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Structural elucidation of metabolites of ritonavir and indinavir by liquid chromatography–mass spectrometry

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Abstract

The structural elucidation of metabolites of ritonavir and indinavir, HIV-protease inhibitor drugs, by liquid chromatography–electrospray ionization mass spectrometry is described. Ritonavir and indinavir were biotransformed separately by incubation with transplant quality human liver microsomes. The incubation mixture was then analyzed by HPLC coupled to ion trap (ITMS) and triple quadrupole mass analyzers. The metabolites retained most of the structural features of the parent molecules. Baseline chromatographic resolution of isobaric species by gradient elution HPLC permitted rapid structural identification of these metabolites. Both drugs were biotransformed primarily by oxidative and hydrolytic pathways to numerous metabolites that retained many of the features of the parent molecules. Triple quadrupole and ion trap mass spectrometry were applied jointly to thoroughly detect and thoroughly characterize these metabolites. Furthermore, retention-time and data-dependent scanning assured acquisition of detailed MS–MS spectra for rapid detection of metabolic pathways of ritonavir and indinavir. Comparison of the ITMS and triple quadrupole data showed qualitative and quantitative differences in the mass spectral patterns, suggesting that these instruments should be used in parallel to ensure comprehensive metabolite detection and characterization by LC–MS.

Keywords: Structural elucidation; Ritonavir; Indinavir

1. Introduction

Since the discovery of HIV in 1983 an arsenal of drugs has been introduced to block its replication within host cells [1,2]. Following the elucidation of the HIV life cycle, researchers have developed agents that disrupt the normal biology of the virus. Two key areas of research have targeted the discovery of inhibitors of reverse transcriptase and protease enzymes [1,3]. Indinavir and ritonavir are two drugs that selectively inhibit the HIV type 1 protease enzyme [4–6]. They prevent proteolytic cleavage of the precursor polyproteins, Pr160 *gagpol* and Pr55 *gag*, to products essential for the maturation of infectious virions. Since their introduction in 1995, ritonavir and indinavir have been used successfully in combination with other anti-HIV drugs to slow and/or prevent the onset of AIDS. Several clinical studies have documented the synergistic effects of reverse transcriptase inhibitors and protease inhibitors in triple drug regimens. Koudriakova et al. demonstrated that ritonavir is a powerful mechanism-based inactivator of CYP3A, thereby explaining its potent inhibition of the elimination of other concurrently administered substrates of CYP3A, including indinavir [7]. Inactivation of intestinal and hepatic

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CYP3A by ritonavir increases the bioavailability and prolongs the half-life of other co-administered protease inhibitors [8–10]. These characteristics of ritonavir may result in a more effective treatment program for HIV because concentrations of drugs are more likely to remain within their effective therapeutic range. This is of particular significance in patients who fail to take their medications exactly at the prescribed intervals.

Largely encouraged by the development of soft ionization techniques (ESI and APCI), mass spectrometry has assumed an increasingly important role in developmental therapeutics, both in industry and academic institutions. API–MS, a technique of unusual versatility, is applied in all stages of drug development including chemical synthesis, protein target identification, library verification, pharmacokinetics, toxicology and many others. Identification and quantification of drugs and metabolites in biological matrices increasingly utilize MS technologies [11,12]. While different combinations of liquid chromatography, UV–DAD, radioactive methods, and NMR are still widely used and applicable, they are extensively employed in conjunction with MS techniques [13,14]. Hyphenated MS techniques are frequently the initial choice for metabolite detection and identification because of their sensitivity and convenience compared to other, albeit very effective techniques such as NMR and radiolabeling [15,16]. Certainly however, it should also be realized that any changes in chirality and racemization will not be observed under typical LC–MS conditions.

This work presents LC–MS(–MS) methods for rapid qualitative identification of metabolites of indinavir and ritonavir. Each drug was metabolized in vitro to numerous metabolites by incubations with human liver microsomes. The incubation mixture was extracted with methylene chloride, evaporated to dryness and the residue redissolved. A 65-min acetonitrile gradient provided a suitable compromise of speed and base line chromatographic resolution of peaks. Since both drugs produced isobaric metabolites, baseline chromatographic separation of these peaks was a necessary prerequisite for identification of products. Structural assignments of metabolites were based on changes in molecular masses and spectral patterns of product ions. In addition, both ion trap and triple quadrupole mass analyzers were used to provide complementary mass spectral information. ITMS provided superior sensitivity in full scan and MS–MS mode while the triple quadrupole, especially when used in combination with in-source fragmentation provided the most structurally informative product ion spectra for these compounds. This dual approach was found to be essential for more comprehensive detection and characterization of metabolites of both drugs by LC–MS. These MS/MS dependencies are demonstrated here for the analysis of specific mono- and dihydroxylated metabolites of ritonavir and idinavir. The detailed characterization of trihydroxylated metabolities of indinavir has been discussed in a previous publication [7].

2. Experimental

2.1. Chemicals

Indinavir, as the sulfate salt (>98% purity), was generously provided by Merck Research Laboratories (West Point, PA, USA). Ritonavir (>98% purity) was a gift from Abbott Laboratories (Abbott Park, IL, USA). HPLC-grade acetonitrile and methanol were acquired from Fisher Scientific Products (Fair Lawn, NJ, USA). Acetic acid and ammonium acetate were purchased from Sigma (St. Louis, MO, USA). House-deionized water was further purified with a Milli-Q water purifying system, Millipore Corporation (Bedford, MA, USA).

