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Revisiting the Metabolism of Ketoconazole Using Accurate Mass

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Abstract: The microsomal metabolism of ketoconazole is revisited using accurate mass LC/MS^n and deuterium labelling. Structures for sixteen metabolites are proposed from rat and human microsomal metabolism of commercial ketoconazole. Thirteen of the proposed structures are well determined and consistent with all data; five of the proposed structures are less certain. Ten of the metabolites are described for the first time. Reaction phenotyping shows that most of the metabolites arise from CYP3A4, the enzyme known to be well inhibited by ketoconazole.

Keywords: Ketoconazole, metabolism, mass spectrometry, deuterium labeling.

1. INTRODUCTION

While investigating the *in vitro* metabolism of an imidazole-containing drug [1] we noticed that the microsomal metabolism of drugs containing this functional group is surprisingly poorly studied given their importance in medicine and as inhibitors of cytochrome P450's. We chose to revisit the microsomal metabolism of ketoconazole as a representative imidazole drug. Ketoconazole is important in commerce as an antifungal [2], is routinely used in the metabolism laboratory as a specific inhibitor of cytochrome P450 3A4 and is the substrate of choice in clinical cytochrome P450 3A4 drug drug interaction studies [3]. Ketoconazole cellular biology continues to be actively studied [4].

Ketoconazole has a long history of use in the lab and the clinic, but a poorly described metabolism. Whitehouse *et al.* [5] describe the isolation and characterization of 9 ketoconazole metabolites from mouse liver after oral dosing. Their major metabolite was shown to be the de N-acetyl metabolite. Their other structures involve piperazine and/or imidazole oxidation. Two of the imidazole oxidation structures drawn in the Whitehouse report were redrawn in a review article [6]. The further metabolism of de N-acetyl ketoconazole by FMO has been described [7]. We describe the identification of human and rat microsomal metabolites of ketoconazole using the modern techniques of accurate mass spectrometry and deuterium exchange.

2. MATERIALS AND METHODS

Ketoconazole was purchased from Sigma-Aldrich (St Louis, MO). Pooled human liver microsomes and male rat liver microsomes were purchased from BD Biosciences (Woburn,MA). Ketoconazole N-oxide was synthesized from ketoconazole by treatment of a 1 mg/ml solution in methanol with a ½ volume of 30% H_2O_2 and sitting at room temperature for 2 days. All other chemicals were purchased from commercial sources and were of the highest purity available. Generic LC/MS/MS and deuterium exchange methods on the Thermo Fisher LTQ Orbitrap, and reaction phenotyping will be published in full in an article in preparation [1].

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Microsomal incubations were conducted at 37◦C in a water bath. Test compounds (20 uM final concentration; predissolved in DMSO at 10 mM)) were mixed with human liver microsomal proteins (1 mg/ml) in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 1 mM GSH (or KCN) and 5 mM $MgCl_2$. The total incubation volume was 1 ml. After 5 min preincubation at 37◦C, the incubation reactions were initiated by the addition of 2 mM NADPH. Reactions were quenched by the addition of 1 mL ice cold acetonitrile after 60 min incubation. Negative control samples contained NADPH with test compound added with the acetonitrile quench.

3. RESULTS AND DISCUSSION

3.1. Product Ion Mass Spectrum of Ketoconazole

The CID of ketoconazole was studied with infusing the compound into the LTQ Orbitrap ion source. The product ion mass spectrum of ketoconazole protonated molecule m/z 531 was also described in Whitehouse *et al.* [5]. In Table **1** we list the m/z values for many of the most important fragment ions, the ppm error and their MS^n relationships as measured on the LTQ Orbitrap. Fig. (**1**) shows the structure of ketoconazole and the key fragmentation pathways along with the MS/MS spectrum. Fragmentation pathway a yielding a neutral ketene and m/z 489 product ion dominates; all of the other fragmentations are observed at much lower levels. Ion b, the fragmentation of the imidazole ring is not observed in the CID of ketoconazole but is included as a pathway for its importance in several metabolites. Fragmentation of the ketal gives the important ion m at m/z 255 with charge retention on the imidazole. Charge retention on piperazine (ion k) is quite insignificant in ketoconazole CID but is observed in metabolites without the basic imidazole. Several minor ions are derived via secondary loss of neutral imidazole from primary ions; in this way ions e, h and o are formed from ions a, d and m, respectively. Ion p, unimportant in CID of protonated ketoconazole becomes more significant in subsequent MS^3 spectra and in spectra of metabolites and has the composition of dichlorobenzyl ion formed via loss of CO from ion o.

