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Identification and quantitative determination of some metabolites of methadone, isomethadone and normethadone

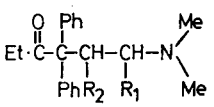
A. H. BECKETT, M. MITCHARD AND A. A. SHIHAB

Department of Pharmacy, Chelsea College (University of London), London, S.W.3, U.K.

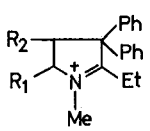
Isomethadone and normethadone are metabolized by microsomal preparations of guinea-pig liver to yield 2-ethyl-1,4-dimethyl-3,3-diphenyl-1-pyrroline and 2-ethyl-1-methyl-3,3-diphenyl-1-pyrroline respectively. The structures of the pyrrolines were established (by comparison with the pyrroline derived from methadone) by thin-layer chromatography and by infrared and nuclear magnetic resonance spectral data. Methadone, isomethadone and normethadone are also metabolized to the corresponding *N*-oxides. A gas chromatographic procedure for the quantitative determination of unchanged drugs, cyclic metabolites and *N*-oxides of methadone, isomethadone and normethadone in microsomal homogenates is described. The *N*-oxides were reduced before analysis.

Normethadone (Ic) has been reported by Vidic (1957), on the evidence of paper chromatography, to be *N*-demethylated in man to the corresponding primary and secondary amines, whilst Yoshida (1958) suggested that rats reduced normethadone to normethadol which was excreted partly as its glucuronide.

However, Beckett, Taylor & others (1968) established that methadone (Ia) is *N*-demethylated in man to a secondary amine which spontaneously rearranges to a pyrroline derivative (IIa) as the major product of metabolism.



Ia) methadone: R_1=Me, R_2=H
b) isomethadone: R_1=H, R_2=Me
c) normethadone: R_1=R_2=H



II a) pyrroline derived from methadone: R_1=Me, R_2=H
b) pyrroline derived from isomethadone: R_1=H, R_2=Me
c) pyrroline derived from normethadone: R_1=R_2=H

The structures of methadone, isomethadone and normethadone are similar, and it is therefore reasonable to assume that the major metabolites of isomethadone and normethadone would also be the corresponding pyrrolines (II).

N-Oxidation is now known to be a significant route of metabolism for some tertiary amines, e.g. propoxyphene (McMahon & Sullivan, 1964), imipramine (Fishman & Goldenberg, 1962), chlorpromazine (Beckett & Hewick, 1967) and chlorcyclizine (Kuntzman, Phillips & others, 1967).

Preliminary work in these laboratories indicated that methadone, isomethadone and normethadone gave *N*-oxides when incubated with hepatic microsomal preparations of some animal species. We have therefore investigated the structures of the

N-demethylated metabolites of isomethadone and normethadone, and developed a method for the quantitative analysis of the parent compounds (Ia, b and c), and their cyclic (IIa, b and c) and *N*-oxide metabolites, for each drug, in biological material.

METHODS

Thin-layer chromatography (*t.l.c.*). Glass plates 20 × 20 cm were spread to a thickness of 0·25 mm with a mixture of Silica Gel G (Merck) and water (1:2). The plates were first allowed to dry at room temperature (20°) for 10 min and then heated for 1 h at 110°. The solvent systems used are listed in Table 1. Dragendorff reagent was used to visualize the spots.

Table 1. *R_F values of methadone, isomethadone and normethadone and their cyclic and N-oxide metabolites on silica-gel plates, using different solvent systems.*

Solvent System	R_F values								
	M	Mc	Mo	I	Ic	Io	N	Nc	No
A	0·72	0·77	0·10	0·70	0·68	0·07	0·75	0·78	0·13
B	0·74	0·76	0·40	0·71	0·68	0·35	0·71	0·73	0·35
C	0·79	0·59	0·74	0·91	0·17	0·79	0·82	0·34	0·65

A = Chloroform–acetone–diethylamine (88:2:10).
B = Benzene–methanol–diethylamine (75:15:10).
C = Methanol–benzene–n-butanol–ammonia (0·88)–water (60:10:15:5:10).
M = Methadone, Mc = methadone cyclic metabolite, Mo = methadone *N*-oxide.
I = Isomethadone, Ic = isomethadone cyclic metabolite, Io = isomethadone *N*-oxide.
N = Normethadone, Nc = normethadone cyclic metabolite, No = normethadone *N*-oxide.