2.2. Microsomes

Samples of human liver were received from the operating room (The Ohio State University Hospitals) or the International Institute for the Advancement of Medicine (Exton, PA). The collection of specimens for research was approved by the Ohio State University Biomedical Sciences Human Subjects Review Committee. Liver microsomes were prepared as previously described [17]. Microsomal protein was determined by the method of Lowry et al. [18]. All microsomal incubations were conducted in a shaking water bath at 37 °C. They contained

liver microsomal protein (2.1 mg/ml), NADPH (1 m*M*), ritonavir (5 μ*M*) or indinavir (10 μ*M*) in 50 m*M* potassium phosphate buffer, pH 7.4. Incubations with no NADPH served as the controls. Total volume of each incubation was 10 ml. The reactions with ritonavir and indinavir were terminated after 30 min and 1 h, respectively. The metabolites were extracted by shaking the incubation mixtures with 20 ml of methylene chloride. After centrifugation, the lower layer was removed and dried under nitrogen at room temperature. The dried residue containing metabolites was redissolved in 300 μl of methanol–water (50:50, v/v) for analysis by LC–MS.

2.3. HPLC

Compounds were separated on either an HP series 1090 or 1100 HPLC equipped with an autosampler and diode array detector (Agilent Technologies, Palo Alto, CA, USA). The different system volumes (ca. 900 μl) between the HPLCs accounted for the ca. 5-min difference in retention times of analytes. The HPLC systems were controlled with the HP ChemStation software running on an IBM compatible PC. A Waters (Milford, MA, USA) Symmetry C_{18} reversed-phase LC column (2.1 mm×150 mm×3.5 μm) was used to separate extracts of the microsomal incubations and to verify purity of parent drugs. Injection volumes were always 15 μl; the pump flow-rate was 210 μl/min, operated in a gradient elution mode. Mobile phase A consisted of 10 m*M* ammonium acetate (adjusted to pH 4.7 with acetic acid) and mobile phase B was acetonitrile.

The evaluation of purity of indinavir and characterization of metabolites used a linear gradient: 15–40% B over 52 min, subsequently adjusted to 65% B in 13 min. The determination of ritonavir purity and characterization of metabolites used a linear gradient of 25–60% B over 52 min, maintained at 60% B for an additional 13 min. The run times in all instances totaled 65 min. The effluent from the column was monitored by UV detection at 210 and 230 nm; DAD spectra were acquired every 0.5 s. The flow from the UV detector (unsplit) was directed into the mass spectrometer with a minimal amount of PEEK tubing (Upchurch, Oak Harbor WA, USA).

2.4. Mass spectrometry

Mass spectrometric detection used either a TSQ 700 triple quadrupole or an LCQ ion trap mass spectrometer (ITMS) from Finnigan (San Jose, CA, USA). The computer that controlled the triple quadrupole was outfitted with a UNIX operating system (version 4F) run with ICIS 8.3 and ICL version 7.5. The LCQ ion trap was controlled by means of an IBM-compatible personal computer equipped with Windows NT and LCQ software, version 1.2. All experiments utilized electrospray ionization in the positive ion mode. Instruments were calibrated with recommended solutions and operated under unit mass resolution conditions (i.e., −1 Da peak width at peak base) except during MS–MS where in-source fragmentation was induced. In these instances, the resolutions on Q1 and Q3 were lowered to improve analyte detection. The electrospray voltage ranged from +3.8 to +4.2 kV and the heated capillary was held at 200 °C. Nitrogen, 25 and 5 p.s.i. of sheath and auxiliary gas, respectively, was used for drying/nebulization.

Contamination of the ESI source was prevented by diverting the column effluent from the first 2.2 min of the run to ‘‘waste’’ by means of an actuating valve. Full scan analysis on the triple quadrupole was conducted from m/z 150 to m/z 900 in 2 s with an electron multiplier voltage of 1000 V. In-source fragmentation was induced by increasing the cone voltage by 40 V. Ion trap full scan analyses were conducted from m/z 150 to m/z 1200 in AGC mode with an upper fill time of 200 ms. A total of three microscans were summed for each macroscan. Collision-induced dissociation (CID) experiments used argon and helium in the triple quadrupole and ion trap, respectively. MS–MS experiments on both the triple quadrupole and ion trap utilized a retention time-dependent LC–MS–MS program into which the retention times and parent ion masses were programmed following review of the full scan LC–MS chromatogram. On the triple quadrupole, metabolite retention times and parent ion masses were programmed into the ULIST for retention time-dependent LC–MS–MS data acquisition. MS–MS experiments on the triple quadrupole used the ESIDAU1 procedure; the mass scans were 2 s each and ranged from 30 Da to the parent mass +30 Da. The

retention-time-dependent MS–MS experiments on the triple quadrupole were programmed into the ICIS software. In a separate analysis, the data-dependent MS–MS scanning feature of the ion trap was used to ensure comprehensive MS–MS information on the metabolites.

3. Results and discussion

The first step in this work involved the characterization of the mass spectral properties of the parent drugs. Both indinavir and ritonavir were determined to be >98% pure by LC–UV–ITMS; no impurities or degradation products were detected. Full scan mass spectral analyses of ritonavir and indinavir showed protonated molecule ions of m/z 721 and 614, respectively. CID analyses of these pseudomolecular ions yielded the mass spectra presented in Fig. 2. The suggested fragmentation patterns are presented in Fig. 1. Determination of the structures of metabolites was facilitated by the fact that both compounds undergo extensive, but well definable fragmentation under MS–MS conditions. The mass spectral patterns (Fig. 2) served as templates in the elucidation of the structures of the proposed metabolites.

