J. Pharm. Pharmac., 1971, **23**, 941–946 *Received April* 26, 1971

The influence of methyl substitution on the *N*-demethylation and *N*-oxidation of normethadone in animal species

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N-Monodemethylation and *N*-oxidation were shown to be the major routes of metabolism of normethadone, (—)-methadone and (—)-isomethadone *in vitro* by hepatic microsomal preparations from rat, rabbit, guinea-pig, mouse and hamster. The rate of *N*-oxidation was decreased and the rate of *N*-demethylation was increased by the introduction of the methyl substituent into normethadone; the configuration of the methyl substituent influenced these processes. K_m and V_{max} values were determined for liver microsomal *N*-demethylation of normethadone, (—)-methadone and (—)-isomethadone by rat and guinea-pig and for the *N*-oxidation of normethadone by guinea-pig. The use of selective inhibitors showed that *N*-demethylation was not preceeded by *N*-oxidation in these compounds.

Rat, rabbit and guinea-pig liver microsomal preparations demethylate methadone (Axelrod, 1956), and (Beckett, Taylor & others, 1968) established that methadone was *N*-demethylated in man to a secondary amine which spontaneously rearranges to a pyrroline derivative. Subsequently, methadone, isomethadone and normethadone were shown to be converted to *N*-oxides and to be demethylated to their corresponding pyrroline derivative by guinea-pig microsomal preparations (Beckett, Mitchard & Shihab, 1971).

It has been suggested that *N*-dealkylation proceeds via an *N*-oxide intermediate which rearranges to form a biochemically unstable *N*-alkylcarbinol (Fish, Johnson & others, 1955; Fish, Sweeley & others, 1956; Petitt & Ziegler, 1963). Stabilized *N*-hydroxymethyl intermediates have since been isolated (McMahon, 1966; Gorrod, Temple & Beckett, 1970), but the formation of the precursor *N*-oxide is disputed by McMahon & Sullivan (1965).

To provide more definitive information, the influence of α- or β-methyl substitution on the rates of *N*-oxidation and demethylation of normethadone by liver microsomes of different species and the effect of selective inhibitors on those processes have been investigated.

METHODS

Young adult male animals (Wistar rats, 250–300 g; Duncan Hartley guinea-pigs, 350–400 g; New Zealand white rabbits, 1·5–2·0 kg; Swiss albino mice, 25 g; golden hamsters, 90–100 g) were killed, the livers rapidly removed and homogenized with an Ultra-turrax (Janke & Kunkel K.G., Stanfeni. Br., Germany) and fortified incubation mixtures prepared as described previously (Beckett & others, 1971).

Substrate studies. (—)-Methadone (National Institute of Health, Bethesda); (—)-isomethadone (Sterling Winthrop Research Institute) and normethadone

(Janssen Pharmaceutica) (5 μmol) were each incubated for 80 min at 37° in a mixture containing, (i) whole liver homogenate, (ii) 10 000 *g* supernatant, (iii) washed and resuspended microsomes and (iv) 140 000 *g* supernatant. In each case the liver preparations contained the equivalent of 0·5 g liver preparation in isotonic KCl (2ml), and the respective preparations were added to separate incubation mixtures to give a final volume of 6 ml. (+)-Methadone and (+)-isomethadone (National Institute of Health, Bethesda) (5 μmol) were incubated with 10 000 *g* supernatant. Methadone *N*-oxide, isomethadone *N*-oxide and normethadone *N*-oxide (prepared by Beckett & others, 1971) (5 μmol) were separately incubated with (i) whole liver homogenate, (ii) 10 000 *g* supernatant or (iii) heat inactivated 10 000 *g* supernatant respectively.

Kinetic studies. The K_m and the maximum rates of metabolism (V_{max}) for microsomal *N*-demethylation of normethadone, (−)-isomethadone and (−)-methadone by rat and guinea-pig and for the *N*-oxidation of normethadone by guinea-pig were determined over the concentration range 5×10^{-5} to 2×10^{-3}M using the washed microsome preparation. The K_m values were calculated from Lineweaver and Burke double reciprocal plots.