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Table 1. Accurate Mass MS^n Data for Ketoconazole

Fragment (Fig. 1)	m/z found	ppm error	Relative abundance in MS^n product ion spectrum xxx=>50% relative abundance; xx=>5% relative abundance; x=detectable					
			531>	531>489	531>446	531>311	531>255	531>255>187>
	531.1572	1.1						
a	489.1454	1.2	xxx					
b	480.1465							
c	463.1184	1.5	x					
d	446.1034	0.9	x	xxx				
e	421.1083	0.7	x	xx				
f	420.0871	2.6	x	xx				
g	418.0715	2.4	x	x	xx			
h	378.0655	2.4	x	x	xx			
i	329.0454	1.8	x	x	x			
j	311.0351	1.0	x	xx	x			
k	277.1546	2.2	x					
l	259.144	2.7	x					
m	255.0088	1.6	xx	xxx	xxx	xx		
n	244.0054	1.6	xx	xx	xx			
o	186.9706	5.9	x			xx	xxx	
p	158.9757	6.9	x		xx	xxx	xxx	xxx

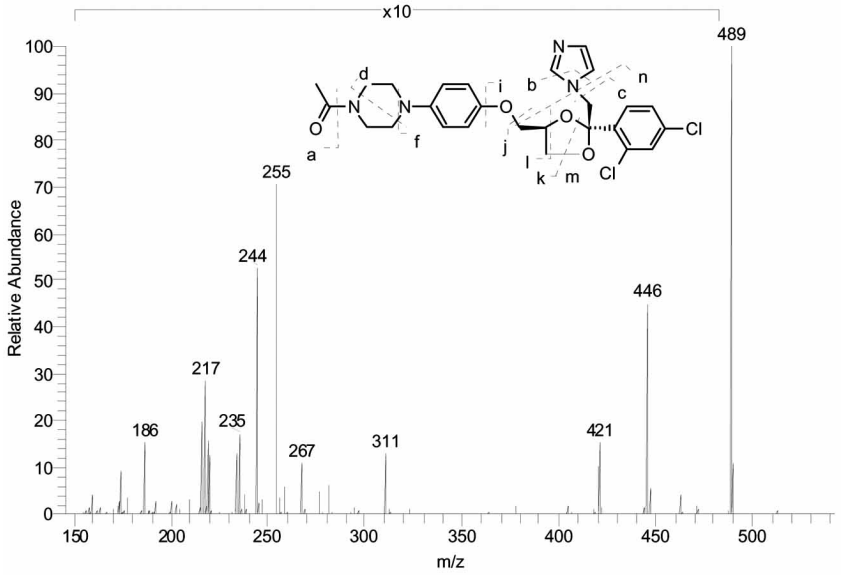
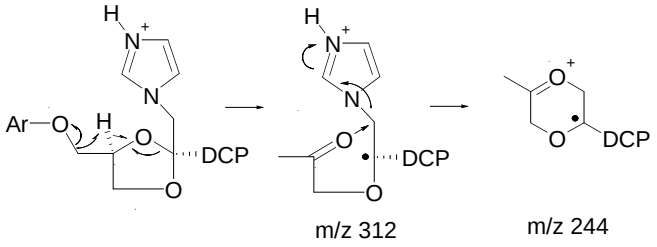


Fig. (1). Product ion spectrum of protonated ketoconazole.