Control microsomal incubations in the absence of NADPH were conducted to exclude analytes/compounds that were unrelated to metabolism of indinavir and ritonavir by CYP enzymes. LC–ITMS base peak chromatograms of these incubations showed a number of products appearing at retention times longer than those of the parent drug (i.e., less polar compounds). These correspond to peaks at 41.5 and 42.3 min in Fig. 3A and 48.4 min in Fig. 3B. Examination of the mass spectra of these compounds did not provide any evidence that they were biotransformation products of either drug. Therefore, they are excluded from this discussion.

Fig. 3 shows the full scan base peak ITMS chromatograms for the metabolites of indinavir and ritonavir extractable in methylene chloride from microsomal incubations. The chromatograms are magnified to illustrate more clearly the profiles of metabolite peaks. Therefore, the unmodified parent drug peaks are left ''off scale.'' The chromatogram of the indinavir metabolites showed the peak of the

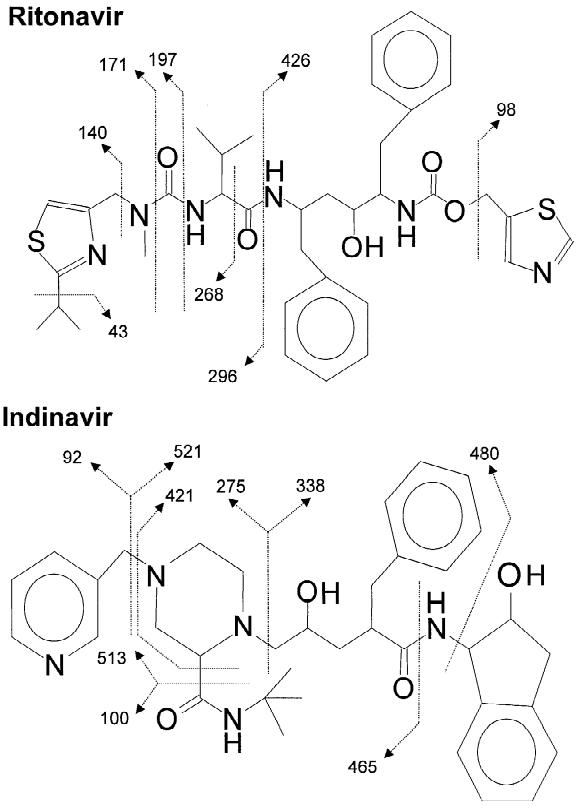

Fig. 1. Structures of ritonavir and indinavir molecules (included in the figure are fragment ions that may be observed under MS–MS and MS^{nth} conditions).

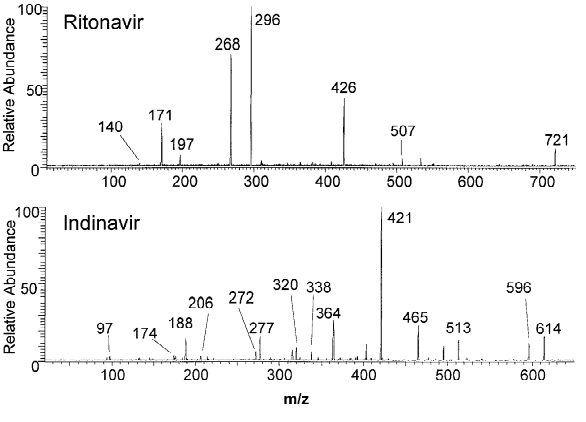

Fig. 2. Product ion spectrum of (A) ritonavir and (B) indinavir generated on a triple quadrupole mass spectrometer.

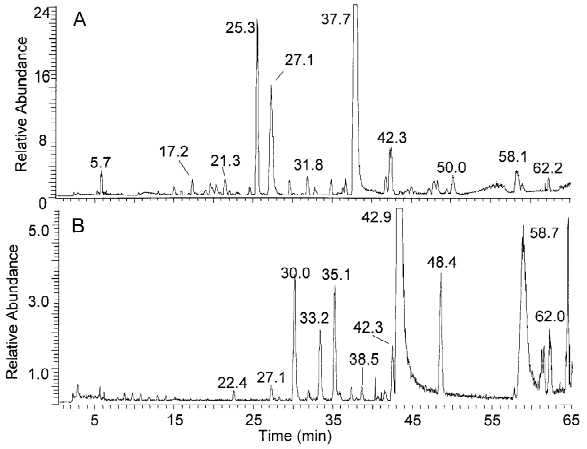

Fig. 3. Full scan base peak ITMS chromatograms for the metabolites of (A) ritonavir and (B) indinavir formed in microsomal incubations.

parent drug (Fig. 3B −42.9 min) and its most intense metabolite peak (30.0 min) at about 4% of the parent drug height. For ritonavir (37.7 min), the most abundant metabolite peak eluted at 25.3 min and its peak area corresponds to about 23% of the parent drug (Fig. 3A). These ratios of parent drug/metabolite(s) are a function of incubation conditions.

