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Characterization of 2-amino-1-benzylbenzimidazole and its metabolites using tandem mass spectrometry

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Abstract

We have investigated the in vitro hamster hepatic microsomal metabolism of the amino-azaheterocycle, 2-amino-1-benzylbenzimidazole (ABB). Three major metabolites were isolated and structurally characterized, using a combination of off-line HPLC, in conjunction with both electron ionization and fast atom bombardment ionization tandem mass spectrometry. ABB was shown to be debenzylated to afford 2-aminobenzimidazole (AB), as well as *N*- and C-oxidized to give 1-benzyl-N^2-hydroxyaminobenzimidazole (BHB) and 2-amino-1-benzyl-hydroxybenzimidazole, respectively. The possible reasons for formation of the exocyclic hydroxylamine BHB are discussed. Furthermore, ABB is proposed as a suitable model compound for investigating parameters that control formation of toxic hydroxylamines derived from amino-azaheterocycles.

Keywords: 2-Amino-1-benzylbenzimidazole; Metabolism; *N*-Oxidation; Hydroxylamine; Tandem mass spectrometry

1. Introduction

The propensity of both exo- and endocyclic nitrogens to undergo metabolic oxidation has attracted a great deal of interest in drug metabolism and toxicological studies. More recently, attention has focused on the amino-azaheterocycles which contain an exo-amino group ortho to the ring nitrogen [1–12]. Numerous amino-azaheterocycles are metabolically oxidized to innocuous *N*-oxides which possess little or no mutagenic and/or carcinogenic activity [2–4,8,13]. Other amino-azaheterocycles are metabolically activated by mixed function oxidase systems to produce extremely genotoxic hydroxylamines [5–7,9–12].

The aminobenzimidazole structure occurs in a large number of commonly used drugs, as well as being produced in the pyrolysis of proteins and amino acids during food preparation [5–7,10–12].

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For instance, Ishida et al. [14] isolated 2-amino-3,4-diethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), from broiled sardines. Both compounds exhibited pronounced mutagenic activity. Furthermore, other workers [11,15] have identified the mutagens 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx) from ground beef.

In this work, the metabolism of 2-amino-1-benzylbenzimidazole (ABB) is reported. ABB was selected as a potential model compound to investigate factors involved in the *N*-oxidation of this group of amino-azaheterocycles. Methods are also described using HPLC and tandem mass spectrometry, which allow investigation of whether or not *N*-oxides and *N*-hydroxylamines are formed in hamster hepatic microsomal incubates of ABB.

2. Materials and methods

2.1. Materials

2-Aminobenzimidazole (2-AB) was purchased from Lancaster Synthesis (UK). All other chemicals used for the preparation of ABB and its potential metabolites were purchased from Aldrich (UK), Sigma Chemical Co. (UK), and British Drug House (UK). Glucose-6-phosphate (G-6-P, disodium salt) and glucose-6-phosphate dehydrogenase (G-6-P-D) were purchased from the Boehringer Corporation Ltd. (UK) and the sodium salt of nicotinamide adenine dinucleotide phosphate (NADP) was purchased from Sigma Chemical Co. (UK). The reversed phase C-18 Sep-Paks were purchased from Waters (Massachusetts, USA). Male Syrian hamsters (body weight 80–110 g) were used in this investigation and were obtained from the King's College animal facilities. Animals were fed a standard diet, as well as water ad libitum.

2.2. Syntheses of standards

ABB was prepared by the method of Kikugawa [16]. Potassium hydroxide (0.9 g) was added to a suspension of 2-AB (0.5 g) in acetone (13 ml) with stirring. After 10 min, methyl iodide (0.5 g) was added to the reaction mixture followed by vigorous stirring for another 10 min. The acetone solution was transferred to a separating funnel containing benzene (120 ml) and subsequently washed with water (1 × 20 ml), followed by saturated sodium chloride solution (20 ml) and then immediately dried with sodium sulphate. The benzene fraction was evaporated to dryness and the product recrystallized from chloroform to give ABB in a yield of 90% (m.p. 193–194°C (lit. 194–195°C)).