Product ion n is unusual in that it is odd electron. The accurate mass of ion n is consistent with the structure formed via loss of imidazole radical from ion j; however MS^3 on ion j (531>311>) does not yield ion n. Alternatively ion n could be formed via initial loss of neutral imidazole to give m/z 463, ion c; again an MS^3 experiment (data not shown) does

not support this. Rather, ion n is formed via an initial homolytic cleavage of the ether bond j to yield a phenoxy radical and a minor (but observed with correct accurate mass) m/z 312 ion. Subsequent loss of neutral imidazole from this ion yields the radical species n drawn as a stabilized oxonium ion, benzyl radical in (Fig. **2**) and first proposed by Miao [8]. This sequence was confirmed by a specific MS^3 (531>312>) experiment. Upon MS^4 (531>489>244>) ion n loses neutral C_3H_6O to give the radical m/z 186 or the neutral radical C_4H_7O to give the dichlorobenzoyl cation, m/z 173.



DCP=2,4-dichlorophenyl

Fig. (2). Proposed fragmentation pathway n.

3.2. Ketoconazole Metabolism in Liver Microsomes

The metabolism of ketoconazole was studied in 8 incubations, rat and human liver microsomes with added cyanide versus added glutathione at time zero and 1 hr. A standard generic acquisition method was used to generate the raw data [1]. This method generates accurate full scan mass spectra as well as accurate MS/MS spectra for the two largest ions in the full scan and low resolution spectra for the two corresponding MS^3 scans. Obtaining data dependent CID data for the two biggest peaks was particularly important for ketoconazole and its metabolites many of which give base peak, doubly charged ions. The LTQ-Orbitrap method also generates low resolution negative ion full scan, MS^2 and MS^3 data, but the negative mode data was not useful in this case. Metabolites were identified by manual comparison of control and treated UV, base peak and extracted ion chromatograms. The metabolites are numbered arbitrarily to put the most important HLM metabolites first. Table **2** lists each metabolite along with its found protonated accurate mass, the ppm error against the postulated structure, the retention time in the chromatograms, the rough prevalence in the HLM and RLM incubations, the MS/MS and MS^3 data and the number of D_2O exchangeable protons. The average mass accuracy for the 16 identified metabolites and 2 impurities was 1.4 ppm. One of the nice features of the LTQ Orbitrap is that much of the mass accuracy error is systematic not random. This drift can be corrected by using a known internal calibrant. If we use the known accurate mass of protonated ketoconazole, the corrected mass accuracies of the metabolites have only an average 0.6 ppm error.

Ketconazole is quite stable to microsomal metabolism. Fig. (**3**) shows the LC/MS chromatogram of the HLM incubation. Of the metabolites, only M2 is readily visible in the MS or UV chromatogram. The postulated structures of the metabolites are shown in (Figs. **4** and **5**) with dotted lines depicting the bonds which would cleave to yield the base peak in their CID. The metabolic reactions can be classified as 1) imidazole cleavages, 2) piperazine oxidations with or without amide cleavage, 3) aromatic oxidation and 4) oxidation or reduction of minor impurities.

M2, previously described as Compound X [5], is the major metabolite in human and rat liver microsomes and formally is formed by addition of 34 Da. The literature product ion spectrum of Compound X is clearly comparable to our M2(but notice that the authors of reference [5] have switched the spectra and structures in their figures relative to the descriptions in the tables and text; we use the designations from the text). Both spectra show a decrease in the loss of ketene (ion a) and complete loss of the typical fragmentations j and m with replacements of ions b and c and k and a base peak due to loss of CO. Whitehead *et al.* surmised this changed CID was due to metabolism of the imidazole ring and drew the structure of Compound X as shown in Fig. (**4a**). But these authors saw major ^1H NMR chemical shifts of the central aromatic ring protons which they ascribed to an N-oxide of the piperazine. A later publication [6] redrew this structure as Compound 198 (comparable to known imidazole oxidation metabolites [9-11]), but this structure has one too many oxygens. To our thinking the presence of intact fragments b, c and k are clear indications that the piperazine is intact and both extra oxygens of M2 are in the imidazole part. The base peak due to loss of CO is diagnostic for N-formyl structures [10]. The strength of the CID fragment b is best justified as cleavage of an amide bond, and the long retention time of M2 plus the lack of doubly charged species in its MS are consistent with the loss of the basic nitrogen. For these reasons we draw ketoconazole's main metabolite as M2 (Fig. **4b**). The presence of 3 deuterium exchangeable protons is consistent with structure M2.