The protonated molecule ions ($[M+H]^{+1}$) and changes in observed mass (ΔM) for these proposed metabolites are presented in Table 1. Hydroxylated metabolites predominate for both indinavir (In1, In4, In5, In6, In7 and In8) and ritonavir (R2, R3, R4 and R6). The presence of numerous isobaric metabolites (m/z 630 for indinavir and m/z 737 for ritonavir, Table 1) emphasizes the importance of baseline resolution of chromatographic peaks. Inadequate chromatographic separation of peaks of isobaric metabolites would obscure MS–MS spectra and render accurate structural determinations impossible. The full scan analyses of the data indicate that the metabolites can be divided into two categories: oxidative and hydrolysis products. The metabolism of indinavir gave rise to three metabolites formed as a result of the elimination of functional groups. These losses represent a net change in mass of −132, −91, and −37 Da for metabolites In2, In3,

Table 1
Product ions for (A) ritonavir and its metabolites and (B) indinavir and its metabolites. Also indicated are the values for protonated molecules ($M+H^{+1}$) and changes in observed mass for the metabolites (ΔM)

R	$[M+H]^{+1}$ 721	ΔM	CID ions:									
			551	525	426	296	268	197	171	140	98	43
Ritonavir												
R1	582	−139		525	426	157						
R2	737	+16			426	312	284	213	187	156		59
R3	737	+16			426	312	284	213	187	156		
R4	737	+16	567		442	296	268		171	140	114	
R5	580	−141			285	296	268	197	171	140		
R6	737	+16			442	296	268	197	171	140		
R7	707	−14			426	282	254		157	140		
R8	709	−12			414	296	268	197	171	140		
R9	634	−87			339	296	268	197	171	140		
In	614		521	513	465	421	380	364	338	133	91	
Indinavir												
In1	646	+32		529	481		380		354			
In2	482	−132			465	289			206			
In3	523	−91		422	374				338			
In4	630	+16		529		437			354			
In5	630	+16		529	465	437		364	354			
In6	630	+16			481		380					
In7	630	+16				437		364				
In8	630	+16			481	421		364	338			
In9	577	−37		476	428		343	327	338	133		

and In9, respectively. Similarly, there are five hydrolysis products of ritonavir, R1, R5, R7, R8, and R9, representing changes in mass of −139, −141, −14, −12, and −87 Da, respectively.

After determination of retention time and molecular mass of the proposed metabolites, retention-time dependent MS–MS scans were programmed (for both ITMS and triple quadrupole) to acquire CID data on-line. A comparison of the product ions of the metabolites with those of the parent drugs reveals a net change of +16 Da in many product ions of metabolites (vis-a-vis product ions of the parent drugs, Fig. 2 and Table 1). The characteristic mass shifts in the product ions help to identify both the nature and site of the modifications. For example, the spectrum of the parent drug ritonavir yields fragment ions of *m/z* 296, 268, 197, 171 and 140. The spectrum of metabolite R2 (Fig. 4A), has fragment ions at *m/z* 312, 284, 213, 187, and 156 thus conclusively narrowing the site of modification to the isopropylmethylthiazole moiety. The presence of the *m/z* 426 product ion confirms that the corresponding portion of the ''right'' half of the molecule remained intact.

Complete structural identification of metabolites may be hindered by the absence of useful product ions in the MS–MS spectrum. Thus, pseudo MS^3 mass spectra, via in-source fragmentation of molecular ions, were used for more precise structural identification of metabolites. For example, comparison of the MS–MS spectra of metabolite R2 and ritonavir determined the presence of an oxidation site (hydroxylation) on the isopropylmethylthiazole moiety. The compounds were collected from HPLC and infused into the mass spectrometer for more extensive mass fragmentation and spectral analysis. The product ion generated in-source by increasing the cone voltage (in-source fragmentation) was mass selected in Q1 (*m/z* 296 or 312) and collisionally dissociated in Q2. The resultant product ions were subjected to mass analysis in Q3. The pseudo MS^3 spectrum of the *m/z* 296 ion (from the parent drug) and the *m/z* 312 ion (from metabolite R2) are shown in Fig. 5A,B, respectively. A careful inspection indicates that many of the product ions of the metabolite (R2) have increased by 16 Da (*m/z* 140→156, 197→213, 268→284). In particular, the ion of *m/z* 43 in the parent drug spectrum has been shifted to *m/z* 59 in that of the metabolite. Therefore, in this instance it was possible to further localize the

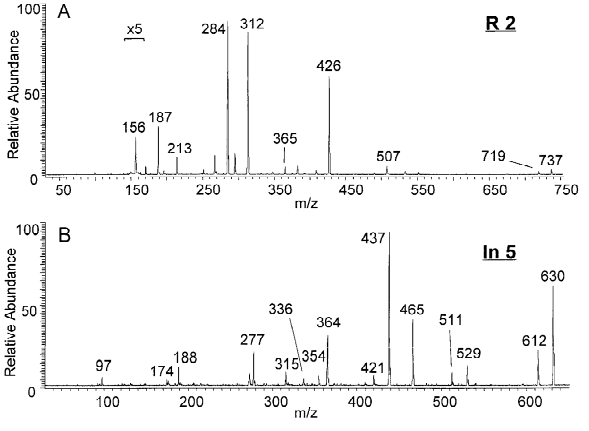

Fig. 4. Product ion spectra of (A) ritonavir metabolite R2 and (B) indinavir metabolite In5.