1-Benzyl-2-chlorobenzimidazole was prepared using the method of Harrison et al. [17]. The 2-chlorobenzimidazole was refluxed with benzyl bromide for 3 h and subsequently recrystallized from water. 1-Benzyl-2-hydroxyaminobenzimidazole (BHB) was synthesized using a modified method of Giner-Sorolla and coworkers [18,19]. Hydroxylamine (5.22 g) was dissolved in 130 ml absolute ethanol, and a solution containing potassium hydroxide (1.22 g) in hot absolute ethanol (4.4 ml) was added. A precipitate was formed and subsequently filtered and washed several times with hot ethanol (10 ml). 1-Benzyl-2-chlorobenzimidazole (0.5 g), dissolved in hot absolute ethanol (8 ml), was added to the solution (total volume 142.4 ml) of hydroxylamine. The mixture was kept for 24 h at 50°C after which it was evaporated to dryness using a rotary evaporator at 40°C. The residue was washed several times with petroleum ether (b.p. 40–60°C) and recrystallized from the same solvent, m.p. 241°C, yield 35%. Elemental analysis: $C_{14}H_{11}N_3O$, calculated: C = 70.28; H = 5.48; N = 17.56%; found: C = 69.92; H = 5.41; N = 17.63%.

2.3. Methods

2.3.1. Preparation of microsomes. Microsomes were prepared from hamster liver as described previously [20]. Washed hepatic microsomes were suspended in phosphate buffer (0.2 M, pH 7.4) at a final concentration of 0.5 g original liver/ml phosphate buffer. Microsomes were usually used within 24 h of preparation or alternatively stored at −80°C, after 20% glycerol was added to the final suspension media. Microsomal protein content was determined by the method of Miller [21].

2.3.2. Microsomal incubations. Incubations were carried out in 25 ml Erlenmeyer flasks maintained at 37°C in a Gallenkamp shaking water bath.

Cofactor solution (NADPH — regenerating), which consisted of NADP (2 μmol), G-6-P (10 μmol), G-6-P-D (1 unit) and magnesium chloride (20 μmol) in phosphate buffer (0.2 M, pH 7.4, 2 ml), was preincubated for 5 min to allow NADPH generation, ABB (5 μmol in 100 μl methanol) was subsequently added, followed by the immediate addition of hepatic microsomes (1 ml). Control flasks using heat inactivated microsomes (90°C for 20 min) containing ABB were also incubated. All incubations were terminated by placing flasks on ice.

2.3.3. Sample preparation for HPLC and mass spectrometry (MS) analysis. The contents of each flask were transferred to screw-capped tubes containing zinc sulphate (100 mg) and shaken in order to precipitate microsomal proteins. The mixture was centrifuged at 3000 rev./min for 15 min and the supernatant passed through an activated reversed phase C-18 Sep-Pak [22]. Excess $ZnSO_4$ was washed off the cartridge by one volume of distilled water. The metabolites were eluted with 2–3 ml freshly distilled methanol and evaporated to dryness by bubbling a slow stream of nitrogen gas through the samples. The residue was reconstituted in 100 μl distilled methanol for HPLC and mass spectrometric analysis.

Peaks corresponding to substrate (ABB) and its metabolites were collected as fractions from the HPLC, and the organic phase evaporated under nitrogen. The remaining aqueous layer was then lyophilized and the residue reconstituted in distilled methanol (100 μl). These fractions along with samples of synthetic standards were individually analyzed by MS.

2.3.4. Control incubations. Control flasks containing heat-inactivated microsomes and ABB were subjected to the same sample clean-up as described above for normal 'test' microsomal incubations. After reversed-phase C-18 Sep-Pak treatment, the analyte mixture was subjected to HPLC, and the subsequent fractions were collected at retention times identical to UV responses observed in test microsomal incubations. These control HPLC fractions were subjected to both MS and tandem MS (MS/MS).

2.3.5. HPLC analysis. An isocratic HPLC system was developed for the analysis of ABB and its microsomally produced metabolites using a 5 μm Nucleosil SA cation exchange column (25 × 0.46 cm i.d.) connected to an LDC UV monitor. Compounds were detected by monitoring at 282 nm and quantitated using a Milton Roy CI-400 computing integrator. The mobile phase consisted of $NH_4H_2PO_4$ (0.25 M) and methanol (55%) at pH 3.6 with a flow rate of 1.0 ml/min.