Several other important microsomal metabolites M3, M4, and M5 are also due to imidazole oxidation. M5, previously described as Compound IX [5] has a 32 Da increase in MW. Its product ion spectrum is dominated by fragment k indicating the intact arylpiperazine not the N-oxide drawn previously. Thus both oxygens must be on the imidazole. M5 is our tentative structure for this metabolite; M5 and the alternative M5' (Fig. **4b**) are both consistent with the observed 2 exchangeable protons. M3 has lost the two carbon piece of the imidazole generating a net -24 Da metabolite, a common imidazole metabolite [9,11,12]. The product ion spectrum of M3 shows charge retention on both sides of a novel cleavage between the ketal oxygens and the quaternary carbon to give m/z 213 and m/z 295; this cleavage is likely stabilized via 5 member ring formation with the amidine. M3 also shows 3 exchangeable protons. Finally M4 has lost all of the imidazole carbons to give a molecule whose product ion spectrum at MH^+=480 is identical to the MS^3 of the ion derived from M2. The presence of 3 exchangeable protons supports structure M4. A reasonable scheme for formation of the imidazole oxidation metabolites is shown as (Fig. **4b**) using ideas from the review [6]. The scheme is initiated with oxidation of the C-C double bond to give an epoxide A which might readily rearrange to B. No mono-oxidized imidazoles were detected in this study although they are described for other imidazoles [9-12]. Hydrolysis of A and B could give C and D. C should tautomerize to M2 while D should tautomerize to glyoxal and M3. Further oxidation of M2 (or D) could yield M5 and M4 could come from hydrolysis of M3.

Table 2. Ketoconazole Metabolites Identified

Literature name and metabolic transformation	M	accurate mass			Abundance		MS/MS confirmatory ions listed by relative abundance	MS^3 base peak	D_2O exchange result
		found	ppm error	RT	RLM	HLM	Nominal mass (assignment, ppm error)		
ketoconazole	P	531.1572	1.1	13.7					1
Cyanide adduct[15] (P+CN-H)	M1	556.1520	0.4	14.4	xx	xx	529(MH^+-HCN,0.6)	487(529-ketene)	No data
Compound X[5] (P+H_2O_2)	M2	565.1630	1.6	15.5	xx	xx	537(MH^+-CO,1.9), 480(b,1.7),277(k,1.4),463(c,2.2)	495(537-ketene)	3
(P-C_2)	M3	507.1577	2.2	13.3	xx	x	213(see text,0.5),295(see text,0.4), 463(c,0.6),480(b,0.4)	151(213-HCN-Cl)	3
(P-C_3NH)	M4	480.1467	2.1	12.9	xx	x	463(c,0.9), 277(k,0.4)	421(463-ketene)	3
Compound IX[5] (P+O_2)	M5	563.1469	0.9	16.2	x	x	277(k,0.4),234(k-C_2H_5N,0.4)	234	2
(P+O_2)	M6	563.1476	2.1	11.0	x	x	435(a-$C_4H_{10}N_2$,0.5),519(MH^+-CO_2,0.4), 311(j,0.3), 521(a,0.2),255(m,0.0)	407(435-CO)	No data
Compound V[5] (P+O-C_2H_6)	M7	517.1054	1.5	13.1	x	x	245(l,0.4), 255(m,0.4)	217(l-CO)	2
(P+O)	M8	547.1519	0.7	13.4	x	x	505(a,2.2),255(m,2.7),329(i,2.1)	462(d+16)	2
(P+O)	M9	547.1521	1.1	13.6	x	x	529(MH^+-H_2O,0.9)	487(529-ketene)	2
(P-C_2H_2)	M10	505.1415	1.2	12.0	x	x	487(MH^+-H_2O,0.0),420(f,0.2) 463(a,0.1)	446(d)	3
(I2+H_2)	M11	257.0251	1.2	10.3	x	x	189(c,2.6)	153(189-HCl)	2
(P+O-H_2)	M12	545.1362	0.6	12.9	x	x	503(a,2.6),527(MH^+-H_2O,2.8),515(MH^+-CH_2O,3.5), 255(m,3.1)	475(a-CH_2O)	1
(P+O)	M13	547.1526	2.0	11.1	x	x	505(a,1.4),530(MH^+-OH,1.5),471(530-C_2H_5NO,1.5),255(m,1.6),529(MH^+-H_2O,1.5)	462(d)	1
Compound II[5] (P-C_2H_2O)	M14	489.1465	1.0	11.3		x	255(m,0.4),446(d,0.7)	187(o)	2
Compound VIII[5] (P-C_6H_9N)	M15	420.0889	1.7	11.0	x		255(m,0.0),244(n,0.4),148(l,4.1)	187(o)	3
(I1-H_2)	M16	343.0256	1.2	11.6	x		159(p,3.1),275(c,1.1),255(m,0.8)	No data	2
Impurity 1	I1	329.0467	2.1	11.3			159(p,2.5),255(m,0.4)	No data	2
Impurity 2	I2	255.0093	0.4	10.7			159(p,3.1),187(o,2.1)	No data	No data