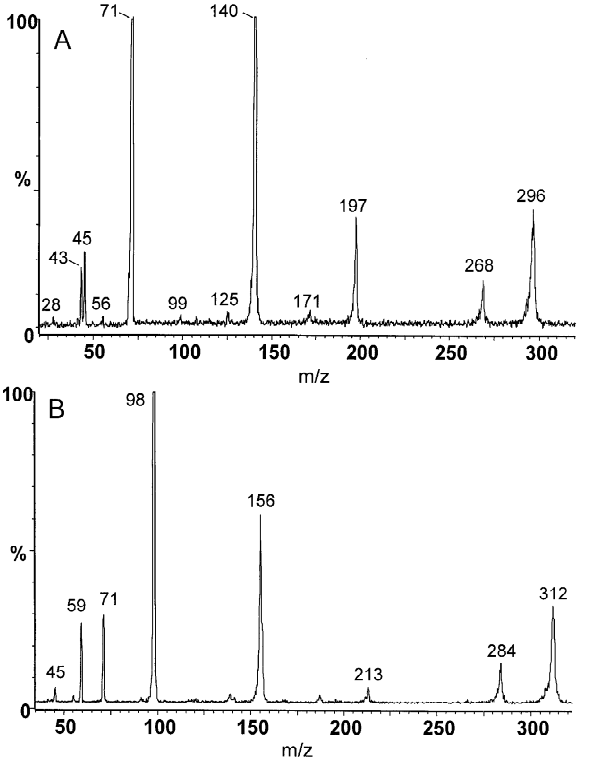

Fig. 5. Pseudo MS^3 product ion spectra for the (A) the ritonavir *m/z* 296 ion and its corresponding (B) R2 metabolite ion at *m/z* 312.

oxidative modification to the isopropyl group attached to the thiazole moiety.

The MS^3 spectra of m/z 296 ion (ritonavir Fig. 5A) and m/z 312 (R1 in Fig. 5B) show some interesting features. Specifically the appearance of m/z 98 in spectrum 5B and the presence of m/z 71 in both spectra should be noted. It would appear that the indicated hydroxylation of the isopropyl group of the side chain enhances the formation of an ion of m/z 98 that is characteristic of the methylthiazole moiety. On the other hand, the m/z 71 ion may be attributed to loss of a neutral 196 fragment from the m/z 268 ion (loss of 213 to from m/z 284 ion in the R1 spectrum). Hence, this ion does not exhibit any mass shift in the spectrum of the metabolite.

Mass spectra of metabolites formed by hydrolysis were examined in a manner similar to those of the oxidation products. The main difference was that the product ion spectra were examined for common mass fragments common to the parent drug and the metabolite (as opposed to increases in masses of fragment ions). Metabolite R1 is an example of hydrolysis in which the isopropylmethylthiazole moiety of ritonavir has been cleaved. This is confirmed by the presence of m/z 525 and m/z 426 product ions in the MS–MS spectrum together with the ion m/z 157. The ion at m/z 157 is analogous to m/z 296 in the parent minus the isopropylmethylthiazole moiety. Similar considerations were employed in the interpretation of the spectra of the other hydrolyzed metabolites. The postulated structural assignments for this and other metabolites of ritonavir are presented in Fig. 6. For reference, relevant product ions of ritonavir and each metabolite are listed in Table 1. The structures of R8 and R9 could not be determined conclusively by mass spectrometry alone, though partial identification was made.

The identification of the metabolites of indinavir was accomplished using procedures similar to those

