanning.

Detection of novel compounds

Cyclophosphamide (1) is a widely used bifunctional anti-cancer alkylating agent[29] forming both mono-DNA adducts, as well as inter- or intra-strand cross-links with DNA.[30] The resulting cross-linked adduct prevents the strand separation of DNA, leading ultimately to cessation of cell division. However, the cross-linked adduct is chemically unstable due to the formation of the diiminium dication formed between 1 and the N-7 position of guanines present in double-stranded DNA.[31] The cross-linked adduct is excised

and ultimately excreted in urine. We were interested in investigating the mechanism of interaction of 1 with DNA, as well as developing methods to quantitate DNA–cyclophosphamide adducts found in urine.

As described previously,[32] we synthesized three putative guanine–cyclophosphamide adducts, namely N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine (NOR-G) (5), N-(2-hydroxyethyl)-N-[2-(7-guaninyl)ethyl] amine (NOR-G-OH) (6) and N,N-bis[2-(7-guaninyl) ethyl]amine (G-NOR-G) (7), and obtained product ion spectra of these synthetic standards as shown in Fig. 2. The product ion spectra of all three standards were simple, with dominant ions at m/z 152 for 5 and 6 (corresponding to loss of the alkyl side chain and concomitant gain of H by guanine to afford $[\text{guanine} + H]^+$) and at m/z 221 for 7 (corresponding to loss of guanine). A double precursor ion scan (m/z 152 and 221) on a mixture derived from the incubation of calf thymus double-stranded DNA with 1 revealed ions at m/z 239, 257 (259, chlorine isotope contribution) and 372 (shown in Fig. 3), confirming the formation and presence of 6, 5 and 7, respectively.[32] We have subsequently utilized this screening method to detect 5 and 7 formed with DNA in rat embryos treated with 1.[33]

Closer inspection of the precursor ion scan shown in Fig. 3 reveals ions at m/z 326 (chlorine isotope ion at 328) and 441. These ions cannot be readily explained from a simple reaction of cyclophosphamide with guanine of DNA. However, product ion data and subsequent synthesis of standards revealed these ions to be molecular ions of guanine–piperazine derivatives, namely G-Pip (8) and G-Pip-G (9), also shown in Fig.

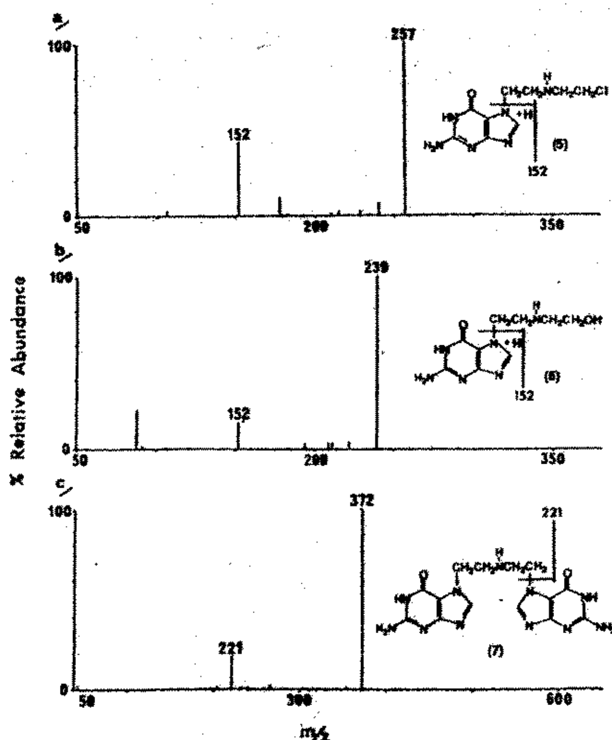


Figure 2. Product ion spectra of the synthetic standards:- (a) NOR-G (5), (b) NOR-G-OH (6) and (c) G-NOR-G (7) obtained at a collision energy of 20 eV at 5×10^{-6} mbar collison gas (air) pressure.

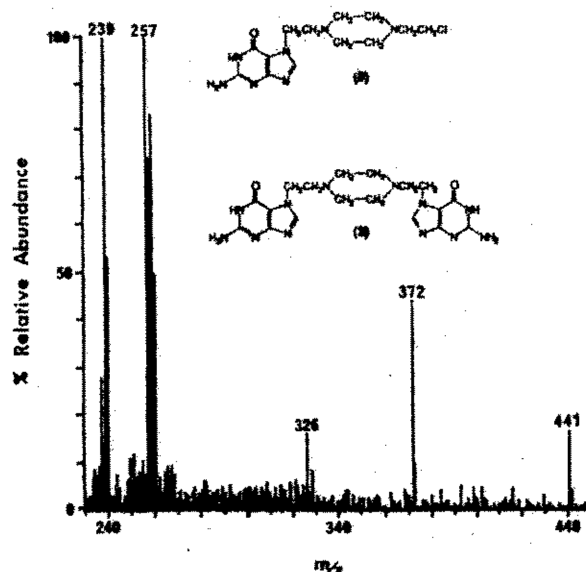


Figure 3. A double precursor ion spectrum of m/z 152 and 221 on a mixture derived from an incubation of cyclophosphamide (1) with calf thymus DNA. Collision cell conditions as for Fig. 2. The ions at m/z 326 (328) and 441 correspond to MH^+ of G-Pip (8) and G-Pip-G (9), respectively. The ions at m/z 239, 257 (259) and 372 are the MH^+ species of NOR-G-OH (6), NOR-G (5) and G-NOR-G (7), respectively.[31]

3.[34] The piperazine ring system has been formed by dimerization of cyclophosphamide or, more likely, by its breakdown product, the nitrogen mustard $(Cl{-}CH_2{-}CH_2)_2{-}NH$). At present, we are investigating the kinetics of formation of the piperazine compound, since detection of the adduct (9) has implications for the mechanism of action of 1. However, in the present context, this example serves to illustrate the utility of precursor ion scanning to detect new adducts/metabolites which were totally unexpected.

Limitations: 'missing' metabolites

Mifentidine (2) is a new H_2-antagonist[35] that represents the prototype of the second generation of histamine antagonists. We have recently demonstrated that *in vitro* guinea pig microsomal incubation of 2 produces at least three metabolites.[9] Our initial strategy was to obtain product ion spectra on the parent drug (2) and a synthetic standard, the formamide cleavage product (10) of 2. In both cases, a prominent fragment ion at m/z 160 was observed, as shown in Fig. 4. A precursor ion scan of m/z 160 on the microsomal incubation mixture revealed ions at m/z 188, 245 and 229 (Fig. 5) corresponding to the formamide (10) and an unusual urea (11) metabolites, plus unreacted parent drug (2), respectively.