Inhibitors studies. The metabolism of normethadone (5 μmol) in incubates containing 10 000 *g* supernatant preparations from guinea-pig liver was investigated in the presence of the following inhibitors: 2-mercaptoethylammonium chloride (cysteamine HCl), *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, 1,10-phenanthroline hydrate (all obtained from BDH), dithiothreitol (P.L. Biochemicals Inc.), SKF 525A (Smith Kline and French) and sodium cyanide (in all cases [I] = 10^{-3}M). Normethadone and the inhibitors were incubated together for 60 min at 37°.

Assay procedure. Normethadone, isomethadone and methadone, their cyclic metabolites and *N*-oxides were extracted and assayed as before (Beckett & others, 1971).

RESULTS AND DISCUSSION

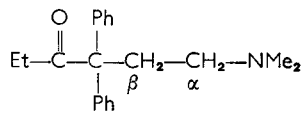
Preliminary studies with fractions from guinea-pig liver indicated that maximal *N*-demethylation and *N*-oxidation of normethadone and its methyl analogues occurred in the 10 000 *g* supernatant (Table 1). Whole liver homogenate and washed microsomes, both fortified with the same co-factors, were less active, whilst no *N*-oxidation and very little *N*-demethylation occurred with the 140 000 *g* supernatant (soluble fraction). The lower activity of the whole homogenate was probably due to the presence of inhibitors since Mitchard (1970) has shown that *N*-demethylation of different substrates by liver microsomal fractions increased as the fraction was purified. The apparent lower activity of the washed microsomes compared with the 10 000 *g* supernatant is due to the recording of results as total activity; purification procedures only increase specific activity.

The influence of α- and β-methyl substitution on the metabolism of normethadone. The 10 000 *g* supernatants of guinea-pig, hamster, rat, mouse and rabbit livers were used to study the effect of species variation on the rate of *N*-dealkylation and *N*-

Table 1. *The percentage recovery of normethadone, (—)-isomethadone and (—)-methadone* (U) *and their respective cyclic metabolites* (C) *and* N-*oxides* (O), *after incubation* (80 *min*) *with different fractions of guinea-pig liver microsomal preparations.* Determination using different livers gave comparable order of results.

	Normethadone			(—)-Isomethadone			(—)-Methadone		
Liver fraction	U	C	O	U	C	O	U	C	O
Whole liver homogenate	51·0	28·5	8·0	48·0	36·0	4·0	41·0	40·0	1·5
Soluble fraction	94·0	3·0	1·0	87·0	2·0	0·0	86·5	5·5	0·0
10 000 *g* supernatant	10·5	39·5	27·0	8·0	63·0	8·5	13·0	65·0	2·5
Washed microsomes and resuspended in isotonic KCl	47·5	20·0	15·0	50·0	27·0	5·0	46·0	34·5	2·0

oxidation of methadones. Methyl substitution reduced the rate of *N*-oxidation in all species; a β-methyl substituent (isomethadone) significantly reduced the rate whilst



Normethadone

an α-methyl substituent (methadone) almost abolished activity (Fig. 1). Conversely, with the exception of the rat, methyl substitution slightly increased the rate of *N*-demethylation, an α-methyl being more effective than a β-methyl substituent. The total metabolism of these compounds by other routes in different species cannot be correlated with the effect of methyl substitution (Fig. 1).