Gas-liquid chromatography (*g.l.c.*). A Perkin Elmer F11 chromatograph with a flame ionization detector was used. Chromatographic Column A was glass tubing $\frac{1}{4}$ inch o.d. and 2 m long, packed with acid washed, DMCS treated Gas Chrom Q (60–80 mesh) coated with OV17 (3% w/w). Column B was stainless steel tubing $\frac{1}{8}$ inch o.d. and 1 m long, packed with acid washed, DMCS treated chromosorb G (80–100 mesh) coated with Carbowax 6000 (2% w/w) and potassium hydroxide (5% w/w). The columns were kept for 24 h under their operating conditions before use, i.e. oven temperature 195° for Column A and 180° for Column B; injection block temperatures about 280°; hydrogen pressure, 20 lb/inch^2; air pressure, 25 lb/inch^2; nitrogen flow rate, 65 ml/min for column A and 36 ml/min for column B.

Isolation of the cyclic metabolites. Isomethadone (Ib) and normethadone (Ic) were each incubated at 37° with the 10 000 *g* microsomal supernatant preparation from male guinea-pig (Duncan Hartley, 400 g) liver homogenates which contained 1 g of liver in 2 ml isotonic KCl; each incubate contained 10 μmol of substrate, 4 μmol NADP, 20 μmol glucose-6-phosphate, 20 μmol $MgCl_2$, 60 μmol nicotinamide and 0·05M phosphate buffer, pH 7·4 in a total volume of 6 ml. The reaction was stopped after 80 min with 6N HCl (1 ml), and the precipitated proteins removed by centrifugation. The pH of each solution was adjusted to between 11 and 12 with sodium hydroxide (20%) and the solutes then extracted with redistilled diethylether (3 × 3 ml). The bulked ethereal extracts were concentrated to about 0·25 ml under reduced pressure. The cyclic metabolites were separated by preparative t.l.c. in the dark. Reference parent compounds were applied near the margins and solvent system C

(Table 1) was used for development. The plate margins were sprayed with Dragendorff reagent and the silica gel between the spots was collected in a glass stoppered centrifuge tube, distilled water (3 ml) and sodium hydroxide (0·2 ml) were added and the solution extracted with diethylether (3 × 3 ml). The ethereal solution was dried over anhydrous sodium sulphate, HCl gas passed, and the precipitated cyclic metabolite chlorides recrystallized from methanol.

The infrared spectra of these hydrochlorides as Nujol mulls were recorded using a Unicam SP200 infrared spectrometer.

The nmr spectra in D_2O were recorded using a Perkin Elmer R-10 nmr spectrometer plus a Northern Scientific 544 CAT with tetramethylsilane as the internal standard.

Isolation of the N-*oxides.* Methadone, isomethadone and normethadone were incubated with the 10 000 *g* liver homogenate described under 'isolation of the cyclic metabolites'. The solutions were extracted with chloroform and the concentrated chloroform extracts were applied to t.l.c. plates. Reference *N*-oxide spots were applied near the plate margins and solvent system B (Table 1) was used for the development. The plate margins were sprayed with Dragendorff reagent to visualize the reference spots and the silica gel between the spots corresponding to the *N*-oxides was collected and divided into two portions. Each portion was transferred to a glass-stoppered centrifuge tube. Distilled water (3 ml) and sodium hydroxide (0·2 ml) was added to one tube and the solution extracted with diethylether (3 × 3 ml). The ethereal solution was concentrated to about 50 μl and 2 μl was injected on to column A. To the other portion was added distilled water (3 ml), 6N HCl (0·3 ml) and 12·5% w/v $TiCl_3$ solution (0·2 ml, technical, Hopkin and Williams Ltd.) and the solution was stored at room temperature for 30 min. The solution was adjusted to pH 11–12, then extracted with diethylether and the solution concentrated and analysed on column A as above.