2.3.6. Mass spectrometry. All mass spectra were obtained on a VG 70-SEQ instrument of EBQ_1Q_2 configuration, where E is an electrostatic analyzer, 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 either: (1) positive ion fast atom bombardment (FAB) mass spectrometry. Xenon atoms from a model B11N saddlefield fast-atom gun (Ion Tech, Teddington, UK) were used at 8.5 keV as the primary ion beam, or (2) direct insertion electron impact (EI), where the probe was heated at 260°C for 30 s in the source. In both cases the ions produced were accelerated to 8 keV from the source region and analyzed in MS_1 using a scan speed of 10 s/decade at a resolution of ~ 1000.

2.3.7. Product ion spectra. Either protonated molecules [MH^+] or radical cation molecular ions [$M^{+\cdot}$] were selected with resolution of ~ 1000 in MS_1 and subjected to collision-induced association (CID) using argon as the collision gas in Q_1. The collision energy was optimized to afford maximum fragmentation but minimize product ion scattering. A collision energy of 20 eV with a gas pressure of ~ 10^{-7} mbar was used throughout the study. The resulting product ions produced were mass analyzed in Q_2 and a product ion spectrum acquired by scanning MS_2 over the mass range 400–40 with 10 scans being obtained in the multichannel analysis mode.

2.3.8. Sample preparation. For FAB-MS and FAB tandem mass spectrometry (FAB-MS/MS), aliquots (1 μl) of each sample were dissolved in 1 μl of the FAB matrix, 3-nitrobenzylalcohol (NBA) which was located on the stainless steel probe tip. In EI-MS and EI-MS/MS studies, the sample (1 μl) was added to the glass vial inserted at the probe

tip. Gentle external heat was used to evaporate the solvent. In both cases (EI and FAB) the probe was subsequently inserted into the MS ion source, and the samples were mass analyzed.

3. Results

3.1. HPLC analysis of microsomal incubations

ABB was incubated with hamster hepatic microsomes for 30 min and the metabolic mixture extracted by solid phase extraction. The extracts were analyzed initially by HPLC. Fig. 1A shows the HPLC chromatogram of a mixture of the 3 synthetic standards, namely, 2-AB, BHB, and ABB. Figs. 1B and 1C show HPLC chromatograms of a test microsomal incubation and heat inactivated microsomal incubation (control) of ABB, respectively. Four UV responses with retention times of 11.73, 13.45, 18.36, and 22 min were observed in test incubates. The peaks at 11.73, 18.36, and 22 min had chromatographic retention times identical to those of the synthetic standards 2-AB, BHB, and the parent compound (ABB), respectively. The metabolite with a retention time of 13.45 min (UNK) did not correspond to any of the known synthetic standards analyzed by HPLC. The control HPLC chromatogram only contained UV responses corresponding to unmetabolized ABB and the internal standard (IS), 2-amino-1-methylbenzimidazole.

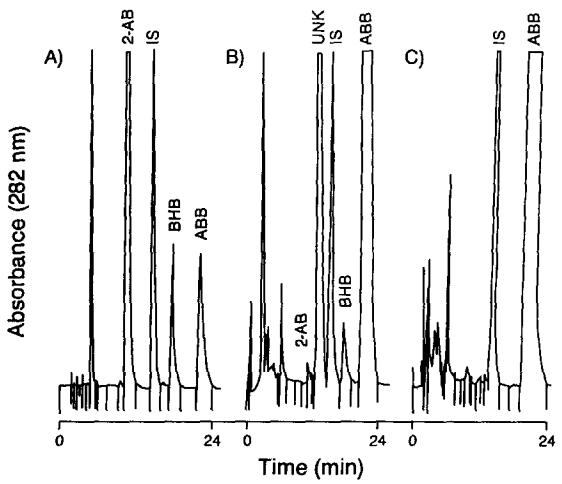

Fig. 1. Isocratic HPLC chromatograms of: (A) synthetic standard mixture of parent compound ABB plus 2-AB and BHB. IS corresponds to an internal standard, namely 2-amino-1-methylbenzimidazole; (B) in vitro hamster hepatic microsomal incubation of ABB. The peak marked UNK does not correspond to any authentic standard retention time and is discussed further in the text; (C) 'control' hamster hepatic microsomal incubation of ABB, where microsomes have been inactivated by heat.

