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Stereoselective Microsomal *N*-Oxidation of *N*-Ethyl-*N*-Methylaniline

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ABSTRACT

The stereoselectivity of metabolic *N*-oxidation of *N*-ethyl-*N*-methylaniline (EMA) was investigated *in vitro* following incubation of the compound (1mM) with fortified hepatic microsomal preparations of both male Wistar rats and New Zealand White (NZW) rabbits. The major metabolites in both species were found to be *N*-ethylaniline, *N*-methylaniline and EMA *N*-oxide. Chromatographic resolution of the *N*-oxide enantiomers was achieved using a Chiralcel OD stationary-phase with a mobile-phase of hexane : ethanol (98 : 2, v/v). Examination of the enantiomeric composition of the *N*-oxide metabolites indicated a predominance of the (-)-(*S*)-*N*-oxide from both species with enantiomeric excesses of 52 ± 2.5 % and 65 ± 2.1 % (n=3) in rat and rabbit tissue respectively. These preliminary observations indicate that the *N*-oxidation of EMA shows product stereoselectivity, the extent of which varies between species.

INTRODUCTION

The extensive use of nitrogen containing organic compounds (agrochemicals, pharmaceutical entities and chemical intermediates) results in human exposure to a large number of these agents under widely varying conditions. *N*-Oxidation is an important route in the metabolism of such molecules and can either effect detoxication or induce formation of cytotoxic species (potential promutagens and procarcinogens) [1].

N-Oxidation of tertiary amines frequently yields *N*-oxides as major metabolic end-products [1]. Whereas a tricoordinate nitrogen atom with three different substituents is not chiral, due to rapid inversion of the two possible antipodes *via* a planar transition state, the corresponding *N*-oxide is chiral (Figure 1). Thus the metabolic formation of an *N*-oxide of this type may show product stereoselectivity or stereospecificity, which may have implications if the metabolite is either pharmacologically or toxicologically active and such activity is selective for one enantiomer. In addition, *N*-oxides may also undergo stereoselective enzymatic reduction. Thus the stereochemical composition of the *N*-oxide measured as an end product of metabolism may result from two independent metabolic processes, differing in terms of their stereoselectivity. There are at present no systematic studies with respect to the chirality of *N*-oxide formation or reduction. Our interest [2,3] in these metabolic transformations has prompted an investigation into methods for the resolution and analysis of such compounds.

In this preliminary report, data are presented which indicate that *in vitro* microsomal *N*-oxidation of *N*-ethyl-*N*-methylaniline (EMA) is stereoselective using both rat and rabbit tissue preparations.

Abbreviations. EMA, *N*-ethyl-*N*-methylaniline; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NADP, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; NZW, New Zealand White; ORD, optical rotatory dispersion; t_R, retention time; and tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

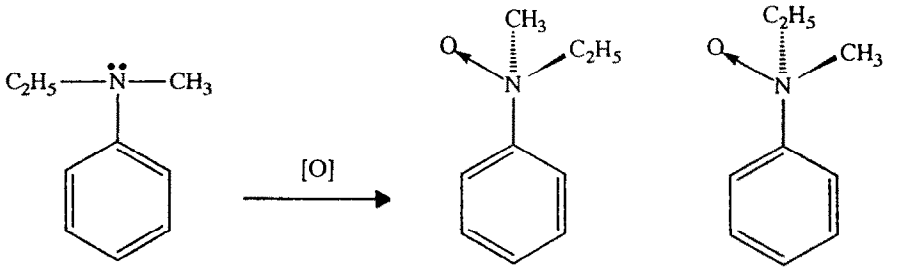

Fig. 1. Formation of enantiomeric *N*-oxides by oxidation of EMA.

MATERIALS AND METHODS

Synthesis of EMA N-Oxide. Racemic EMA *N*-oxide was prepared by oxidation of EMA (Aldrich, Gillingham, U.K.) using *m*-chloroperoxybenzoic acid (Sigma, Poole, U.K.) in dichloromethane solution (24hrs, room temperature, 1.0 equivalent). The *N*-oxide product was separated from remaining reactants by silica column chromatography and converted to its hydrochloride salt by treatment with HCl gas. The recrystallized HCl salt (m.p. 121°C; lit. value 120-121°C, [4]) was characterised by ^{1}H-NMR, MS and elemental analysis. [^{1}H-NMR (90 MHz, $CDCl_3$), δ.ppm., 1.22 (t, 3H, CH_2-C<u>H</u>$_3$), 4.2 (s, 3H, N-C<u>H</u>$_3$), 4.3-4.65 (m, 2H, C<u>H</u>$_2$-CH_3), 7.5-8.2 (m, 5H, Ar-<u>H</u>) and 13.1-13.8 (br s, 1H, N-O<u>H</u>); MS: (EI, 35ev) *m/z* (relative intensity %), 151 ($M.^+$, 10), 136 (24), 135 (48), 134 (11), 122 (8), 121 (33), 120 (100), 107 (19), 106 (63), 105 (7), 79 (7), 77 (14), 36 (29); Elemental Analysis: Found (%), C, 57.48; H, 7.34; N, 7.53; Calculated (%) for $C_9H_{13}NO.HCl$, C, 57.60; H, 7.52; N, 7.46].