The effect of configuration of the methyl substituents. *S*-(+)-Methadone and *R*-(+)-isomethadone were *N*-monodemethylated (Fig. 2) by the 10 000 *g* rat liver supernatant slightly more rapidly than their enantiomorphs. However, the 10 000 *g*

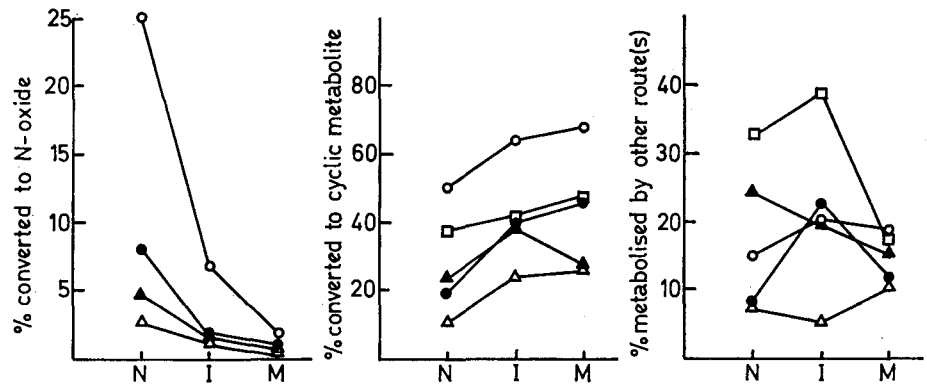


FIG. 1. The effect of methyl substitution in normethadone on *N*-oxidation, *N*-monodemethylation and metabolism by other route(s), using liver microsomal preparations (10 000 *g*) from guinea-pig —○—, hamster —●—, rat —▲—, mouse —△— and rabbit —□—. The linking lines have no significance and serve only to emphasize the trends seen in each species. *N*-oxidation as measured by *N*-oxide formation, *N*-monodemethylation as measured by cyclic metabolite formation. Incubation time 80 min. N = normethadone, I = (—)-isomethadone, M = (—)-methadone.

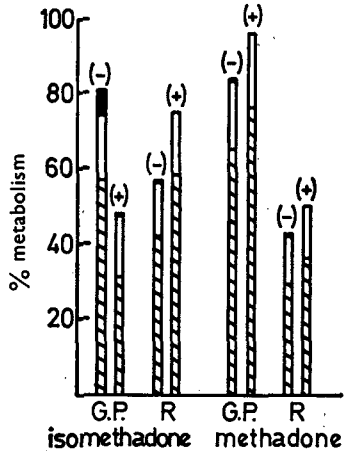


FIG. 2. The effect of stereochemistry of the methyl group in methadone and isomethadone on *N*-oxidation (solid columns), *N*-monodemethylation (hatched columns) and metabolism by other routes (open columns) using liver microsomal preparations (10 000 *g* supernatant) of rat and guinea-pig. Incubation time 80 min. GP = guinea-pig, R = rat.

guinea-pig liver supernatant, although *N*-dealkylating *S*-(+)- more than *R*-(−)-methadone, demethylated *S*-(−)-isomethadone more rapidly than its isomer.

The differences between the rates of metabolism of the isomers were greater with a β- than with an α-methyl substituent. *N*-Oxidation of methadone was only a minor route of metabolism. More *N*-oxide was formed with *S*-(−)- than with *R*-(+)-isomethadone using guinea-pig and to a lesser extent rat liver preparations.

Enzyme substrate characteristics. The guinea-pig and rat enzyme-substrate characteristics (V_{max} and K_m) are presented in Table 2. It was possible only to determine the K_m value ($5{\cdot}4 \times 10^{-4}$M) for the *N*-oxidation of normethadone with the guinea-pig liver preparation because, in all other cases, rates of *N*-oxidation were too slow to permit accurate measurement; this value is very similar to the corresponding K_m value ($5{\cdot}0 \times 10^{-4}$M) for the *N*-demethylation of normethadone.

Metabolism of the N-*oxides.* There was negligible metabolism of the *N*-oxides by the 10 000 *g* supernatants from rat, guinea-pig, mouse and hamster, incubated at 37° for 80 min. The heat inactivated preparations gave similar results. Although

Table 2. *Enzyme-substrate characteristics* (K_m *and* V_{max}) *for* N-*monodemethylation and* N-*oxidation of normethadone,* (−)-*isomethadone and* (−)-*methadone by guinea-pig and rat liver microsomes* (140 000 *g*). The rates were linear up to 15 min; V_{max} represent nmol g^{-1} liver min^{-1}.