3.2. MS analysis of standards and metabolites

Initial studies focused on the most suitable and sensitive ionization technique using the synthetic standards as model compounds. FAB-MS analysis of the parent compound ABB (MH^+ = 224) and BHB (MH^+ = 240), in NBA as matrix, revealed MH^+ ions at a signal:noise of ~ 3:1 for ~ 100 pmol of each compound. However, the FAB-MS analysis of 2-AB (MH^+ = 134) was not particularly sensitive, and ~ 1 nmol of compound was needed to observe a protonated molecule signal. We have observed previously that for low molecular weight compounds (< 200 Da) FAB-MS is not particularly sensitive [23]. Analysis of 2-AB by EI-MS allowed detection limits of ~ 200 pmol for the radical cation molecular ion ($M^{+\cdot}$ = 133).

The test microsomal incubation was subjected only to a reversed-phase C-18 Sep-Pak clean-up prior to analysis by FAB-MS and EI-MS. The FAB-MS analysis of the test mixture revealed ions at m/z 224 and m/z 240, indicating the tentative presence of unmetabolized parent compound ABB and the *N*-hydroxylamine, BHB, respectively. In the control incubation, only an ion at m/z 224 corresponding to ABB was detected. EI-MS analysis of the test mixture revealed ions at m/z 223, 239, and 133 corresponding to $M^{+\cdot}$ of ABB, BHB, and 2-AB, respectively. Analysis of the control incubation mixture only revealed a significant ion at m/z 223.

3.3. Product ion spectra of synthetic standards ABB, BHB, and 2-AB

Product ion spectra of ABB and BHB were obtained using FAB-MS as the primary ionization method, whereas EI-MS was used for 2-AB. Molecular (precursor) ions were selected using MS_1 and subjected to CID in Q_1 by collision with argon. The resulting product ions were mass

analyzed in MS_2 (Q_2), and these results are summarized in Table 1.

3.4. Product ion spectra of HPLC-purified metabolites derived from microsomal incubations

Tandem MS analysis of HPLC fractions 22 (MH^+ = 224), 18.36 (MH^+ = 240) and 11.73 min ($M^{+\cdot}$ = 133) afforded product ion spectra (Fig. 2A-C) that were almost identical to spectra obtained on synthetic standards of unmetabolized parent ABB (FAB-MS/MS), BHB (FAB-MS/MS), and 2-AB (EI-MS/MS), respectively (Table 1). HPLC fractions of a control incubation with identical retention times to fractions containing metabolites from test incubations were also subjected to MS/MS analysis. 'Control' MS/MS spectra were acquired by isolating specific molecular ions for the appropriate HPLC fraction and then subjecting them to CID. Ions observed in both control and test incubation MS/MS spectra are labelled as 'C' in Fig. 2A-D.

The FAB-MS/MS product ion spectrum of MH^+ = 224 (HPLC retention time 22 min), corresponding to unmetabolized ABB from a test incubation, was very simple with only 2 product ions observed at m/z 146 and 91. These ions arise from cleavage of the phenyl-methylene bond and the $\rangle$N-C methylene bond to afford a methylene-aminobenzimidazole cation and the stable tropylium cation at m/z = 91 [$C_7H_7^+$], as shown in Fig. 2A. The FAB-MS/MS product ion (MH^+ = 240) of the HPLC test incubation fraction (retention time 18.36 min) afforded a series of specific fragment ions at m/z 223, 162, 149, 134, and 91. These ions can be ascribed to fragmentation pathways of BHB and are shown in Fig. 2B and are identical to those found in the product ion spectrum of MH^+ = 240 for the synthetic standard BHB (Table 1). The EI-MS/MS product ion spectrum of $M^{+\cdot}$ = 133 HPLC fraction (retention time 11.73 min) gave a series of product ions (Fig. 2C) afforded by multiple bond cleavages, resulting from higher internal energy deposition by EI-MS [23].