Previous work,[36] based on UV spectroscopy and comparison of HPLC retention times, had tentatively concluded that both the amine (12) and the N-hydroxylamine (13) metabolites (shown below) were produced after a microsomal incubation of 2. Clearly, the amine (12) would not be detected in the precursor

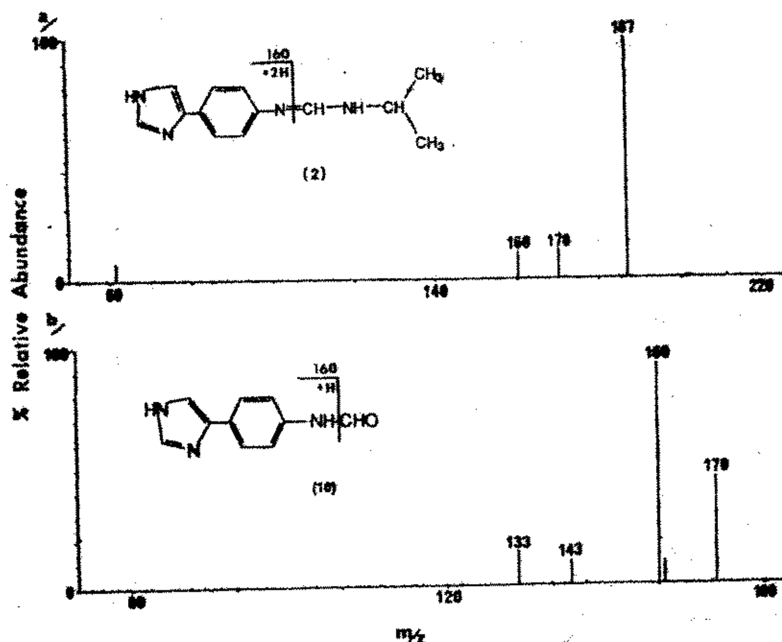


Figure 4. Product ion spectra of (a) the parent drug mifentidine (2), and (b) the synthetic formamide derivative (10), obtained at a collision energy of ~30 eV at 5×10^{-4} mbar collision gas (argon) pressure. For clarity, the protonated molecular ions at *m/z* 229 (for (2)) and 188 (for (10)) are not shown, and spectra are normalized to the most abundant product ion. It should be noted that the structures of the product ions at *m/z* 160 in both spectra (a) and (b) do not show the site of protonation to afford the MH^+.

ion scan of *m/z* 160 since this corresponds to its protonated molecular ion (MH^+). However, it appeared unusual that we were not detecting a protonated molecular ion for the *N*-hydroxylamine compound (13) using the precursor ion scanning method.

We obtained a product ion spectrum of the protonated molecular ion (MH^+) from a synthetic standard of 13, and this is shown in Fig. 6. A fragment ion at *m/z*

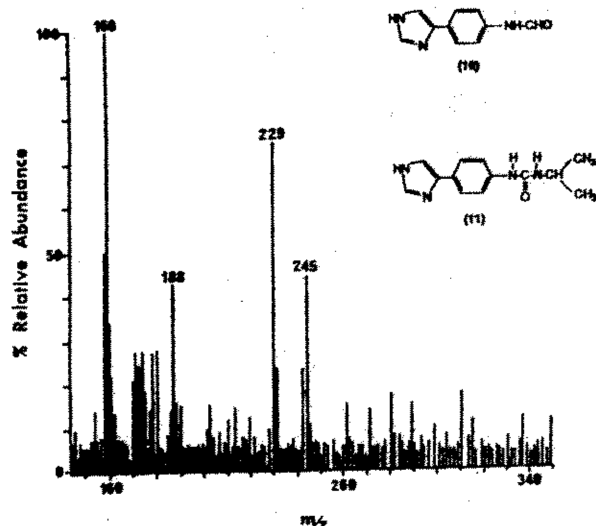


Figure 5. Precursor ion scan of *m/z* 160 on a microsomal incubation mixture derived from mifentidine (2). Collision cell conditions as for Fig. 4. The ion at *m/z* 229 is unreacted 2, whereas ions at *m/z* 188 and 245 correspond to the formamide (10) and urea (11) metabolites as shown.

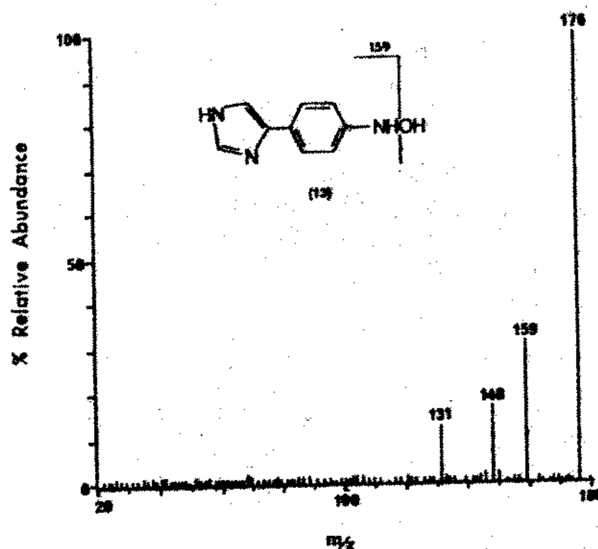


Figure 6. Product ion spectrum of synthetic *N*-hydroxylamine standard (13) of 2 on *m/z* 176. Collision energy was ~220 eV at $\sim 10^{-5}$ mbar of collision gas (argon) pressure. The site of protonation to afford MH^+ at *m/z* 176 is not shown.

159 is observed corresponding to an unusual loss of hydroxy radical (·OH) from MH^+ in a collision-induced dissociation spectrum. This same loss is observed in the FAB mass spectrum of a wide variety of *N*-hydroxyarylamines.[37]

In this instance, using the method of precursor ion scanning, two major metabolites produced in the microsomal incubation of mifentidine were missed. In particular, the *N*-hydroxyarylamine (13) was not detected using this methodology (we have subsequently demonstrated

the presence of this metabolite[38]). This is particularly important, since this class of metabolites is commonly recognized as being responsible for the toxic effects of aromatic amines, causing mutagenic and carcinogenic activity, as well as general cell damage.[39]

Artifacts

Cimetropium bromide (3) is a quaternary ammonium salt that possesses anti-spasmodic and anti-muscarinic activity and has been used as an analgesic in the treatment of irritable bowel syndrome.[40] The product ion spectrum of the parent drug (3) ($M^+ = 358$) contains a fragment ion at *m/z* 304 corresponding to a neutral loss of 54 Da. This is due to loss of the —CH_2—cyclopropyl side chain with concomitant gain of H by the bridgehead nitrogen.[10] Previously, we had tentatively concluded that the likely site of metabolic activity was on the ester side chain,[41] therefore using CNL scanning (54 Da) we were able to rapidly detect the presence and ultimately identify nine metabolites of 3.[10] Furthermore, by using this strategy, we have demonstrated differences in the metabolism of 3 by various animal species.[26]

