THE *IN VITRO* HEPATIC MICROSOMAL METABOLISM OF N-BENZYLADAMANTANAMINE IN RATS

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SUMMARY

The metabolism of N-benzyladamantanamine (NBAD) was studied *in vitro* using rat hepatic microsomal preparations. The substrate and proposed metabolites were synthesized and characterized using spectroscopic techniques and separated using a reverse phase HPLC system. NBAD was incubated with rat microsomal preparations, extracted into DCM in the presence of NaCl and evaporated under a stream of nitrogen. The results from HPLC studies showed that NBAD produced the corresponding nitrone and hydroxylamine. This experiment also revealed that dealkylation occurred. No metabolites were observed which corresponded to authentic amide or oxaziridine. The reactions required a microsomal enzyme source and NADPH as a cofactor. The results indicate that the nitrone observed as a metabolite of NBAD is not an intermediate leading to the formation of an oxaziridine and hence an amide, under careful experimental conditions excluding light.

KEY WORDS

benzyladamantanamine, nitrones, microsomes, rat, metabolism, oxaziridines

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INTRODUCTION

Adamantanamine (1-aminoadamantane) is commonly used as an antiviral agent. Since it has a highly basic primary amine function, it is susceptible to chemical reactions in the organism to form either alkylated products, i.e. secondary amines, or it may react with endogenous aldehydes to form imines. The secondary amines and imines thus formed are susceptible to further transformation into reactive intermediates, i.e. the corresponding hydroxylamine, nitrone, oxaziridine and amide, either metabolically or chemically in the body /1-6/. These reactive intermediates may react with endogenous macromolecules to produce carcinogenic, mutagenic and immunogenic effects. A literature search revealed that there was no metabolic study on N-substitute derivatives of adamantanamine. We, therefore, aimed to study the *in vitro* hepatic microsomal metabolism of N-benzyladamantanamine (NBAD), a model secondary amine derived from adamantanamine, to investigate the possible formation of the intermediates proposed above.

Previous studies have revealed that amides and nitrones are metabolites arising from certain secondary aromatic amines /1-3/. It was later established that nitrones are not metabolic intermediates in the formation of amides and they may be involved in the chemical production of amides via oxaziridines during analysis /4/. It was later demonstrated that certain imines were chemical artifacts arising from secondary aromatic amine metabolism /5/ and that these imines could be intermediates in amide formation /6/. The *in vitro* metabolism of N-benzyl-*tert*-butylamine (NBTBA) has recently been studied /7/. It was shown that no metabolites were observed which corresponded to authentic amide or oxaziridine. The proposal was that the nitrone was not an intermediate leading to the formation of an oxaziridine and hence an amide, under careful experimental conditions excluding light /7/.

Although the diaryl oxaziridines are known to be unstable /8-10/, the oxaziridine derivatives of adamantanamine were reported to be stable /11/. This was another reason for selecting NBAD as substrate for the present study. We report here the results of a study of the *in vitro* oxidative microsomal metabolism of NBAD with hepatic washed rat microsomes fortified with NADPH.