Preparation of hepatic microsomes. Livers were excised from male Wistar rats (250 g) and male NZW rabbits (2.5 kg), washed and homogenised in tris/KCl buffer (0.25 M, pH 7.4). The microsomal fractions were isolated by differential centrifugation and resuspended in tris/KCl buffer to a final volume equivalent to 0.5 g of original liver/ml. All procedures were carried out at 0°C.

In vitro incubations. EMA HCl (2 μmole/0.5 ml water, final concentration 1 mM) was incubated in the presence of hepatic microsomal suspension (1 ml) containing 5-10 mg protein and appropriate cofactors [NADP (2 μmole) and glucose-6-phosphate dehydrogenase (1 unit), (BCL, Lewes, U.K.), glucose-6-phosphate (10 μmole) and magnesium chloride (20 μmole), (Sigma, Poole, U.K.) in phosphate buffer pH 7.4, 0.2 M (0.5 ml)] at 37°C for up to 60 minutes. The incubations were terminated by the addition of concentrated HCl (15 μl) and stored on ice until extraction. Immediately prior to extraction, the incubates were neutralised by addition of saturated sodium hydroxide solution (15 μl) and an internal standard was added (50 μg *N-i*-butylaniline).

Solid-phase extraction of samples. The microsomal incubation samples were divided into 2 x 1 ml aliquots, centrifuged and each extracted using either a C8 or a C18 100 mg solid-phase extraction cartridge (Jones Chromatography, Mid-Glamorgan, U.K.). Extraction of the first aliquot was carried out using a C8 cartridge which allowed the isolation of the *N*-oxide with the *N*-dealkylated metabolites. After application of the samples (1 ml) the cartridges were washed with phosphate buffer (pH 7.4, 0.2 M, 1 ml) and the analytes eluted with 200 μl of acetonitrile : methanol (1 : 1, v/v; acidified with phosphoric acid, 5 μl/ml eluent). The eluted sample was then diluted with an equal volume of the HPLC mobile-phase buffer (see below) and an aliquot (100 μl) analysed using reverse-phase HPLC.

The second aliquot (1 ml) was extracted using a C18 cartridge which allowed selective isolation of the *N*-oxide metabolite. Following sample application the cartridge was washed with methanol : water (1 : 9 v/v,

0.5 ml) and the analyte eluted with methanol : water (2 : 3, v/v, 0.5 ml). The eluent was lyophilised, the residue reconstituted in hexane : ethanol (3 : 1, v/v, 40 μl) and analysed (20 μl) using a chiral HPLC stationary-phase to determine the enantiomeric composition of the *N*-oxide.

Chromatographic analysis of the extracts. Quantitative analyses of the extracts from the C8 cartridges were performed using a Spherisorb 5 ODS1 (25 cm x 4.6 mm i.d.) reverse-phase HPLC column (LDC, Staffs, U.K.). The mobile-phase consisted of acetonitrile (Rathburn, Walkerburn, U.K.): citric acid/phosphate buffer (0.015 M, pH 8.0), (53 : 47, v/v), with a flow rate of 1 ml/min, monitoring the eluent at 210 nm. Standard curves were prepared from microsomal media for the *N*-dealkylated metabolites (*N*-ethylaniline, *N*-methylaniline and aniline) using authentic material (Aldrich, Gillingham, U.K) and for the *N*-oxide metabolite.

The enantiomeric composition of the *N*-oxide metabolite was determined using a Chiralcel OD (25 cm x 4.6 mm i.d.) HPLC column (HPLC Technology Ltd, Macclesfield, U.K.). The mobile-phase consisted of hexane : ethanol (Rathburn, Walkerburn, U.K.), (98 : 2, v/v), at a flow rate of 1 ml/min, monitoring the eluent at 210 nm. All analyses were performed at ambient temperature.

RESULTS AND DISCUSSION

Following incubation of EMA with hepatic microsomal preparations from both the rat and rabbit, the major routes of oxidative metabolism in both species were *N*-demethylation, *N*-deethylation and *N*-oxidation. *N*-Oxide formation was similar for both species, *ca* 8 % and 10 % being recovered as the *N*-oxide after an incubation period of 60 minutes with the rat and rabbit tissues, respectively.

The chromatographic resolution of the synthetic enantiomeric *N*-oxides of EMA was achieved using the chiral HPLC stationary-phase described above (t_{R1} = 49.59 min; t_{R2} = 58.16 min; separation factor, α = 1.18, Figure 2a). The elution order of the two enantiomers was determined by partial resolution of the racemate by complexation with either (+)-(*R*)- or (-)-(*S*)-2,2′-dihydroxy-1,1′-binaphthol according to the method of Toda *et al* [5]. Using these enriched samples, the chromatographic elution order of the *N*-oxide enantiomers was determined to be *R* before *S*.