The FAB-MS/MS product ion spectrum of MH^+ = 240 (HPLC fraction retention time 13.45 min of test incubation) gave rise to ions at m/z 162, 149, and 91 as shown in Fig. 2D. Ions observed at m/z 212, 194, and 166 were also observed in the product ion spectrum of MH^+ = 240 from an HPLC fraction from a control incubation with identical retention time (13–14 min) to the test fraction. In order to obtain more structural information on the UNKNOWN (at retention time 13.45 min), the HPLC fraction was also subjected to EI-MS/MS ($M^{+\cdot}$ = 239) and compared with the EI-MS/MS of standard BHB ($M^{+\cdot}$ = 239) in order to investigate the location of the oxidation process, and these results are summarized in Table 2. Of particular note was the absence of m/z 223 in the product ion spectrum of UNKNOWN, related to the loss of –OH from an *N*-hydroxylamine. The product ions produced in both EI-MS/MS and FAB-MS/MS indicated oxidation of the imidazole phenyl ring had occurred giving rise to a phenol, as shown in Fig. 2D. Furthermore, treatment of

Table 1
Product ions observed in the CID MS/MS spectra of synthetic standards ABB, BHB and 2-AB

Molecular and product ions observed	Fragment lost (m/z)	Possible structure of ion detected
(1) ABB		
224 (100)[a]	—	MH^+ [b]
146 (10)	78, [C_6H_6]	[AB-H_2C]$^+$ [c]
91 (50)	133, [AB][c]	[C_7H_7]$^+$
(2) BHB		
240 (100)	—	MH^+ [b]
223 (80)	17, [-OH]	[ABB]$^+$ [d]
162 (10)	78, [C_6H_6]	[AB-CH_2 + OH]$^+$
149 (15)	91, [C_7H_7]	[AB + OH]$^+$
134 (10)	106, [C_7H_7 + OH]	[AB]$^+$
91 (55)	149	[C_7H_7]$^+$
(3) 2-AB		
133 (100)	—	$M^{+\cdot}$ [b]
106 (90)	27, [HCN]	—
91 (15)	42, [CH_2N_2]	[C_6H_5N]$^+$
79 (15)	54	—

[a]Values in parentheses represent relative ion abundances.
[b]Note that the primary ionization mode for ABB and BHB was FAB-MS which affords protonated molecules (MH^+). 2-AB was ionized using EI-MS which produces a radical cation molecular ion ($M^{+\cdot}$).
[c]AB — [2-aminobenzimidazole-H].
[d]ABB — [2-amino-1-benzylbenzimidazole-H].