The CNL spectrum shown in Fig. 7 contains ions at *m/z* 210, 328, 340, 344, 374, 390 and 406, which have all been shown to be metabolites of 3 (the ion at *m/z* 358 is unmetabolized parent drug (3)).[10] However, the ions observed at *m/z* 192, 208 and 238 also appear in a 'control' incubation CNL spectrum where 3 had been 'incubated' in the presence of heat-inactivated microsomes. A subsequent close inspection of the positive ion FAB mass spectrum of pure 3 also revealed ions at *m/z* 192, 208 and 238, which were difficult to detect due to the high background noise common in FAB mass spectra. It appears that these ions are formed from 3 during the FAB ionization process and have arisen from either complete loss of the ester side chain to afford the alkene (*m/z* 192 (14)), facile acyl cleavage of the ester side chain (*m/z* 208 (15)) or fragmentation of the (—O—C(O)—R) bond (*m/z* 238 (16)), and these structures are shown in Fig. 7. The ion at *m/z* 448 is still under investigation but is believed to arise from a reaction of a metabolite, produced during microsomal incubation, with unreacted parent drug (3).[10]

This example highlights the problem associated with having relatively large amounts of unreacted parent drug in the reaction mixture, which under FAB conditions can undergo a variety of reactions, such as reduction and dehydration,[42,43] which are similar to observed metabolic processes. Furthermore, it emphasizes the necessity for careful control experiments to be run in conjunction with attempts to rapidly screen drug metabolite mixtures using CNL or precursor ion scanning, as has been suggested recently by Vrbanac *et al.*[28].

Combination approach

Haloperidol (4) is a neuroleptic drug used in current clinical practice.[44] However, in some patients, as well as animals, it produces a number of neurological side effects such as tardive dyskinesias, and can induce Parkinsonian symptoms.[45,46] Therefore, there is great

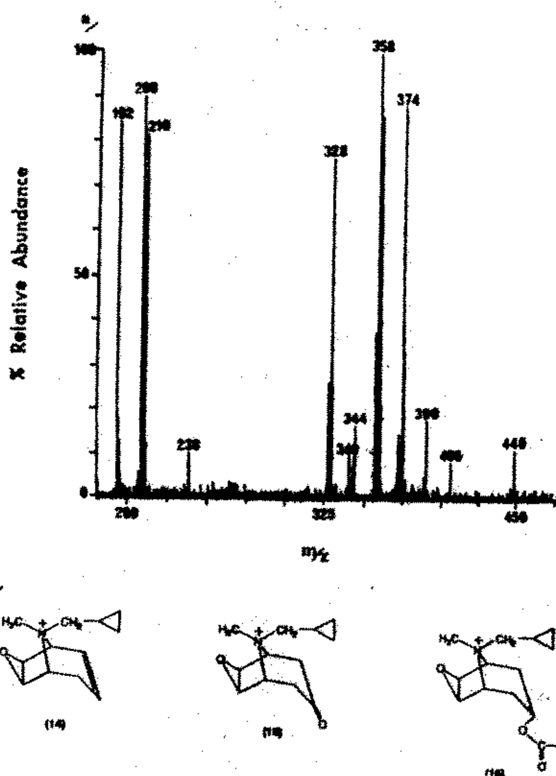
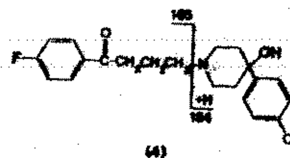


Figure 7. (a) Constant neutral loss (Δ54 Da) spectrum of a microsomal incubate of cimetropium (3). Collision energy was ~20 eV at 10^{-5} mbar collision gas (argon) pressure. The abundant ion at *m/z* 358 is unreacted parent drug (3), whereas ions at *m/z* 210, 328, 340, 344, 374, 390 and 406 are metabolites.[10] (b) Structures of artifacts produced from 3 during the FAB process: (i) *m/z* 192 (14); (ii) *m/z* 208 (15); (iii) *m/z* 238 (16).

interest in identifying those metabolite(s) producing such side effects. We and others, in particular Castagnoli and coworkers, have carried out both *in vitro* and *in vivo* studies of the metabolic fate of 4.[47–51]

Our initial studies of the *in vitro* metabolism of 4 utilized off-line HPLC in conjunction with FAB mass spectral and product ion spectral analyses.[47] However, we have subsequently developed a modified strategy based on our previous experiences of the limitations of simply using precursor ion and CNL scanning to detect drug metabolites of 4.

As in our previous studies of other drugs, we obtained a product ion spectrum of the parent drug (4). Only two useful, prominent ions at *m/z* 165 (90% of protonated molecular ion abundance) and 194 (25%) were detected. The ion at *m/z* 165 corresponds to a charge-initiated fragmentation of the aliphatic methylene carbon–piperidinol nitrogen bond with expulsion of the piperidinol nitrogen-containing moiety as a neutral. The ion at *m/z* 194 is brought about by the cleavage of the same carbon–nitrogen bond but with charge retention on the chlorophenyl moiety, as outlined below.



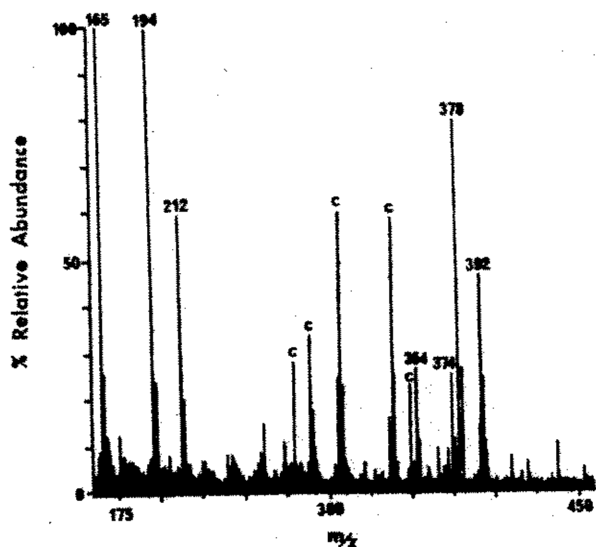
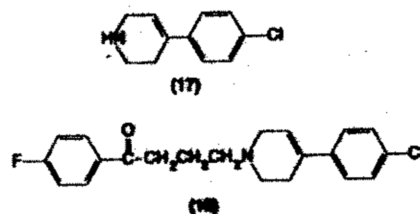


Figure 8. A double precursor ion spectrum of m/z 165 and 194 on a mixture derived from a microsomal incubation of haloperidol (4). Note that unreacted parent drug was removed by HPLC. The ions at m/z 212, 354, 374, 378 and 392 are metabolites. Collision energy was ~220 eV with a collision gas (argon) pressure of ~10^{-5} mbar; c = ions in control spectrum.