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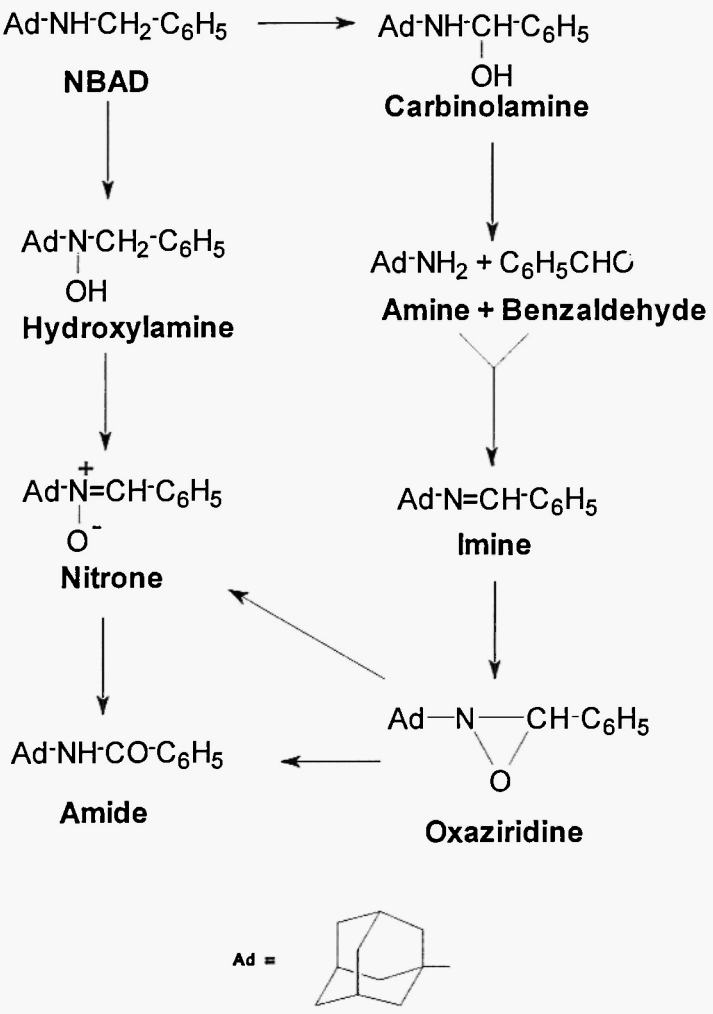

Fig. 1: Possible intermediates involved in the metabolism of N-benzyl-adamantanamine (NBAD).

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MATERIALS AND METHODS

Synthesis and characterization of NBAD and its potential metabolites

Chemicals

Adamantanamine, benzyl and benzoyl chlorides, m-chlorperbenzoic acid (m-CPBA), lithium aluminium hydride, sodium borohydride and benzaldehyde were all purchased from Aldrich Chemical Company, UK. All the chromatographic solvents and plastic-backed TLC plates precoated with silica-gel $60F_{254}$ were obtained from E. Merck, Darmstadt, Germany. Silica-gel 60; particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck, Darmstadt, Germany) was used as column chromatography stationary phase.

Synthesis of N-benzyladamantanamine (NBAD)

The substrate, NBAD, was prepared by reduction of the corresponding imine, BDAD /12/, using the following procedures. The corresponding imine, BDAD, was prepared by refluxing equimolar amounts of 1-aminoadamantane and benzaldehyde (0.004 mol) in toluene at 150°C for 45 hours as described by Drukker /12/ according to the modified method of Amal *et al.* /13/. After the reaction was complete, toluene was evaporated and the yellow residue was recrystallized from methanol. Its melting point matched that recorded in the literature. Mass spectra showed molecular ion peaks and correct fragmentation patterns for imines (Table 1).

NBAD was also prepared by the method of Drukker /12/. BDAD (0.001 mol) was dissolved in methanol. To this mechanically stirred solution was added equimolar $NaBH_4$ in small portions over 15 min and the solvent evaporated to dryness. Water was added to the residual solid in order to decompose excess $NaBH_4$. The crude product was then subsequently washed with water and extracted with diethylether. After evaporation, the oily product obtained was treated with HCl gas to form the HCl salt of the substrate. Mass spectra showed molecular ion peaks and correct fragmentation pattern (Table 1).

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TABLE 1

Analytical and spectral data of NBAD and some of its potential metabolites.

Compound (Abbreviation)	M.w.	Yield (%)	Description & m.p. (°C)	Major mass spectral fragments m/z (% relative abundance)	TLC Rfx100 values	HPLC retention time (min)	UV (maximum absorbance) (nm)
N-Benzy adamantanam ne (NBAD HCl)	277.5	68	wh te powder 211-213	242(100), 241(2), 184 (0.5), 152 (0.5)	43.9 (Base; 0 (HC)	6.5	211-213
N-Benzyl-N-hydroxy adamantanamine (BHAD)	257	40	white cry 77-80	257(4), 256(17.5), 242(100), 152 (1), 135 (0.5), 106 (0.5)	61.5	13.3	192
N-(Adamantanyl)-α-phenyl oxaziridine (ADPO)	255	26	yellow powder 101-103	256(5), 255 (0.5), 240(100), 239 (1), 199(2), 197(1), 169(1), 150(37), 135(2), 103(4)	63.2	18.2	206, 280
N-(Adamantanyl)-α-phenyl nitrone (ADPN)	255	57	cream powder 270	256(100), 240(11.5), 152(2), 135 (0.5), 106 (1)	47.3	7.3	205, 293
N-benzoy adamantam ne (BZAD)	255	75	whi e powder 127-129	256(100), 255(2), 198(1), 152 (1)	49.5	9.1	203, 225
N-benzylidene adamantanamine (BDAD)	239	48	white cry 60	240(10?), 169(2), 152(98), 135(3.5), 106(2)	54.9	4.5	206, 247