Abundance: xx EIC peak height exceeds 1% of parent drug; x peak height <1% but detectable.

The *in vivo* metabolism of ketoconazole is dominated by amide cleavage [5,7,13,14], but in these microsome incubations, M14 is a minor metabolite readily identified in that its product ion spectrum matches the MS^3 (531>489>) of ketoconazole protonated molecule and it shows 2 exchangeable protons. M14 was compound II in Whitehouse *et al.* [5,14]. Its further oxidation products [7] have been implicated in the cytotoxicity of ketoconazole.

The piperazine N-oxide of ketoconazole has not been previously reported but we prepared it simply by treatment at room temperature of a methanol solution of ketoconazole with 30% H_2O_2. After 2 days, the mixture was 2/3 N-oxide and 1/3 starting material. Structure proof by mass spectrometry was unequivocal showing ions a and d moved by 16 Da and m unchanged. There were also two radical ions at m/z 530 due to loss of hydroxyl radical and m/z 471 due to subsequent loss of acetamide; such odd electron species are

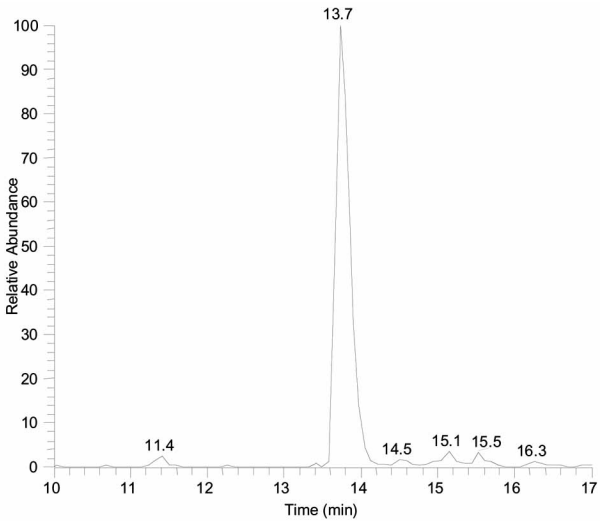
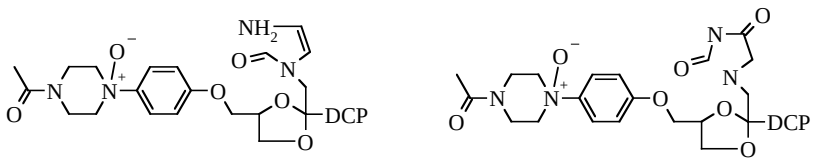


Fig. (3). LC/base peak MS chromatogram for the HLM 1 hr sample.

common for anilines but would not be preferred pathways for any other possible structure for this N-oxide. This N-oxide was also detected (identical in all respects) as a minor HLM and RLM metabolite of ketoconazole, M13. M13 has 1 exchangeable proton.