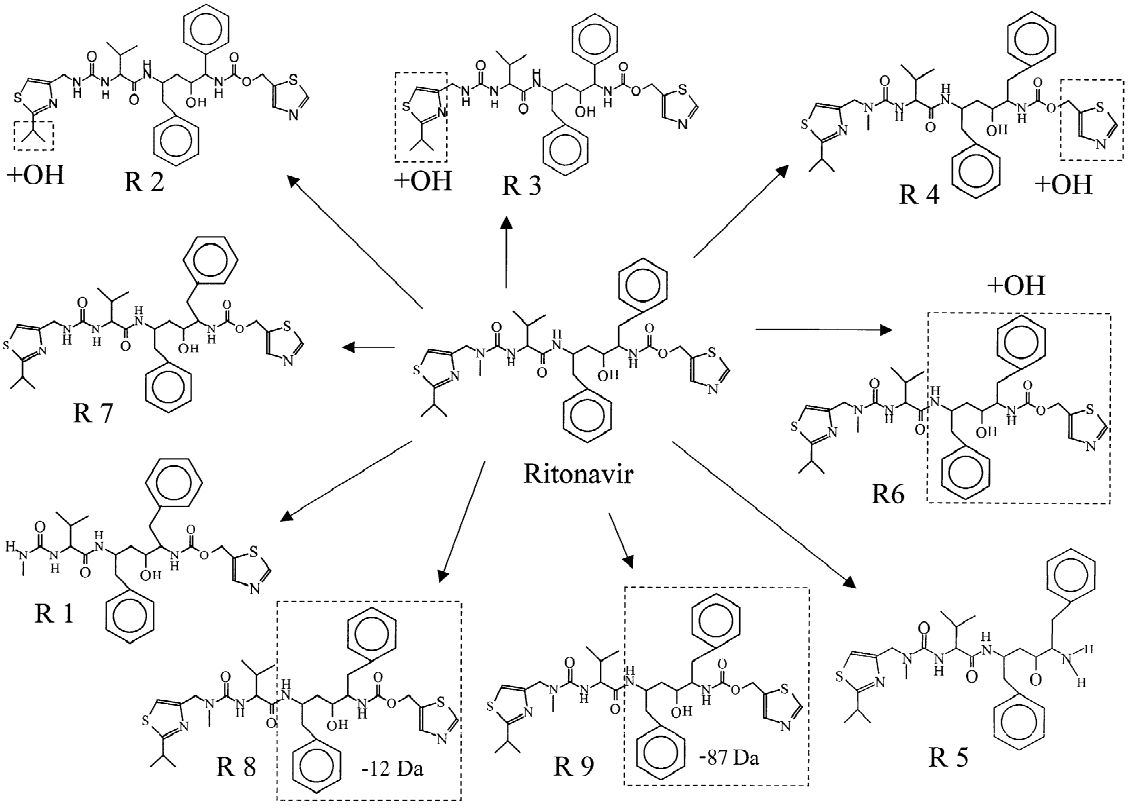

Fig. 6. Structural assignments made for ritonavir metabolites.

described above. The structural elucidation of metabolites of indinavir was more difficult than for ritonavir because the patterns of fragmentation were more complex due to product ions that result from multiple (≥ 2) bond cleavages. For example, the m/z 421 ion (Fig. 4B) is formed by cleavage of both the trimethylamide and methylpyridine moieties. However, once fully clarified, such multiple bond cleavages significantly assisted in establishing more precisely the site and nature of metabolic modifications on the parent indinavir molecule.

Many spectra, however, were very informative and permitted at least partial identification of individual metabolites. For example, In5 has a protonated mass of m/z 630. The MS–MS spectrum of this metabolite compared to the MS–MS of indinavir reveals that oxidation occurred on the indanyl moiety. This assignment can be confirmed from the presence of several different and relevant product ions (Fig. 4B). The presence of the ion at m/z 465 indicates that this portion of the molecule is unchanged while ions at m/z 529, 354, 437, 364 shows definitively that the oxidation is on the indanyl portion of the molecule. The structural assignments for indinavir metabolites are given in Fig. 7 and are based on the observed masses in Table 1. In previous work carried out in our laboratory, we reported that as many as three multiple phase I hydroxylations can occur simultaneously on indinavir, leading to the formation of more polar trihydroxylated metabolites [7]. However, as noted earlier, since the present study focused on the MS/MS characteristics of the less polar CH_2Cl_2-extractable compounds, the former metabolites are not considered in this report.

3.1. Comparison of QQQ versus ITMS

The major goal of this project was to detect and characterize the microsomal metabolites of ritonavir and indinavir. This required examination and selec-

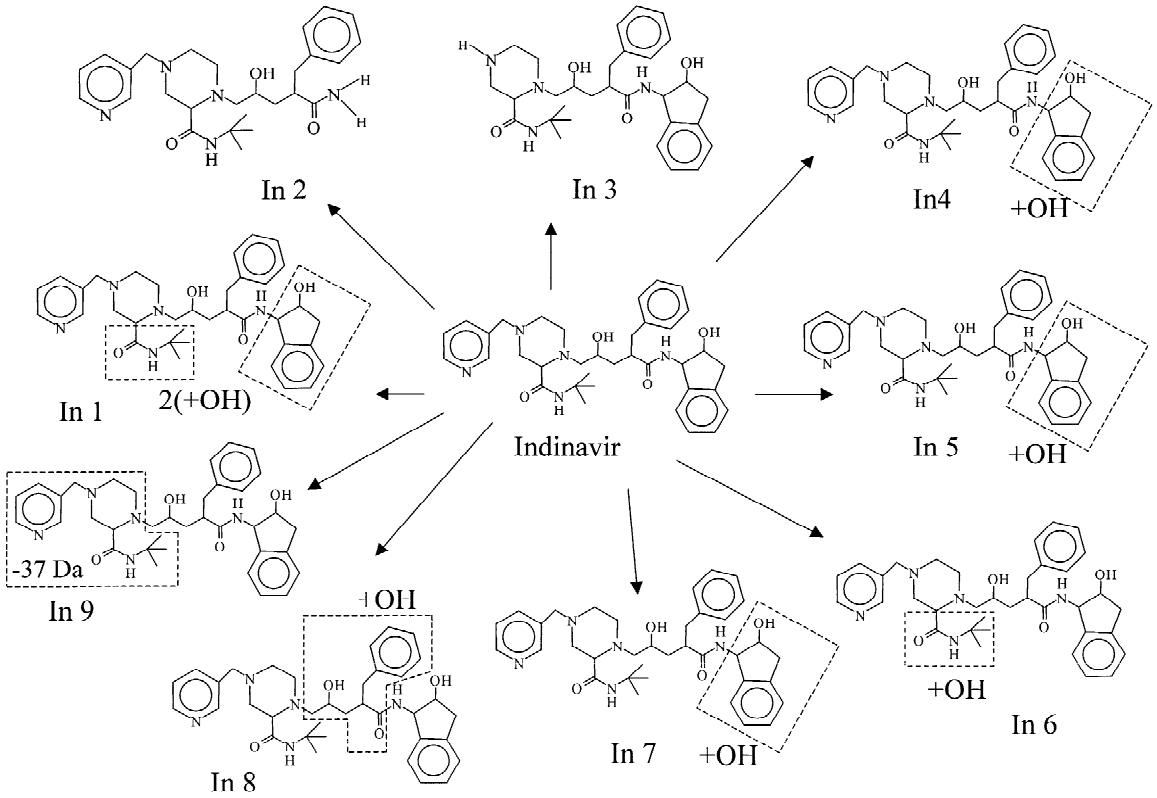

Fig. 7. Structural assignments made for indinavir metabolites.