UV spectra were recorded on a Kontron Uvikon 860 UV spectrophotometer. EI-mass spectra were determined by direct insertion of samples on a mass spectrometer with 70 eV ionisation potentials. Solvent system for silicagel TLC: petroleum ether*: acetone (70:30, v/v) (*b.p. 40-60°C). For HPLC conditions see text.

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Synthesis of N-adamantanyl-α-phenylnitrone (ADPN)

Synthesis of the required nitrone was achieved by the m-CPBA oxidation of NBAD /14/. NBAD (0.005 mol) was dissolved in dry acetone and the flask kept in the dark. To this stirred and cooled solution was added m-CPBA (0.0065 mol in acetone) dropwise over 30 min. The reaction was continued for a further 1.5 h and terminated by removal of the solvent under reduced pressure. The residual solid was dissolved in DCM (25 ml) and this solution was subsequently washed with aqueous K_2CO_3 (0.5 M, 3 x 25 ml), dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to leave the crude nitrone. Trituration of the solid with ice-cold diethylether yielded ADPN in a pure state (Table 1).

Synthesis of N-benzyl-N-hydroxyadamantanamine (BHAD)

The general procedure for the preparation of N-substituted phenylhydroxylamines by the reduction of parent nitrones was employed /14/. ADPN (0.001 mol) was dissolved in previously dried, hot diethylether. This solution was added dropwise to a mechanically stirred ethereal suspension of $LiAlH_4$ over 30 min. The mixture was stirred for a further 20 min and the reaction terminated by the addition of water dropwise, 15% NaOH and additional water to yield a granular precipitate which was washed with warm diethylether (3 x 5 ml). The combined ethereal solution was dried with aqueous Na_2SO_4 and evaporated under reduced pressure. The crude BHAD was recrystallized from petroleum ether (b.p. 40-60°C) (Table 1).

Synthesis of N-benzoyladamantanamine (BZAD)

The corresponding amide, BZAD, was rapidly prepared in a pure state from adamantanamine (0.002 mol) and an equimolar amount of benzoyl chloride by the Schotten-Baumann reaction /15/. The crude amide was recrystallized from ethanol. The amide had the correct mass fragmentation pattern (Table 1).

Synthesis of N-adamantanyl-α-phenyloxaziridine (ADPO)

To prepare ADPO, we utilized the method of Pews /16/ in which m-CPBA (0.001 mol) in dichloromethane (DCM) was added to an equimolar amount of imine, BDAD, in DCM dropwise over thirty min.

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The solution was filtered to remove the m-CPBA and washed with dilute sodium sulphite solution, followed by dilute sodium carbonate solution, and dried over sodium carbonate. The DCM was evaporated at room temperature using a rotary film evaporator. The reaction mixture was examined by TLC. Following spraying with Dragendorff reagent, it was shown that a new substance was present which immediately gave an orange colour in contrast to the authentic imine which did not respond to this reagent. The mixture was analysed by mass spectrometry which showed a M+16 molecular ion peak. The UV spectra showed that the maximum absorbance of this peak was different from the corresponding nitrone and amide (Table 1).

High performance liquid chromatography

The substrate and its potential metabolites were analysed using a reverse-phase isocratic HPLC system which comprised a model 302 Gilson pump, a model 7125 syringe loading sample injector valve fitted with a 20µl sample loop, a SpectroMonitor III Model 1204A LDC variable wavelength UV detector and a Tekman recorder. The analytical column (Phase Separations Ltd., Deeside, UK) contained Spherisorb 5µm ODS (250 x 4.6 mm i.d.) and the pre-column material was co-pellicular ODS (Whatman International Ltd., Maidstone, Kent). The mobile-phase composition was acetonitrile:water (60:40, v/v) at a flow rate of 1 ml/min. The substrate and its potential metabolites were detected at 254 nm. HPLC retention times of the substrate and its potential metabolites are shown in Table 1.