Argoti *et al.* [15] describe a cyanide adduct of a piperazine iminium species. We also observed this metabolite as M1, the major HLM metabolite, only when potassium cyanide was included in the incubation. The product ion spectrum of M1 shows exclusively loss of HCN. In MS^3 a further loss of ketene is observed. The monohydroxy cyanide adduct previously described [15,16] was observed at a low level but spectra were not obtained. Without cyanide present microsomal metabolism presents M9 (with 2 exchangeable protons) as a very minor hydroxylation metabolite which loses water substantially in source during electrospray ionization to give a species whose CID matches that of the iminium ion derived from the cyano adduct M1. Synthetic M13 also displays a small fragment ion at m/z 529 (of correct accurate mass) corresponding to water loss from the proto-

a. Literature proposed structures for M2



Compound X Compound 198

b. Proposed pathway for ketoconazole imidazole oxidation

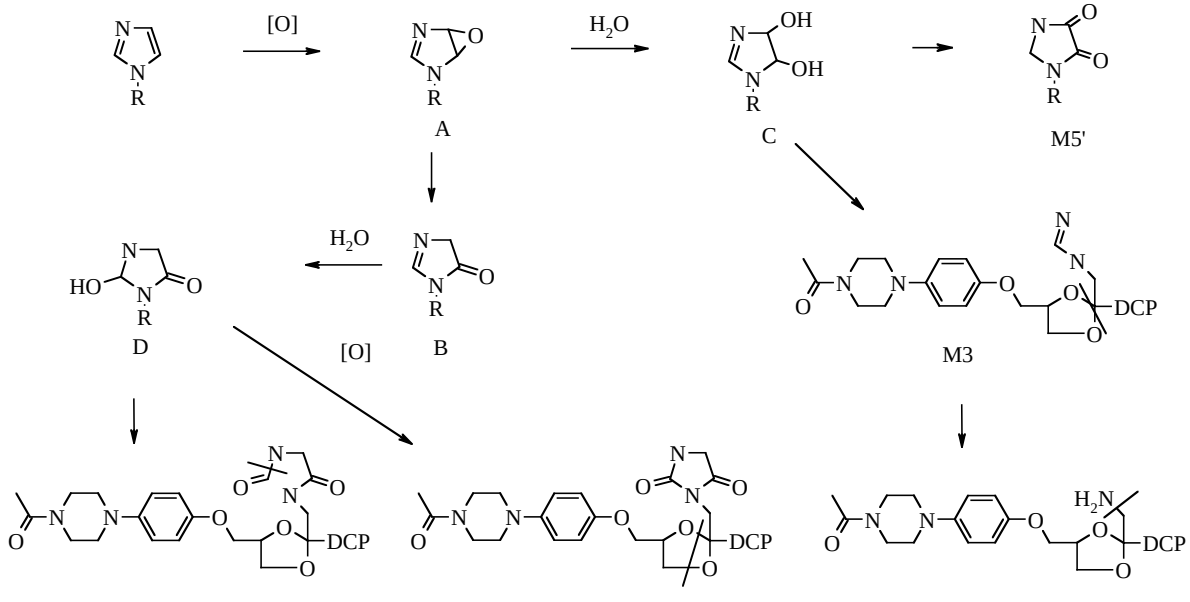


Fig. (4). Metabolic oxidation of the ketoconazole imidazole moiety.