Based on well-developed principles of predicting sites of phase I metabolic activity,[2] it is likely that modification may occur at either end of the parent drug (4). Therefore, a double precursor ion scan of m/z 165 and 194 should detect a large percentage of metabolites produced. However, based on our previous experience with the production of artifacts by the FAB process, when large amounts of unreacted parent drug are present, we inserted an additional, rapid gradient HPLC step. This allowed the removal of unreacted parent drug (4) from the microsomal mixture. The resulting precursor ion scan of m/z 165, 194 for guinea-pig liver microsomal incubation of 4 is shown in Fig. 8. Ions at m/z 212, 354, 374, 378 and 392 have been shown to be metabolites of 4 as described previously, using off-line HPLC and product ion spectral analysis.[47]

As described for mifentidine (2), it is possible to fail to detect important metabolites when using the simple screening techniques of precursor ion (or CNL) scanning. In the case of haloperidol metabolites still containing the chlorophenyl ring functionality, the use of the chlorine isotope contribution can be utilized as a general screening method in a simple FAB mass spectral analysis of the metabolite mixture. This has been utilized by other workers[52–55] as a useful method of screening xenobiotic and drug metabolite mixtures.

A positive ion FAB mass spectrum of the metabolite mixture derived from microsomal incubation of 4 after HPLC is shown in Fig. 9. Ions at m/z 194 and 358 are clearly distinguishable as having a distinct chlorine isotope contribution at m/z 196 and 360, respectively. These ions were not detected in the precursor ion scan shown in Fig. 8, and by comparing their product ion spectra with those of synthetic standards (results not shown), we propose structures (17) [MH^+ = 194] and (18) [MH^+ = 358] as shown below. We are still investigating why we did not detect an ion at m/z 358 in the double precursor ion scan.



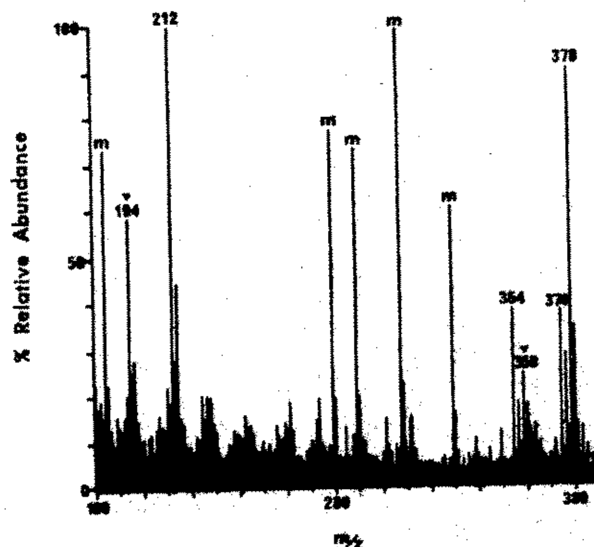


Figure 9. A positive ion FAB mass spectrum on a mixture derived from microsomal incubation of haloperidol (4). Ions marked ▼ were not detected in the CNL spectrum shown in Fig. 8; m = matrix ions.

CONCLUSIONS

The use of precursor ion or CNL scanning in screening complex mixtures and, in particular, drug metabolite mixtures, is now a routine analytical tool. The techniques have, potentially, removed the necessity of time-consuming chromatographic steps, as well as offering, when used in conjunction with product ion spectral data obtained on 'detected unknowns', a rapid method of obtaining considerable structural data on a large number of compounds. However, it is clear from the work presented here that, although such an approach can be extremely useful in detecting novel compounds as exemplified by the detection of G-Pip (8) and G-Pip-G (9), it is also readily obvious that such an approach also has limitations and problems associated with it. In particular, by using only such a strategy, it is likely that certain important metabolites will not be detected as described above in the case of the amine (12) and *N*-hydroxylamine (13) metabolites of 2. This same conclusion was reached by Vrbanac *et al.*[18] in their recent study of the metabolic fate of the anti-anxiety–sedative drug 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)-imidazo-[1,5-α]quinoxalin-4(5*H*)-one. Furthermore, such methods, and in particular, CNL scanning, exclude the possibility of novel metabolites produced at unusual sites of reactivity. For instance, if metabolism had occurred at either the bridgehead quaternary nitrogen or the cyclopropyl functional group of

cimetropium bromide (3), these metabolites would not be detected using a CNL scan of 54 Da. Another problem associated with this approach, as also exemplified by the cimetropium bromide (3) study, is the production of artifacts when large amounts of unreacted drug substrate are present.

It is clear that such a simple approach to complex mixture analysis is limited, and the advantage afforded by the rapid nature of this screening strategy is offset by a number of problems. With the advent of routine HPLC/mass spectrometry and capillary electrophoresis/mass spectrometry interfaces, it is likely that screening methods, such as precursor ion and CNL scanning, will be utilized in conjunction with such on-line techniques in order to circumvent the problems discussed above.

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Utility of the Parent–Neutral Loss Scan Screening Technique: Partial Characterization of Urinary Metabolites of U-78875 in Monkey Urine

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Metabolites of an antianxiety–sedative drug candidate (U-78875; 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)-imidazo[1,5-α]quinoxalin-4(5*H*)-one (I)) present in the urine of monkeys were detected using tandem mass spectrometry (MS/MS) by application of parent ion scans and characterized or partially characterized by performing daughter ion scans of the pseudo-molecular ions of suspected metabolites. The use of liquid secondary ion mass spectrometry ionization of crude urinary extracts in combination with tandem quadrupole MS/MS analyses using parent ion scans of *m/z* 69 and subsequent daughter ion scans characterized unmetabolized I and *N*-dealkyl I (U-85466) and partially characterized aryl hydroxyl, aryl hydroxyl-*N*-dealkyl, aryl *O*-glucuronide, aryl *O*-glucuronide-*N*-dealkyl, aryl *O*-sulfate and aryl *O*-sulfate-*N*-dealkyl metabolites. From these data it was concluded that some of the metabolic pathways involved in the biotransformation of U-78875 include *N*-dealkylation, aryl hydroxylation and conjugation of aryl hydroxides. Several other metabolites of U-78875 not detected using this analytical approach were subsequently identified by alternative mass spectrometric approaches. These data clearly demonstrated both the utility and, just as important, the limitations of the parent–neutral loss scan screening technique in detecting drug metabolites in complex biological milieux.