tion of the most appropriate LC–MS instrumentation and methods. Up to this point we have deliberately shown full scan chromatographic data from the ITMS and only product ion spectra from the triple quadrupole. Prior to the introduction of ion trap MS, both quantitative and qualitative metabolic studies had been conducted on triple quadrupole mass spectrometer. The following data illustrate an example where triple quadrupole MS is not necessarily the most suitable analyzer for the application. The results demonstrate the specific advantages/disadvantages of ITMS and triple quadrupole mass spectrometers and show that neither method possesses general superiority over the other.

Fig. 8 shows a comparison of extracted ion chromatograms of m/z 646 and 630 from a triple quadrupole and an ITMS. Both chromatograms show injections of 15 μl of metabolite solution onto virtually identical LC–MS systems (the only difference being in the mass analyzers themselves). Using software correction features, the triple quadrupole chromatogram has been reformatted to allow for alignment of peaks between the mass chromatograms. Note that the peak widths and shapes are comparable suggesting similar chromatographic performance between the two HPLC systems. Hence, the following conclusions regarding MS sensitivity were drawn.

Examination of Fig. 8 shows significantly different signal/noise ratios of peaks in the ITMS and triple quadrupole chromatograms. Metabolites In1 and In7 are barely visible as real peaks in the triple quadrupole chromatogram and would have been overlooked by automated and/or manual detection methods. In contrast, the ITMS chromatogram renders these peaks more easily visible and readily identifiable as genuine metabolites requiring further investigation by MS–MS. These differences in signal/noise ratios can be explained by the disparity in duty cycle between the two types of mass analyzers [19]. When scanning over a wide mass range, a beam instrument like the triple quadrupole is only capable of detecting a small fraction (<1%) of the overall ions entering the MS. On the other hand, trapping instruments like the ITMS have increased sampling efficiency when acquiring full scan mass spectra. Indeed, similar reports on differences in instrument sensitivity have been reported elsewhere [20].

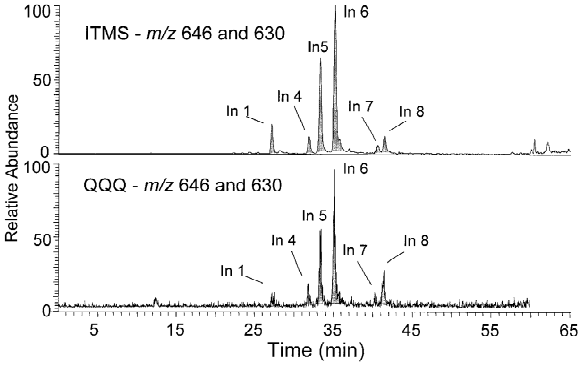

Fig. 8. Extracted ion chromatograms for hydroxylated metabolites of indinavir (m/z 646 and 630) as obtained by ion trap (IT) MS and triple quadrupole (QQQ) MS. (QQQ chromatogram adjusted 5 min for peak alignment.)

The above example serves to emphasize the use of a trapping instrument when scanning over a wide mass range. In fact, in the above example the ion trap was actually scanned over a wider mass range (specifically 300 Da wider range) than the triple quadrupole. Sole reliance on the triple quadrupole data for detection would have resulted in failure to detect many metabolites. Not unexpectedly, similar trends were also observed in the base peak chromatograms of these two instruments. It is noteworthy that the intensities of the base peaks from the ITMS (Fig. 3) are significantly greater than corresponding peaks from the triple quadrupole (Fig. 9). Regardless of whether automated or manual peak detection methods are used, this example demonstrates that the ion trap is more sensitive in full scan MS mode [19].

The scanning properties of the triple quadrupole can also have a negative impact on data-dependent experiments for acquisition of MS–MS spectra. In full scan mode, analytes may escape MS–MS detection because their signal strengths are below preprogrammed threshold intensities (i.e., below programmed signal/noise ratios). For such an application it is critical to select mass spectrometers suited for full scan analysis. When using full scan mass spectra to direct data-dependent experiments, a mass analyzer with high duty cycle characteristics over a wide mass range should be used, e.g., time of flight or ion trap MS. These types of mass analyzers can mimic the sensitivity of a quadrupole instrument in SIM mode while still acquiring full scan mass

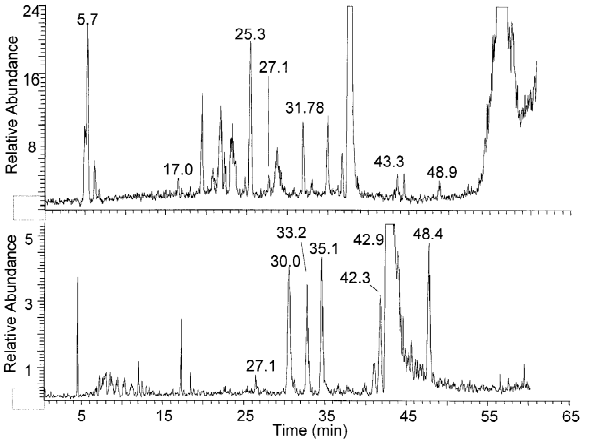

Fig. 9. Full scan base peak QQQ chromatograms for the metabolites of (A) ritonavir and (B) indinavir formed in microsomal incubations. (QQQ chromatogram adjusted 5 min for peak alignment.)