	N-Monodemethylation of						*N*-Oxidation of	
	Normethadone		(−)-Isomethadone		(−)-Methadone		Normethadone	
Species	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Guinea-pig ..	$5{\cdot}0 \times 10^{-4}$M	38	$2{\cdot}5 \times 10^{-4}$M	43	$3{\cdot}5 \times 10^{-4}$M	56	$5{\cdot}4 \times 10^{-4}$M	27
Rat	$2{\cdot}5 \times 10^{-4}$M	33	$2{\cdot}2 \times 10^{-4}$M	45	$2{\cdot}1 \times 10^{-4}$M	38	—	—

Table 3. *The effect of various inhibitors on* N-*monodemethylation and* N-*oxidation of normethadone by guinea-pig* (10 000 *g*) *liver microsomes.* Incubation time was 60 min at 37°C. Metabolism in absence of inhibitors considered as 100%.

1×10^{-3}M inhibitors	Exp.	Percentage metabolism by different routes *N*-Monodemethylation	*N*-Oxidation	Other route(s)
SKF 525A	1	15	105	43
	2	13	105	40
2-Mercaptoethyl ammonium chloride	1	60	10	90
	2	65	12	87
Sodium cyanide	1	76	105	125
	2	80	100	125
-Chloromercuribenzoic acid	1	82	76	150
	2	75	68	140
N-Ethylmaleimide	1	77	97	100
	2	83	92	100
1,10-Phenanthroline hydrate	1	39	63	100
	2	41	62	100
Dithiothreitol	1	88	17	120
	2	90	16	125

some *N*-oxide (12%) was metabolized by the whole homogenate, this observation alone is insufficient to implicate *N*-oxide formation in the process of *N*-dealkylation particularly as neither cyclic metabolites nor tertiary amines were detected when the *N*-oxides were incubated with the 10 000 *g* supernatant or heat inactivated 10 000 *g* supernatant.

Inhibitors studies. Enzyme inhibitors studies provided further evidence that *N*-oxidation and *N*-dealkylation are independent processes. The percent *N*-oxidation and *N*-demethylation of normethadone after incubation with a number of inhibitors is shown in Table 3. The enzymic reactions were inhibited to different extents by all the inhibitors studied, but in particular SKF 525A specifically inhibited *N*-dealkylation and was without effect on *N*-oxidation whereas dithiothreitol was a specific inhibitor of *N*-oxidation and only slightly (10%) inhibited *N*-dealkylation. Selective inhibition of *N*-oxide and cotinine formation from nicotine has also been observed (Gorrod & Keysell, unpublished observation). Although the data obtained from inhibitor studies with SKF 525A do not preclude the possibility of the *N*-oxide forming as an intermediate in the process of *N*-dealkylation, data obtained from studies with dithiothreitol can only be explained if the two processes of *N*-oxidation and *N*-dealkylation occur independently.

N-*Oxidation and* N-*dealkylation as separate metabolic pathways*

The above isomeric substrate studies showed that the steric features which promoted *N*-oxidation reduced the rate of *N*-dealkylation. Methyl substitution in the vicinity of the nitrogen atom of normethadone significantly inhibited *N*-oxidation but the same substituent slightly increased *N*-dealkylation. *N*-Dealkylation but not *N*-oxidation reaction showed stereo-selectivity. The kinetic data therefore indicate that the two reactions proceed independently.

The metabolic studies with *N*-oxides as substrates failed to demonstrate demethylation and the inhibitor studies established different inhibitor profiles for *N*-oxidation and *N*-demethylation.

The results support therefore the conclusion that *N*-dealkylation of normethadone and its analogues is not preceded by *N*-oxidation in accord with similar conclusions of McMahon & Sullivan using propoxyphene (1965).

Acknowledgements

We thank The Medical Research Council for a grant in support of this work. One of us (A.A.S.) is indebted to The Calouste Gulbenkian Foundation for a scholarship.

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