Examination of the *N*-oxide metabolites using the chiral HPLC system indicated that *N*-oxidation of EMA with both rat and rabbit microsomes was stereoselective, affording predominantly the (-)-(*S*)-enantiomer (Figures 2b and 2c). Differences in the enantiomeric ratios were demonstrated, with measured enantiomeric excesses of 52 ± 2.5 % and 65 ± 2.1 %, (n=3, obtained using 3 separate microsomal preparations), with respect to the (-)-(*S*)-enantiomer, using rat and rabbit tissue respectively.

These preliminary observations with EMA are significant as they show that *N*-oxidation can be a quantitatively important route of metabolism, that this process is subject to product stereoselectivity and that species differences exist with respect to the extent of stereoselectivity of the *N*-oxidation. There are limited data in the literature with respect to the stereoselectivity of enzymic *N*-oxidation of prochiral tertiary amines. Ziegler *et al* [6], as part of a review, presented data on EMA *N*-oxide isolated from rat urine following administration of EMA and from incubations of EMA with purified porcine hepatic flavin-containing monooxygenase. The authors demonstrated, by use of ORD measurements, that the (-)-isomer of the *N*-oxide appeared to predominate, a result in agreement with our *in vitro* data. Experimental data were lacking in this review [6] and no attempt was made to determine either the enantiomeric composition or the absolute configuration of the predominant *N*-oxide enantiomer.

We are aware of only one other report of stereoselective oxidation of a prochiral nitrogen centre, an as yet unpublished meeting abstract by Lindeke *et al* [7] who examined the *in vitro* metabolic *N*-oxidation of the irreversible monoamine oxidase inhibitor pargyline (*N*-benzyl-*N*-methyl-2-propynylamine) using rat hepatic microsomal preparations. The metabolic *N*-oxide produced was shown to consist of a single enantiomer by chromatographic analysis using a protein based chiral HPLC stationary-phase known to resolve the synthetic racemic material. The absolute configuration of the metabolite was however not

reported.

Systematic studies on the *N*-oxidation of prochiral tertiary amines are imperative for structure metabolism studies, since the stereochemical nature of the active site of the enzyme(s) responsible can best be understood by use of substrates, or the formation of products, containing one or more chiral centres. A judicious choice of congeners may facilitate a clearer understanding of the active site(s) of the tissue and species specific isoforms of the flavin-containing monooxygenase, which is primarily responsible for the direct two electron *N*-oxidation of tertiary aliphatic, alicyclic and *N*,*N*-dialkylaryl amines [3]. Such investigations are currently in progress.

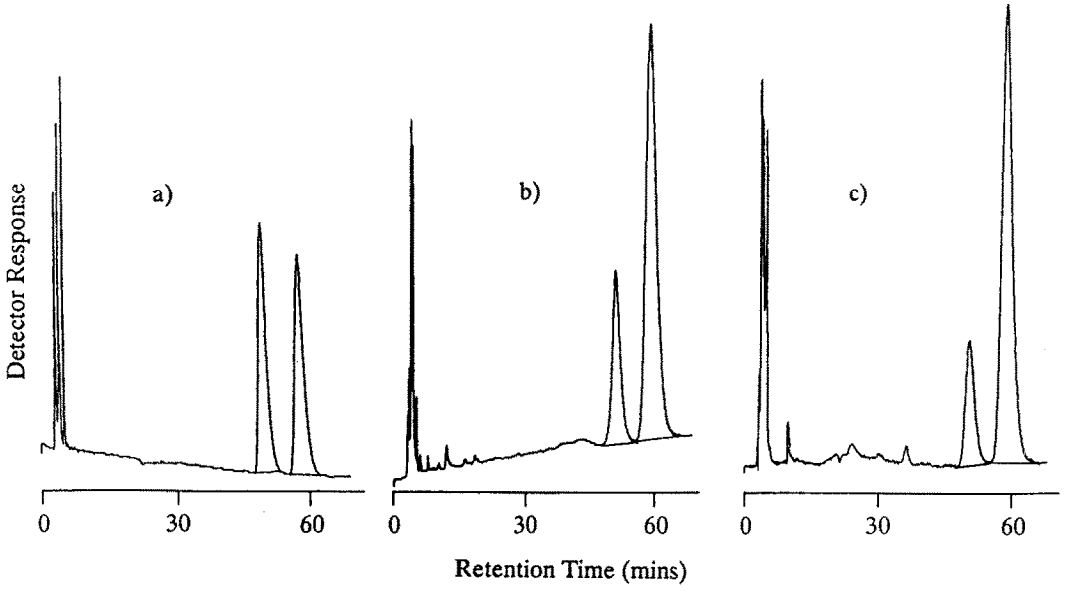

Fig. 2. Chromatographic resolution of EMA *N*-oxide enantiomers using the Chiralcel OD stationary-phase.
a). Synthetic racemic EMA *N*-oxide
b). Metabolic products from incubation of EMA with rat hepatic microsomes
c). Metabolic products from incubation of EMA with rabbit hepatic microsomes

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