INTRODUCTION

Drug metabolite identification in animals and human subjects is important from both a scientific and product development perspective. The development of drugs in the pharmaceutical industry is a complex interactive process that can be roughly divided into three stages, although considerable overlap and interaction blur such a division. Potential drugs from synthetic and biological sources are first tested in pharmacological screens to detect desirable biological activity. After the initial screening process, selected compounds then enter preclinical development. The third stage in this complex process is clinical evaluation of drugs using normal and targeted human subjects. The determination of drug metabolite structures has traditionally occurred after a compound has been selected for extensive preclinical toxicological evaluation since drug disposition studies are expensive, relatively time consuming and require the synthesis of a radioisotope-labeled drug. It has been suggested that tandem mass spectrometry (MS/MS), utilizing parent and neutral loss scans, can be employed to characterize or partially characterize many drug metabolite structures relatively rapidly.[1–5] This is accomplished by first obtaining a daughter ion mass spectrum of the molecular ion, or more commonly, the pseudo-molecular ion. Characteristic neutral losses and daughter ions are noted in the daughter ion mass spectrum. This information is then used to screen for drug metabolites using appropriate neutral loss and parent ion scans, hereafter referred to as the parent–neutral loss scan screening technique. Daughter ion mass spectra are then obtained for drug metabolite candidates. Interpretation of these spectra may characterize or partially characterize drug metabolite structures and may also suggest a second series of neutral loss and/or parent ion scans to screen for further drug metabolites. Using this approach it is possible to obtain important early metabolism information on drug candidates.

U-78875 (3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)-imidazo[1,5-α]quinoxalin-4(5*H*)-one) is a non-benzodiazepine antianxiety–sedative agent.[6–10] The structure of U-78875 (I) is shown in Fig. 1. The purpose of the present study was to identify metabolites of *I* present in monkey urine by liquid secondary ion mass spectrometry (LSIMS) ionization and tandem quadrupole mass spectrometry. This was to be accomplished by obtaining high-resolution electron ionization data for I, collisionally activated dissociation (CAD) spectra of I and structural analogs and by the judicious use of parent and/or neutral loss scans determined from the daughter ion mass spectrum of I. This paper

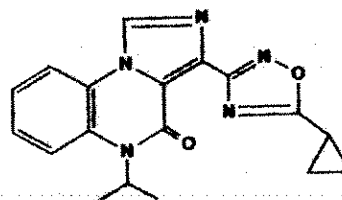

Figure 1. Structure of the non-benzodiazepine antianxiety–sedative I, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)-imidazo[1,5-α]quinoxalin-4(5*H*)-one).

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describes the results obtained using this approach in combination with partial purification of urine samples using gradient high-performance liquid chromatography (HPLC) coupled with fraction collection and LSIMS ionization of the sample.

EXPERIMENTAL

Materials

Silanized glass or polypropylene handling materials were used in these analyses. Glass was silanized by reaction with 5% dimethyldichlorosilane in toluene for 20 min and then washing with methanol. Solvents were obtained from Burdick and Jackson (Muskegon, Michigan). Octadecylsilane (ODS) sample preparation cartridges were obtained from Waters (Millipore Corporation, Milford, Massachusetts).

Sample extraction and purification

ODS sample preparation cartridges were washed with 10 ml methanol followed by 10 ml water before use. Urine samples were obtained from a I-treated monkey (100 mg kg^{-1}, oral administration, 0–4 h). The dose administered was five to ten times greater than the pharmacodynamic 'threshold' dose. The control sample was collected prior to drug administration. Samples (10 ml, control and I-treated) were centrifuged at 3000 r.p.m. for 5 min and then were extracted without any adjustment of the pH. Urine was pushed through the extraction cartridges using a silanized glass syringe at a rate of approximately two to three drops per second. The cartridges were then washed with 5 ml water and adsorbed material eluted with 3 ml methanol. Radiolabeled I was not available to determine recovery. The methanol eluate was dried under a stream of nitrogen. The resulting residue was dissolved in water and chromatographed on ODS using gradient elution reversed-phase HPLC. Fractions were collected every 5% change in solvent composition. Sample fractions were dried under a stream of nitrogen at ambient temperature and stored at −20 °C. Samples were dissolved in 20 μl of methanol–water (1:1) prior to analysis by mass spectrometry.

Mass spectrometry

High-resolution mass spectral data were recorded using a VG ZAB-2F high-resolution mass spectrometer and direct probe electron ionization (EI). Accurate mass measurements were performed by the peak-matching technique using the peaks of perfluorokerosene as reference masses. The resolution was set to 10000.

Low-resolution CAD mass spectra were obtained using a Finnigan MAT TSQ-70 tandem quadrupole mass spectrometer. EI spectra were recorded using 70 eV. Tuning of the ion optics and calibration of the mass axis in the EI mode of operation were performed using perfluorotributyl amine (PFTBA). LSIMS mass spectra were recorded using an Ion Tech (Teddington, UK) B21340 saddle-field source and B50 power supply unit and 8 keV xenon atoms. Tuning of the ion optics and calibration of the mass axis in the LSIMS mode of operation were performed using PFTBA and an EI ion source (the LSIMS source was installed after tuning). The LSIMS matrix was 2-hydroxyethyl disulfide (2-HED). CAD mass spectra were obtained using argon as the collision gas at 1 mtorr. Optimization of the ion optics in the MS/MS mode of operation was accomplished using PFTBA and EI. Tuning of ion optics and calibration of the mass axis were first done using computer algorithms followed by manual inspection of the tune parameters and adjustment of various parameters.

RESULTS

High-resolution EI mass spectrum

I (Fig. 1) is a highly aromatized four-ring system molecule with isopropyl and cyclopropyl side chains. Thus the fragmentations that can occur in the mass spectrometer under EI conditions cannot be predicted in advance. The EI mass spectrum of I is shown in Fig. 2. Attempts to interpret low-resolution and CAD data can lead to ambiguities or errors as far as structural assignments are concerned. For example, the ions appearing at m/z 266, 225, 210, 90 or 69 can be represented by several structures. Thus, to ascertain if each of these ions can be represented by a single structure, high-resolution mass measurements were made. A summary of the high-resolution data follows.

The ion that appears at m/z 293 is due to the loss of C_3H_6 and can be interpreted in two ways. First, it might be the loss of the cyclopropyl group and a hydrogen radical which could come from the isopropyl group. An alternative, and more appealing, explanation as far as mass spectral fragmentation is concerned is that this ion is produced by a McLafferty rearrangement involving the isopropyl group. One of the hydrogens of the two methyl groups migrates to the carbonyl function of the neighboring lactam with elimination of propene.