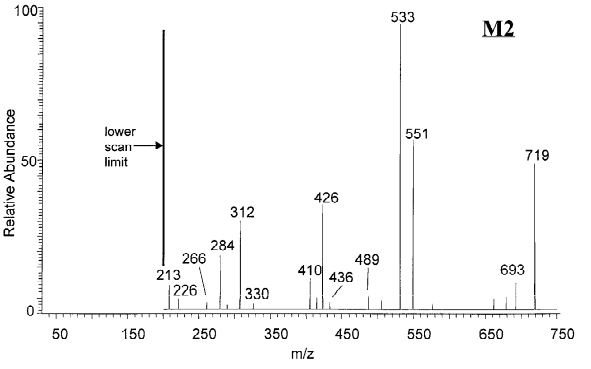

Fig. 10. Product ion spectrum of metabolite R2 obtained on an ion trap mass spectrometer.

spectra. Therefore, informative full scan mass spectra can be retrieved without significantly sacrificing sensitivity. On the other hand, the scanning properties of the triple quadrupole can also be extremely advantageous. Constant neutral loss or parent ion scan detection methods can filter out unwanted chemical noise arising from matrix components so that only analytes (potentially) related to the parent compound are detected. These types of MS–MS features unique to the triple quadrupole produce specific and sensitive results not available on an ion trap.

Dissimilarity in full scan MS capabilities was not the only practical difference between ITMS and triple quadrupole data that arose during this work. Fig. 10 shows the product ion spectrum of metabolite R2 obtained by ITMS. Here many of the product ions previously noted by triple quadrupole MS–MS (Fig. 4A) are present. Most importantly, this spectrum contains the relevant product ions necessary for structure elucidation of R2: m/z 426, 312, 284, and 213. However, fundamental limitations in the function of the specific ion trap used in this work do not permit detection of product ions with masses less than 1/3 of the parent ion. Thus, the highly informative 156 ion in the QQQ spectrum, which revealed the modification on the isopropylmethylthiazole group is missing from the ITMS spectrum. Additional MS^3 experiments on the m/z 312 ion would have been necessary to reveal the presence of the meaningful m/z 156 ion. This example demonstrates that under certain conditions of pseudo MS^3, the triple quadrupole MS can be more informative than MS^3 on the ITMS. This is especially true when the masses of relevant product ions are less than 1/3 of the parent ion. More specifically, the inherent inability of our ITMS system to scan below m/z 50 would have precluded our ability to pinpoint the hydroxylation of R2 on the isopropyl moiety of ritonavir (Fig. 5, shift of m/z 43→m/z 59). Normally however, MS^3 on an ITMS system would be the preferred approach for MS^3 analysis because of its higher sensitivity compared to pseudo MS^3 on a triple quadrupole.

The utility of MS^n on an ITMS is well documented for a variety of applications, including structural analyses of biopolymers of DNA, proteins, and oligosaccharides [21–23]. Our work demonstrates some of the limitations in MS–MS capabilities of ion trapping instrumentation. It is useful to recognize that ITMS is not always more, or less, suitable for MS–MS experiments than triple quadrupole mass spectrometry. The examples given here have illustrated that the triple quadrupole MS is not well suited for full scan MS analysis due to lack of sensitivity. This is particularly true when scanning at unit mass resolution over an extended mass range (e.g., >800 Da). However, it is widely accepted that considerable variation in sensitivity exists not only among different instrument models but also among different manufacturers of mass spectrometers. Our results should not be interpreted as universally applicable to all classes of compounds and struc-

tures. Rather, different mass analyzers should be used to complement each other and to take advantage of specific properties of individual MS instruments.

4. Conclusions

An LC–MS(–MS) method for the detection and qualitative identification of human microsomal metabolites of the protease inhibitors, ritonavir and indinavir, is presented. Microsomal incubations further evaluated by LC–MS(–MS) revealed extensive biotransformation and the detection of nine metabolites for each drug. The method provided chromatographic resolution for each isobaric species thereby permitting collection of unambiguous MS–MS spectra. MS–MS spectra allowed for specific structural determinations of hydrolytic products of metabolism. Although the structure of oxidative metabolites could not be precisely determined in all instances, MS–MS spectra provided enough information to pinpoint modifications to a confined area of the molecule. Further investigation of these metabolites by NMR will aid in determining the exact locations of these modifications.

Qualitative comparisons between an ion trap and triple quadruple mass spectrometers highlighted differences in the quality and abundance of spectral information derived from each instrument. Not surprisingly, ITMS proved to have superior sensitivity in full scan mode, better signal-to-noise ratios, and consequently improved capacity to detect analytes. Although not a problem encountered in our work, the type of mass analyzer used may negatively impact automated- or signal-dependent scanning procedures. The triple quadrupole provided the most structurally informative data at similar stages of MS–MS data acquisition. In addition, the most precise structural determinations used pseudo MS^3 scanning procedures on the triple quadrupole. However, without complementary use of the ion trap and triple quadrupole, metabolites of both drugs would have been overlooked and/or incompletely identified. These data suggest that neither triple quadrupole nor ITMS should be used exclusively for detection and identification of metabolites by MS. Instead, the systems should be regarded as synergistic and when used together, offer significant advantages.

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