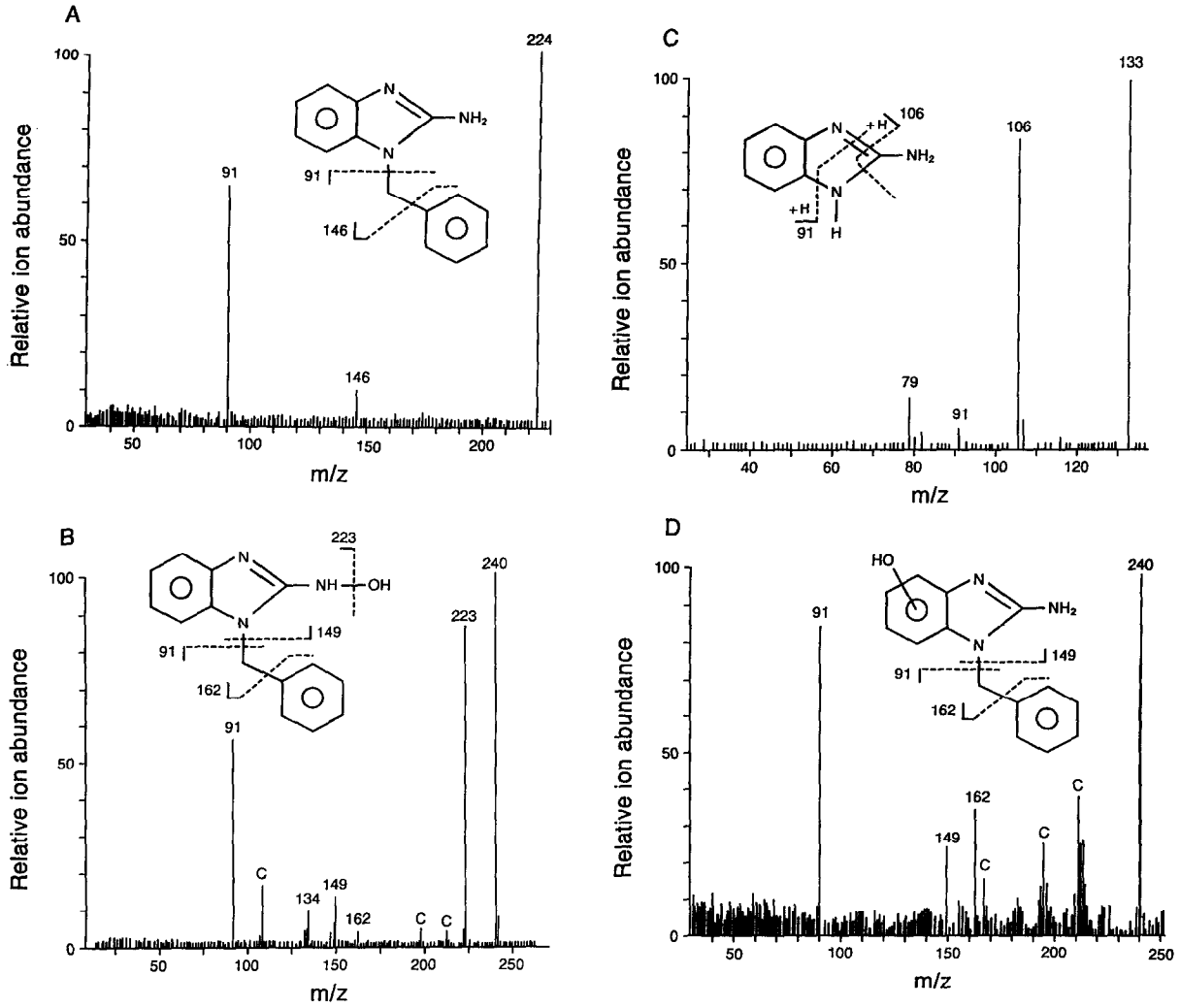

Fig. 2. The tandem mass spectral analysis of metabolites derived from a microsomal incubation of ABB, purified and collected off-line by HPLC. (A) FAB-MS/MS of parent compound ABB where the precursor ion (MH^+) m/z 224 was subjected to CID. (B) FAB-MS/MS of the precursor ion (MH^+) m/z 240 identified as BHB. (C) EI-MS/MS of the precursor ion (M^+) m/z 133, identified as 2-AB. (D) FAB-MS/MS of the precursor ion (MH^+) m/z 240 derived from HPLC UNK peak. Identified as the 2-amino-1-benzyl-hydroxybenzimidazole compound shown.

this compound with diazotised *p*-nitroaniline/Na_2CO_3, a reagent specific for phenolic groups, resulted in the characteristic deep purple color affirming the presence of such functionality. This colorimetric assay was negative when used with all synthetic standards and other metabolites produced.

4. Discussion

ABB was metabolized by an NADPH-dependent hamster hepatic microsomal enzyme system to 3 metabolites. Test metabolic extracts were purified by HPLC and isolated fractions were subjected to MS and MS/MS analyses using either

Table 2
Product ions produced in the EI-MS/MS product ion spectrum of the synthetic standard BHB and a structurally unknown metabolite isolated from HPLC

Molecular and product ions observed	Fragment lost (m/z)
(1) BHB-synthetic standard	
239 (100)	—
224 (15)	15, [-OH + 2H]
223 (10)	16, [-OH + H]
222 (10)	17, [-OH]
161 (10)	78, [C_6H_6]
148 (95)	91, [C_7H_7]
91 (55)	148, —
(2) UNKNOWN	
239 (100)	—
212 (25)	27, [HCN]
211 (40)	28, [-HCN-H]
161 (30)	78, [C_6H_6]
148 (25)	91, [C_7H_7]
128 (45)	111, —
91 (30)	148, —