The ion occurring at m/z 266 originates from the molecular ion by loss of 69 Da. The exact mass mea-

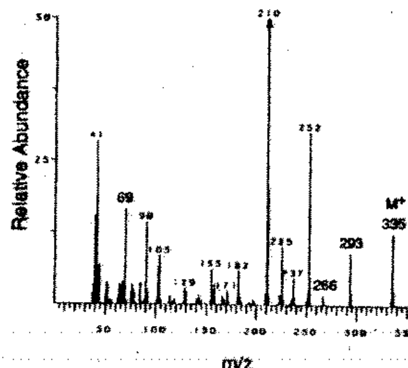

Figure 2. EI mass spectrum of I. High-resolution data were obtained for selected ions. All mass spectral data are positive ion.

surement for this ion indicates that this loss corresponds to C_4H_5O. Thus, the most likely candidate for this is the cyclopropyl group with the adjacent carbon and the oxygen bound to it. This will be called the cyclopropylcarbonyl loss. The loss of the cyclopropyl with the adjacent carbon and the nitrogen bound to it plus two hydrogens can be ruled out on the basis of the elemental composition of this ion ($C_{14}H_{12}N_5O$).

The ion that occurs at *m/z* 252 has an elemental composition of $C_{14}N_{12}N_4O$. Thus, it amounts to the loss of $C_4H_5N_1O$ from the molecular ion. This can be interpreted to be cleavage of the oxadiazole ring in such a way as to leave a nitrile function attached to the rest of the molecule.

The elemental composition of *m/z* 225 is $C_{11}H_7N_5O$. It is clear from the structure of I that no such fragment can be lost as single entity. Therefore, we have to conclude that this ion is the result of the loss of two fragments: the loss of the cyclopropylcarbonyl with hydrogen rearrangement and the propene by the McLafferty rearrangement discussed above.

The ion at *m/z* 210 has an elemental composition of $C_{11}H_6N_4O$ and differs from the elemental composition of the molecular ion by $C_7H_{11}N_1O$. This again cannot be due to the loss of a single fragment from the molecular ion, but is due to the loss of two fragments. As in the previous case, the origin of this fragment is a combination of the loss of propene from the *m/z* 252 ion or the cleavage of the oxidiazole ring from the *m/z* 293 ion or a simultaneous loss of both of these fragments from the molecular ion.

Finally, the ion at *m/z* 69 appeared as a doublet. The exact masses indicate that the more abundant ion of this doublet has the elemental composition of C_4H_5O. Thus, this is the ionized part of the cyclopropylcarbonyl fragment, a cyclopropylacylium ion. The other ion of this doublet has the elemental composition of C_5H_9 and cannot be directly generated from the structure of the molecular ion. Either this is due to some deep-seated rearrangement, or it is due to some impurity. The latter is the most probable case.

Tandem quadrupole daughter ion mass spectra

The CAD mass spectrum of I obtained using the tandem quadrupole instrument is displayed in Fig. 3. Daughter ions observed at *m/z* 294, 268, 226 and 69 were also observed to occur in the EI mass spectrum (correcting for differences in the number of hydrogen atoms). The ions at 294, 268 and 226 represent neutral losses of 42, 68 and 110 Da, respectively. No other structurally significant ions were observed at higher or lower collision energies. The ion at *m/z* 294 could result from loss of propene originating from the isopropyl group (via a McLafferty rearrangment as discussed above) or the cyclopropyl group and a hydrogen radical. As was previously discussed, the ions at *m/z* 268 (neutral loss of 68) and *m/z* 69 could result from two different rearrangements involving the cyclopropyl group and the oxadiazole ring system. Thus, the low-resolution data did not allow for the unambiguous assignment of the structure of either of these ions. The

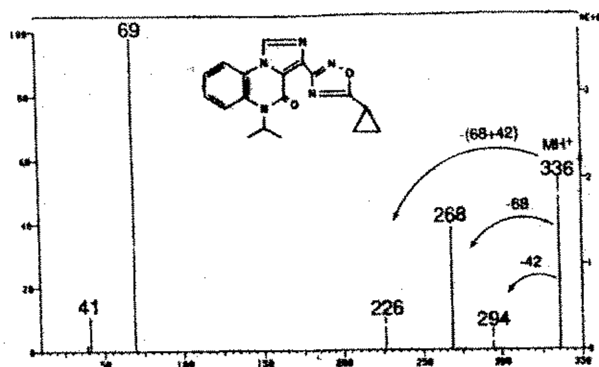

Fig. 3. Daughter ion mass spectrum of *m/z* 336 of I (MH$^+$, pseudo-molecular ion) obtained using a tandem quadrupole mass spectrometer and LSIMS ionization. CAD parameters were 1.0 mtorr collision gas pressure and 10 eV collision energy. All CAD mass spectral data are positive ion.

ion at *m/z* 226 could be interpreted as successive neutral losses of 42 and 68 Da.

Figure 4 displays the CAD mass spectrum obtained for U-82440. U-82440 is a structural analog of I where the isopropyl group has been replaced by a cyclopropylmethyl group. It is significant to note that MH$^+$ − 42 is no longer observed in this daughter ion mass spectrum and the neutral loss of 42 Da has now shifted to 54 Da. The CAD mass spectrum of another analog where R$_1$ is a different aliphatic group (a 3-methyl-2-butenyl group) also exhibited an absence of the 42 Da neutral loss and exhibited a neutral loss representative of the shift in mass of the R$_1$ group. The successive losses of 42 and 68 Da from MH$^+$ seen in Fig. 3 are observed as successive losses of 54 and 68 Da (*m/z* 226; Fig. 4). These observations, taken together with the high-resolution data, indicate that the high-mass ions in the daughter ion mass spectrum of I (Fig. 3) result from neutral loss of propene originating from the isopropyl group (42 Da; *m/z* 294), neutral loss of the cyclopropylcarbonyl group (68 Da; *m/z* 268), and successive loss of both groups (110 Da; *m/z* 226).

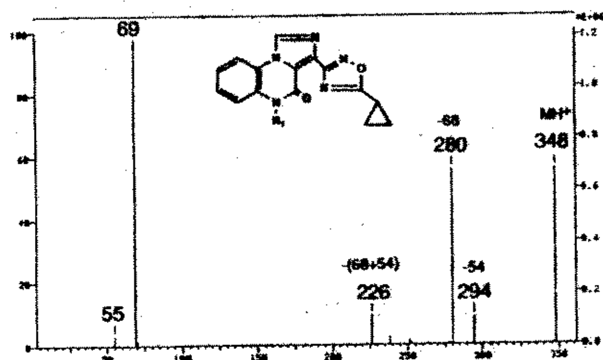

Figure 4. Daughter ion mass spectrum of *m/z* 348 (MH$^+$, pseudo-molecular ion) of a structural analog of I where a cyclopropyl methyl group has been substituted for the isopropyl side chain. Data were obtained using a tandem quadrupole mass spectrometer and LSIMS ionization. CAD parameters were 1.0 mtorr collision gas pressure and 10 eV collision energy.