Synthesis of the N-*oxides* (*Ia, b and c*). The *N*-oxides of methadone, isomethadone and normethadone were prepared by the method published by Upjohn & Co. (1958) for the preparation of methadone *N*-oxide from methadone.

Stability of the tertiary amines (*Ia, b and c*) *to chemical reduction.* Methadone, isomethadone and normethadone (20 μg) as the hydrochlorides in distilled water (5 ml) were added separately to glass-stoppered centrifuge tubes; HCl (1 ml) and granular zinc (0·5 g) were added and the tubes then heated on a water bath at 60° for 0·25–3 h. At the end of the reaction, a solution (1 ml) of tripelannamine (10 μg) as internal standard was added to each tube and the solution adjusted to pH 11–12 with sodium hydroxide. The solution was extracted with ether and analysed by g.l.c. as above. The same procedure was repeated using $TiCl_3$ (0·2 ml) and HCl (0·3 ml) instead of granulated zinc and the solution stored in the dark at room temperature. The solution was extracted and analysed as above.

Quantitative analysis of the parent drug in the presence of its cyclic metabolite and N-*oxide.* Methadone (Ia), its cyclic metabolite (IIa) and its *N*-oxide (20 μg each) as the hydrochlorides in distilled water (4 ml) were placed in glass-stoppered centrifuge tubes together with tripelennamine (10 μg) as internal standard. Heat inactivated (10 000 *g*) liver homogenate (1 ml) was added. The solution was adjusted to pH 11–12 and extracted with diethylether (3 × 3 ml); the extract was concentrated and the unchanged drug and cyclic metabolite analysed using column A as above. The

extracted alkaline solution was made acid with HCl and the *N*-oxide reduced with $TiCl_3$ for 30 min as described above. The solution was then made alkaline, immediately extracted with ether and analysed as for parent drug. Retention times are in Table 2.

Table 2. *Retention times (min) of methadone, isomethadone and normethadone and their cyclic metabolites.*

	Methadone		Isomethadone		Normethadone	
Column	Unchanged drug	Cyclic metabolite	Unchanged drug	Cyclic metabolite	Unchanged drug	Cyclic metabolite
A	12·0	8·2	12·2	10·4	10·7	8·5
B	12·0	7·5	11·4	9·2	12·1	8·5

The same procedure was repeated with isomethadone (Ib) and normethadone (Ic) in the presence of their cyclic metabolites (IIb and c respectively) and their *N*-oxides.

N-*Demethylation of the tertiary amines (I b and c).* Isomethadone and normethadone (5 μmol each) were separately incubated with the 10 000 *g* liver homogenate previously described, to which semicarbazide (75 μmol in 1 ml of 0·05M phosphate buffer pH 7·4) had also been added. The reaction was stopped by the addition of $ZnSO_4$ (2 ml; 20%) and 2 ml saturated solution of $Ba(OH)_2$ and the precipitate removed by centrifugation. The amount of formaldehyde produced was determined by the method of Nash (1953).

RESULTS AND DISCUSSION

Incubation of isomethadone and normethadone with guinea-pig liver homogenate yielded formaldehyde which indicates that these compounds were *N*-demethylated in a manner similar to methadone.

Structure of the pyrroline metabolites. The ethereal extract of the solutions obtained from the isomethadone incubate yielded two spots on t.l.c. The R_F value of one spot corresponded with that of isomethadone, and the other spot gave R_F values in systems A and B that were similar to the R_F values obtained for the pyrroline derivative of methadone (see Table 1); t.l.c. was carried out in the dark since the metabolite was photosensitive.

Analysis by g.l.c. (Column A and B) of the ethereal extract of isomethadone gave two peaks, one of which corresponded to isomethadone whilst the other was of shorter retention time. The structure of the isomethadone metabolite giving the second spot or peak was proved to be the substituted pyrroline chloride (IIb) by the following evidence.