Incubation and extraction procedures

β-Nicotinamide dinucleotide phosphate (disodium salt, NADP) and glucose-6-phosphate (disodium salt, G-6-P) were purchased from BDH. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml; G-6-PD) was obtained from Boehringer Mannheim Corporation (London). Dichloromethane was obtained from Merck.

Adult male Wistar rats were used in this study. The animals were deprived of food overnight prior to sacrifice, but were allowed water *ad libitum*. They were previously fed on a balanced diet. Hepatic washed microsomes were prepared as described by Schenkman and Cinti /17/.

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Incubations were carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of NADP (2 μmole), G-6-P (10 μmole), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 μmole) in phosphate buffer (0.2 M, pH 7.4, 2 ml) at pH 7.4. Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmol) in methanol (5 μl). The incubation was continued for 30 min, terminated and extracted with dichloromethane (2 x 5 ml). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 200 μl of methanol for HPLC. Because of the known chemical lability of nitrone and oxaziridine functional groups /8-10/, incubations were carried out in the dark and light was rigorously avoided during subsequent extraction and concentration procedures. These conditions were used to prevent the chemical conversion of any oxaziridine present to the isomeric amide during analysis. The reconstituted extracts were analysed using the reverse-phase isocratic HPLC system described above.

RESULTS AND DISCUSSION

Following the *in vitro* metabolism of NBAD, no metabolites were observed which corresponded to authentic amides and oxaziridines (Figure 2). However, the corresponding nitrone and hydroxylamine were detected. These metabolites were not observed in control experiments either in the presence of denatured microsomes or in the absence of co-factors. In addition to these metabolites, another metabolic product which had a retention time of 4.5 min was also observed (Figure 2). This had the same retention time as both authentic benzaldehyde and authentic imine, BDAD. The formation of imines from the dealkylation products of secondary aromatic amines is a known reaction in experimental animals /5/. However, as the mobile phase employed in the present experiment failed to separate benzaldehyde and the corresponding imine, no conclusion can be made at this stage indicating that BDAD was formed. These preliminary results only show that dealkylation occurred. Experiments are in progress in our laboratories to examine the involvement of imine following NBAD metabolism. Figure 2 shows an HPLC chromatogram following NBAD metabolism by rat microsomes.

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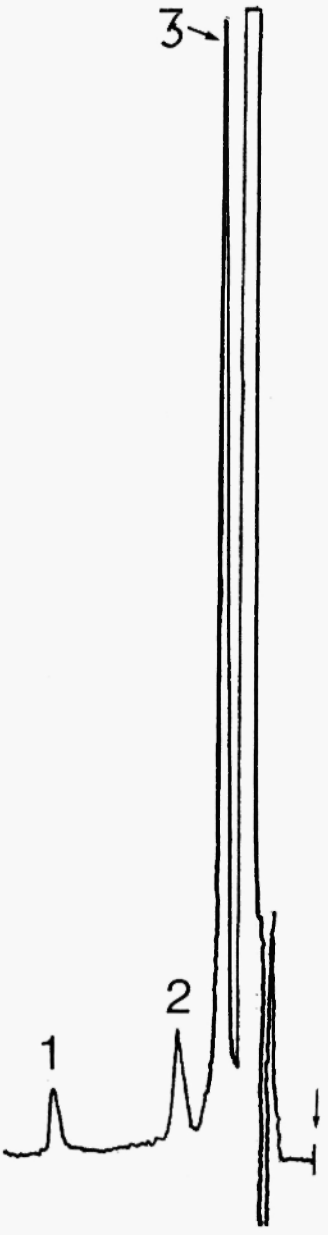

Fig. 2: HPLC chromatogram obtained following extraction of male rat microsomal incubation mixture with NBAD as substrate. 1 = BHAD; 2 = ADPN; 3 =benzaldehyde and/or BDAD (see text for abbreviations).

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