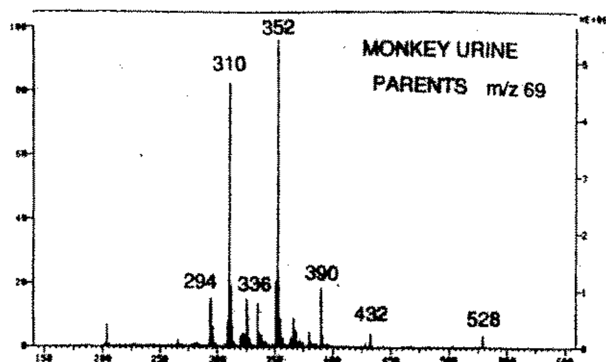

Figure 5. Parent ion mass spectrum of m/z 69 that was observed for a urine sample obtained from a I-treated monkey. Data were obtained using a tandem quadrupole mass spectrometer and LSIMS ionization. CAD parameters were 1.0 mtorr collision gas pressure and 10 eV collision energy.

Tandem quadrupole analysis of urine samples

Based upon the above data, it was hypothesized that metabolites of I not involving the 5-cyclopropyl-oxadiazole moiety would also form the cyclopropylacylium ion (m/z 69) as a daughter ion upon collisional activation or exhibit a neutral loss of 68 Da. The only neutral loss or parent scan suggested by the CAD mass spectrum of I useful to screen for metabolites of I is a neutral loss of 42 Da. Besides being of low intensity, the 42 Da loss is a highly non-specific neutral loss. Also note that the formation of m/z 69 is preferred to a neutral loss of 68 Da. Therefore, to detect metabolites of I, it was concluded that a parent scan of m/z 69 was the only practical scan function to utilize. A collision energy of 10 eV and a collision gas pressure of 1.0 mtorr were found to be optimal.

Single-stage mass spectral analysis (using the second analyzer) and parent scans of m/z 69 were performed on each of the urine samples described above using LSIMS ionization. Single-stage mass spectral analysis provided little useful information and will not be discussed. However, parent ion scans of m/z 69 provided information that was useful to characterize or partially characterize a number of metabolites of I. The mass spectrum obtained using parent ion scans of m/z 69 for the HPLC fraction that was observed to contain the largest number of parent ions that could rationally be related to possible I drug metabolites is shown in Fig. 5. The corresponding parent ion mass spectrum obtained for the control urine fraction exhibited none of the ions labeled in Fig. 5. Interpretation of the positive daughter ion mass spectral data obtained for parent ions m/z 336, 294, 352, 310, 432 and 528 was as follows.

Phase I metabolism. The positive daughter ion mass spectrum obtained for m/z 336 was m/z 336 (24; % of base peak), 294 (2), 268 (38), 226 (8), 69 (100) and 41 (11). This daughter ion mass spectrum is virtually identical to that obtained for reference I at the same CAD conditions.

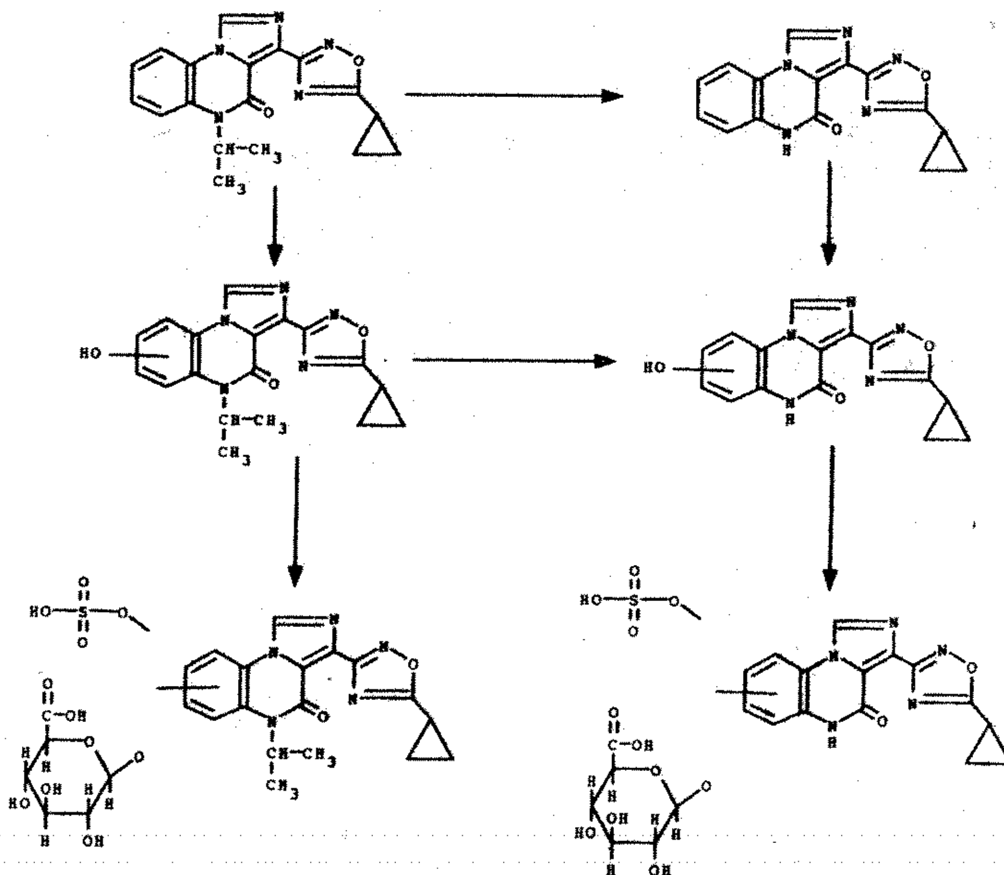

Figure 6. A summary of the metabolic transformations of I characterized by use of the parent–neutral loss scan screening technique.

These data confirmed the presence of unmetabolized I in monkey urine.

The positive daughter ion mass spectrum obtained for m/z 294 was 294 (21), 226 (16), 69 (100) and 41 (13). These data were interpreted to indicate the presence of an *N*-dealkyl metabolite of I. A reference standard was available for this compound (U-85456) and the positive daughter ion mass spectrum of this compound was observed to be identical to this spectrum.

The positive daughter ion mass spectrum of m/z 352 was 352 (19), 310 (22), 284 (9), 266 (3), 242 (18), 69 (100) and 41 (7). These data indicate the presence of an aryl hydroxyl metabolite of I. The position of the hyroxylation cannot be ascertained from the CAD mass spectrum.

The positive daughter ion mass spectrum of m/z 310 was 310 (7), 242 (25), 69 (100) and 41 (19). These data, taken together with the confirmed presence of an *N*-dealkyl metabolite and the highly probable presence of an aryl hydroxylated metabolite, indicate that this compound is most likely an aryl hydroxy-*N*-dealkyl metabolite.