(i) The infrared spectrum of the chloride showed no $\overset{+}{N}$-H band corresponding to that of tertiary amine salt. (ii) The unusually high intensity of the adsorption band at 1665 cm^{-1} suggested a C=N stretching frequency rather than C=C. (iii) There was no absorption band at 1710 cm^{-1} corresponding to the carbonyl group in the parent compound. (iv) The nmr signal at 6·3τ gave integral for three protons which indicated one *N*-methyl substituent. (v) The triplet at 9·3τ indicated the presence of an endocyclic alkene, whereas the lack of signal at about 2·3τ indicated the absence

of an olefinic proton and thus the absence of the exocyclic double bond. (vi) No signal for vinyl hydrogen was obtained.

It is therefore concluded that metabolic monodemethylation followed by cyclization and dehydration occurs for isomethadone in a manner similar to that described for methadone (Beckett & others, 1968).

Similar ethereal extracts of the products of metabolism of normethadone also gave two spots on t.l.c. (and two peaks by g.l.c.), one of which corresponded to that of the unchanged drug and the other to the cyclic structure (IIc) as indicated by evidence similar to that presented for isomethadone. The double bond of the salt was again endocyclic (IIc).

Assay procedure for determining the tertiary amines and their metabolites. Synthetic methadone *N*-oxide was reduced quantitatively to methadone with $TiCl_3$/HCl. This treatment did not reduce methadone itself, nor did the presence of extracts from liver microsome fractions interfere. Similar results were obtained using isomethadone and normethadone. However, reduction with Zn/HCl was unsatisfactory as both the carbonyl and *N*-oxide were reduced. There was also some decomposition of the tertiary amines, when the reduction mixtures were stored after the acidic $TiCl_3$ was made alkaline.

Table 3. *Percentage recovery of methadone, isomethadone and normethadone and their corresponding cyclic and* N*-oxide metabolites after addition of each drug with its corresponding metabolites to heat-inactivated guinea-pig liver homogenate.*

Compound	Recovery		
	Unchanged drug	Cyclic metabolite	*N*-Oxide
Methadone	94·6	95·6	85·5
Isomethadone	96·4	97·5	86·2
Normethadone	94·0	98·4	91·2

A chloroform extract of a methadone incubation mixture gave spots on t.l.c. (solvent system A) the R_F values of which corresponded to those of synthetic methadone *N*-oxide, cyclic metabolite and methadone. The presence of the *N*-oxide was further confirmed by extraction from the t.l.c. plate followed by $TiCl_3$/HCl reduction to give methadone as confirmed by g.l.c. analysis.

Analysis by g.l.c. of an ethereal extract of the spot corresponding to *N*-oxide in the thin-layer chromatogram showed that methadone was absent before reduction. Ethereal extraction could therefore be used to remove unchanged methadone and its cyclic metabolite from an incubate to leave the *N*-oxide in the aqueous alkaline solution. Reduction of the *N*-oxide remaining in solution by the method described in the experimental section reformed methadone quantitatively, which was subsequently extracted and analysed by g.l.c. The product was analysed immediately after the solution was made alkaline because some decomposition of the tertiary amine ketones occurred under alkaline conditions. Similar results were obtained when isomethadone and normethadone were used.

A quantitative method for the analysis of unchanged methadone, its cyclic metabolite and its *N*-oxide was therefore developed as described in the experimental section. The method involved extracting the drug and its cyclic metabolite from an alkaline solution into ether and subjecting the extract to quantitative g.l.c. analysis using tripelennamine as internal standard. The *N*-oxide remaining in the alkaline solution was reduced with $TiCl_3$ after acidification with HCl to yield the parent amine which was extracted and analysed by g.l.c. The percentage recovery of these three compounds added to heat-inactivated liver homogenate is shown in Table 3. The method was also applied successfully to the analysis of isomethadone and its cyclic metabolite and *N*-oxide and to normethadone and its corresponding metabolites when present together in biological material.

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