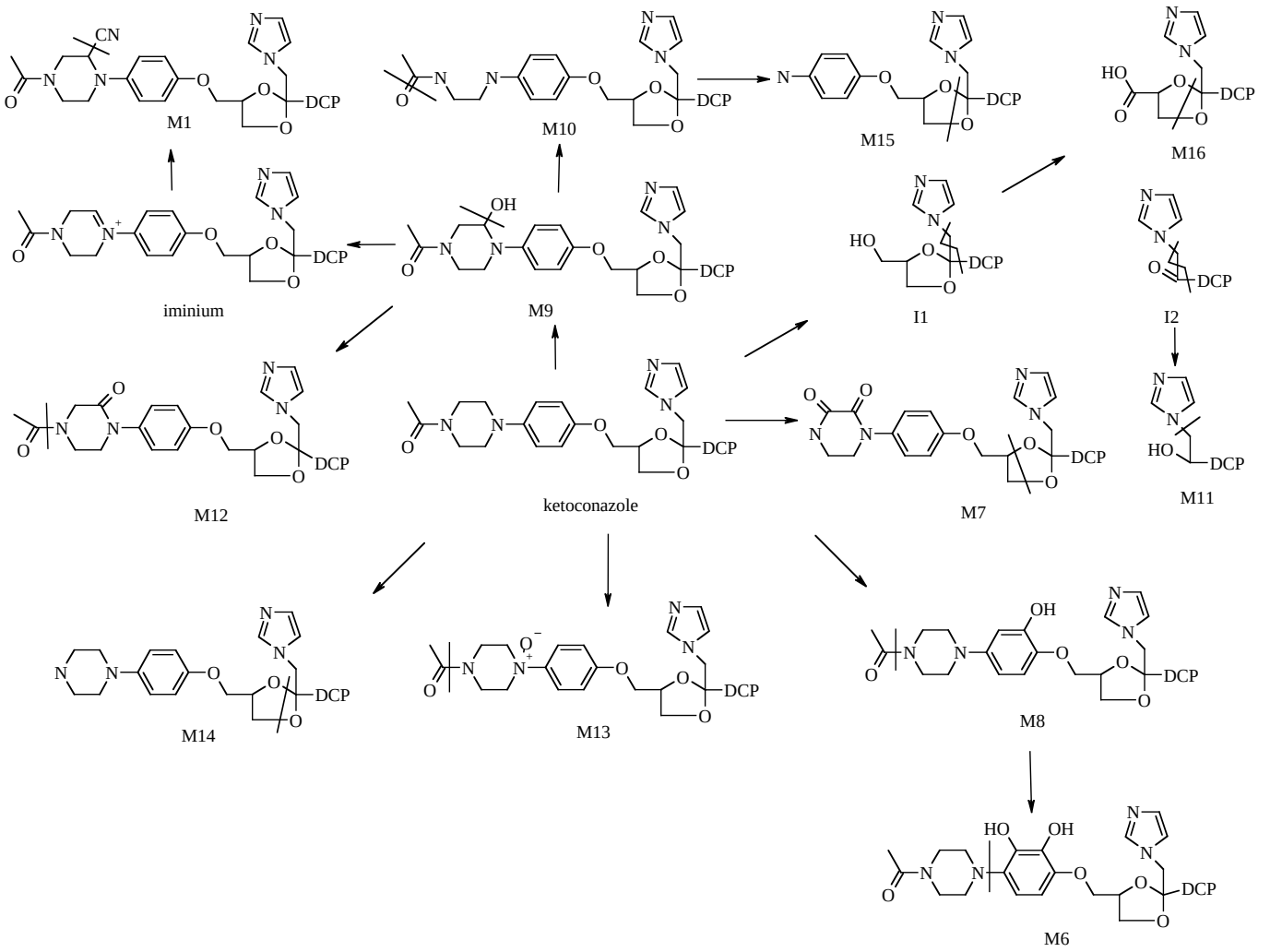


Fig. (5). Other ketoconazole microsomal metabolites.

nated molecule. The subsequent MS^3 of this ion (547>529>) is identical both to the MS^3 (556>529>) of cyanide M1 and the MS^3 (547>529>) of 2-hydroxypiperazine M9. This result proves the structure of M1 and M9. We did not observe the "iminium" compound or its 2,3-dehydropiperazine tautomer [17] as metabolites in this work. The latter is described as an impurity of ketoconazole [18].

M12 is a very minor +14 Da metabolite. Its product ion spectrum shows loss of ketene and/or formaldehyde and also shows ion m. M12 has 1 deuterium exchangeable proton. It is drawn as the lactam [19] but this structure is not consistent with the formaldehyde loss.

M10 is a minor metabolite with loss of two carbons from the piperazine. In CID protonated M10 loses water or ketene and shows major ion f and a small m. The importance of the water loss in CID may be best explained by the tautomeric cyclic aminol structure. The increase in deuterium exchangeable protons to 3 is strong support to this structure. M15, previously compound VIII [5], is a minor aniline metabolite formed via complete piperazine oxidation. M15 corresponds to ketoconazole CID fragment f, gives m and n and an ion m/z 148 corresponding to fragmentation l on CID. The increase in deuterium exchangeable protons to 3 is also strong support to this structure.