EI or FAB as the primary ionization technique. Based on a comparison of HPLC retention times and MS/MS spectral data with synthetic standards, ABB was debenzylated to 2-AB and *N*-oxidized at the exo-amino group to afford BHB, as shown in Fig. 3. *N*-oxidation of ABB by microsomal enzymes to the corresponding hydroxylamine is supported by the previous work of Hashimoto et al. [12]. They demonstrated that 2-amino-6-methyl-dipyrido [1,2-a:3′,2′-d] imidazole and 2-amino-dipyrido [1,2-a:3′,2′-d] imidazole, both resulting pyrolysis products of glutamic acid, are metabolized at their exo-amino group to toxic hydroxylamines.

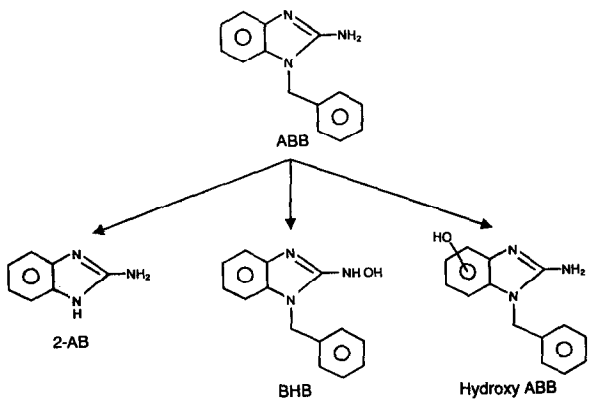

Fig. 3. Metabolic pathways of ABB when subjected to hamster hepatic microsomal incubation.

The UNKNOWN metabolite (retention time 13.45 min) did not correspond to a known retention time of authentic standards. FAB-MS/MS analysis revealed product ions at m/z 162, 149, and 91 (Fig. 2D). Comparison with the product ion spectrum of synthetic BHB (Table 1) revealed similar product ions at m/z 162, 149, and 91 and clearly indicate that oxidation of the UNKNOWN had occurred somewhere on the aminobenzimidazole ring system. However, a key difference in the 2-product ion spectra is that the BHB authentic standard contains a product ion at m/z 223 corresponding to the loss of the OH radical, characteristic of the presence of *N*-hydroxylamine functionality [24,25] whereas the UNKNOWN does not possess such a product ion.

In the EI-MS/MS product ion spectrum of UNKNOWN (Table 2) an ion at m/z 212 corresponding to loss of HCN from the exo-amino group is observed. This corresponds to a similar product ion observed in the EI-MS/MS spectrum of synthetic 2-AB (Table 1). These results strongly indicate that oxidation of the exo-amino group has not taken place. The only other locations for oxidation to occur is either at the methylene bridge carbon or the aromatic ring of the benzimidazole functionality. Presence of a product ion at m/z 149 in the FAB-MS/MS spectrum (Fig. 2D) and m/z 148 in the EI-MS/MS spectrum (Table 2) indicate that oxidation has occurred on the benzyl ring to afford the hydroxybenzimidizole as shown in Fig. 2D. The metabolic products and pathways for metabolism of ABB by hamster liver microsomes are summarized in Fig. 3.

5. Conclusion

Hydroxylamines are weaker bases (pK_a 3–6) than their parent amines and are often unstable being either further oxidized to nitroso derivatives

or reduced back to the parent amine. They are capable of covalently binding proteins and, in contrast to their parent amines, are potent carcinogens. As a consequence, analysis and identification of hydroxylamines usually is a cumbersome task. In this paper we have shown that solid phase extraction of metabolic mixtures of ABB followed by their analysis either pre- or post-HPLC analysis by tandem mass spectrometry allows rapid isolation and structural characterization of the metabolites. Furthermore, ABB should serve as a useful model compound to investigate the factors determining the formation of toxic hydroxylamine metabolites derived from amino-heterocycles.

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