Phase II metabolism. The positive daughter ion mass spectrum of m/z 432 was 432 (46), 390 (68), 310 (46), 284 (20), 242 (19) and 69 (100). A neutral loss of 80 Da, which is characteristic of aryl *O*-sulfate conjugates in the positive mode of ionization,[3] was observed in combination with neutral losses of 42 and 68 Da. This mass spectrum indicates the presence of an *O*-sulfate conjugate of the aryl hydroxy metabolite.

The daughter ion mass spectrum of m/z 528 was 528 (31), 460 (8), 352 (87), 310 (11), 284 (100), 242 (17) and 69 (37). These data indicated the presence of an *O*-glucuronide conjugate of a hydroxylated metabolite. The spectrum is dominated by neutral losses of 176 Da as expected.[3]

In addition to the above data, the presence of aryl *O*-sulfate-*N*-dealkyl and aryl *O*-glucuronide-*N*-dealkyl metabolites was suggested by parent ion and daughter ion scan data and by the observation that *N*-dealkylation is apparently a major metabolic pathway for I in the monkey. A summary of the metabolic transformations of I suggested by use of the parent–neutral loss screening technique is displayed in Fig. 6.

The above data characterize or partially characterize several metabolites of I. It is clear from the above data that unmetabolized I and *N*-dealkyl I are present in monkey urine. It was not possible to fully characterize the other metabolites since the position of oxidation could not be obtained by interpretation of the daughter ion mass spectra. Complete characterization of these metabolites would require further physicochemical analysis.

DISCUSSION

A critical stage in the drug development process is at the interface between discovery and development where the choice of a specific compound(s) for development is made from a group of structurally related compounds with similar pharmacodynamic properties. This decision point precedes a second stage of time-consuming and expensive drug safety, drug metabolism studies (addressing absorption, distribution, metabolism and excretion issues), and ultimately very expensive phase I, II and III clinical trials. Success in the discovery and development of new pharmaceutical products is associated with early thoughtful characterization in animal models. The probability of success in the clinical trials was increased by the recognition of potentially fatal flaws that may exist in the compound. A major function of drug metabolism groups involved in drug discovery and development is to identify unacceptable biopharmaceutical/pharmacokinetic and intrinsic toxicity attributes of drug candidates. Drug metabolism research may effect the selection of drug candidates with respect to problems associated with high first-pass metabolism, formation of active metabolites and formation of toxic metabolites (e.g. reactive chemical intermediates). Therefore, rapid lead-finding drug metabolism studies are of great utility in selecting, from a group of analogs, drug candidates that will have the best overall biopharmaceutical, pharmacokinetic and toxicological attributes in man. The success of drug metabolism research groups in rapidly characterizing the metabolic profiles of a group of compounds in various species resides in the utilization of state-of-the-art mass spectrometry technology. The data presented above demonstrate the utility of the parent–neutral loss scan screening technique in rapidly elucidating drug metabolite structures present in complex biological milieux. The above data also illustrate the value of obtaining high-resolution data as well as CAD spectra of structural analogs, and underscore the complementary nature of high-resolution mass spectral data and comparative EI or CAD mass spectral data of structural analogs.

Control samples were included in this study and controls are routinely analyzed in this laboratory. It has been our observation that inclusion of controls in the overall analytical scheme is well worth the extra effort, whatever the mass spectra analyses performed. Collection of control samples allows analysis by single-stage mass spectrometry and comparison of control and drug data obtained by on-line chromatography mass spectral analysis (liquid chromatography/mass spectrometry or gas chromatography/mass spectrometry). In general, we have observed that this approach is far more successful in detecting and characterizing drug metabolites in biological samples than the parent–neutral loss scan screening technique. In fact, in many situations the use of parent and/or neutral loss scans suggested by the daughter ion mass spectrum of a drug may give little or no information concerning the metabolism of the drug, if, for example, the metabolism is highly complex. Figure 7 shows the structure of U-74006. This molecule contains two distinct domains—a steroid domain and a complex heterocyclic domain—and the low-energy CAD daughter ion mass spectrum of this compound is dominated by cleavages between the steroid and heterocyclic domains. Based upon what is known about the metabolism of steroids and the metabolism of pyrrolidinyl groups, extensive and highly complex metabolism would be expected to occur in both regions, and this has in fact been noted to occur. Application of parent

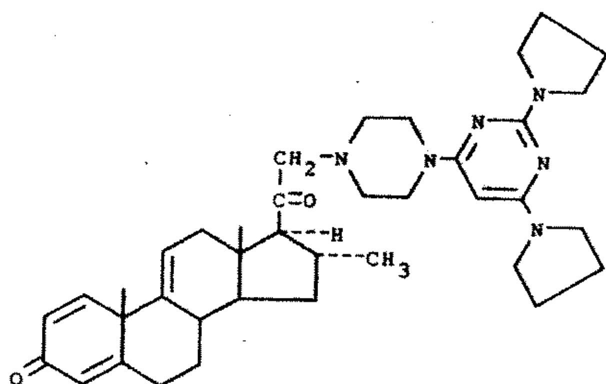

Figure 7. Structure of U-74006.

and/or neutral loss scans suggested by the daughter ion mass spectrum of this compound would provide minimal information concerning the metabolism of this molecule. This clearly represents a situation where careful comparison of data obtained from on-line chromatography/mass spectral analysis (either single-stage mass spectrometry or followed by MS/MS analysis) of an appropriate control sample and a drug-related sample would provide the greatest amount of information. In the present study, radio-labeled drug was not administered, and it is therefore impossible to obtain balance data, and an unknown number of metabolites, possibly major metabolites, may have been undetected using the parent–neutral loss scan screening technique. In fact, more recent studies have shown that a number of important metabolic transformations of I involving the cyclopropyl-oxadiazole moiety were not detected using this approach alone. Clearly, the success of the parent–neutral loss scan screening technique in elucidating drug metabolite structures is highly compound specific.

Summary

The use of LSIMS ionization in combination with tandem quadrupole MS/MS analyses using parent ion scans has characterized or partially characterized the following compounds in the urine of monkeys treated with I: unmetabolized I, *N*-dealkyl (U-85466), aryl hydroxyl, aryl hydroxyl-*N*-dealkyl, aryl *O*-sulfate, aryl *O*-sulfate-*N*-dealkyl, aryl *O*-glucuronide and aryl *O*-glucuronide-*N*-dealkyl metabolites. These data accurately illustrate both the utility and the limitations of the parent–neutral loss scan screening technique in the elucidation of drug metabolite structures without the use of radioisotopes in certain situations. General hypotheses concerning the metabolism of I were offered based upon these data; however, balance information and a complete metabolic profile were absent.

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