M7 is a minor metabolite likely the same as compound V from the earlier work [5]. Ion m and the ion m/z 245 corresponding to cleavage l are consistent with the 2,3-diketopiperazine structure drawn by Whitehouse *et al.* [5] as is the presence of 2 exchangeable protons but other structures are possible. Whitehouse *et al.* [5] also report an isomer of compound V, called IV, and identified as the N-formyl analog of ketoconazole. We also observed this compound as a minor chromatographic peak but it appeared to be equally present in treated and control incubations and may be an artifact of transamidation with formic acid containing mobile phase.

Two metabolites are derived from oxidation of the aminophenol central ring. Metabolite M6, isomeric with M5, is a +32 Da dihydroxylation by accurate mass. The presence of ions j and l reflect an intact imidazole. The accurate masses in product ion spectrum , the generic MS^3 (563>435>) and separate non-data dependent MS^3 experiments (563>521>;563>407>;data not shown) prove the CID sequence: MH^+ to lose ketene followed by $C_4H_{10}N_2$ followed by CO followed by $C_5H_4O_2$ to yield ion j. This sequence is most consistent with the shown 2,3-dihydroxy structure. Alternative 2,5 or 2,6 dihydroxylations would be less likely to lose CO_2, as this molecular species does to give the odd m/z

519 ion. The MS^3 of this isolated ion (563>519>)only yields ion j. Metabolite M6 was not observable in the D_2O exchange analysis. M8 is drawn as the aromatic hydroxylation because its spectrum includes ions a+16, d+16 and intact m. M8 also shows an ion at m/z 329.0453, assigned as i, which must be due to a Ar-O bond cleavage most likely in the catechol substitution pattern. M8 shows the predicted 2 exchangeable protons.

No O-dealkylation metabolites of ketoconazole have been described and we do not report any here. But I1 and I2 were two impurities present in this commercial batch of ketoconazole. They are present at roughly 1% in both time zero and 1 hr incubations. The alcohol I1 corresponds to CID fragment i. Protonated I1 yields product ions m and p upon CID. M16 is a minor metabolite which corresponds to oxidation of I1. M16 shows the imidazole loss in CID to give m/z 275 and ion p. The second impurity, I2, is the ketone comparable to ketoconazole fragment ion m. The reduction product of I2 is M11. In CID M11 loses the imidazole to give m/z 189. M11 and M16 could arise directly from multistep processes from ketoconazole but this is unlikely. Inclusion of these minor impurities and their metabolites adds little to the ketoconazole metabolism story, but does provide additional support for the ion structures utilized herein.

CYP phenotyping showed M2 to be formed primarily via CYP3A4 oxidation with a minor involvement of CYP2D6. M3, M6, M7, M8 and M13 are also observable in CYP3A4 incubations. The breadth of metabolites observed is a surprise given the supposed tight binding of the imidazole group of ketoconazole to the heme group of CYP3A4. However there is evidence that ketoconazole can fit into the active site in multiple ways [20].

The power of modern accurate mass spectrometry and deuterium exchange in metabolite identification is demonstrated. In this study we redetected 5 of the metabolites observed by Whitehouse *et al*. [5] assigning altered structures to two of their metabolites. Four of the metabolites described in that article (III, IV, VI and VII) were not detected in this work. Of the 16 metabolites and two impurities detected, strongly supported consistent structures could be proposed for 13. Structures for the other 5 (M5, M6, M7, M8 and M12) were less certain. The major microsomal metabolites M1 and M2 might be amenable to isolation and NMR structure confirmation but for all of the others this would be a very difficult undertaking.

These generic LC/MS^n methods are often also sufficiently sensitive for detecting trapped reactive metabolites. We characterized the known major cyano adduct of oxidized ketoconazole, M1, observed the hydroxycyano adduct but did not observe the GSH adduct previously described [15]. More sensitive, targeted analyses with neutral loss and precursor scanning offer advantages to full scan mass spectrometry for detection of trace reactive metabolite adducts [21].

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ABBREVIATIONS

LC = Liquid chromatography

MS = Mass spectrometry

UV = Ultraviolet spectroscopy

CID = Collision induced dissociation

NMR = Nuclear magnetic resonance

CYP = Cytochrome P450

FMO = Flavin-containing monooxygenase

RLM = Rat liver microsome

HLM = Human liver microsome

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