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COMPARISON OF THE OXIME AND THE HYDROXYLAMINE DERIVATIVES OF 4-CHLOROAMPHETAMINE AS DEPLETORS OF BRAIN 5-HYDROXYINDOLES

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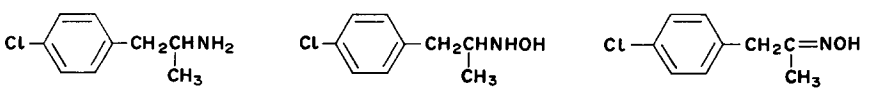
Abstract—Brain 5-hydroxyindole levels were measured 6 hr after the i.p. injection of 4-chloroamphetamine and two of its possible metabolites—the oxime and the hydroxylamine. Both 4-chloroamphetamine and *N*-hydroxy-4-chloroamphetamine at 0·1 m-mole/kg caused marked reduction of serotonin and of 5-hydroxyindoleacetic acid levels, whereas the oxime had only a slight effect on serotonin levels. The *N*-hydroxy derivative appeared to be almost completely reduced to 4-chloroamphetamine *in vivo*, for it produced brain levels of 4-chloroamphetamine equivalent to those produced by 4-chloroamphetamine itself and lowered brain 5-hydroxyindole levels as much as did 4-chloroamphetamine at three different dose levels. Our results make it seem unlikely that 4-chloroamphetamine's depletion of brain serotonin and 5-hydroxyindoleacetic acid is mediated by the conversion of 4-chloroamphetamine to these metabolites.

4-CHLOROAMPHETAMINE depletes the brain of both serotonin and its major metabolite, 5-hydroxyindoleacetic acid. The action of 4-chloroamphetamine on brain serotonin is probably complex,[1] but the reduction of both serotonin and 5-hydroxyindoleacetic acid levels could come about primarily through inhibition of tryptophan 5-hydroxylation. Sanders-Bush *et al.*[2,3] have reported that 4-chloroamphetamine injection into rats leads to a marked decrease in cerebral tryptophan hydroxylase levels, despite the inability of the drug to inhibit tryptophan hydroxylase *in vitro*. Whether the decline in tryptophan hydroxylase levels *in vivo* represents a specific inactivation of the enzyme or a generalized destruction of serotoninergic nerve terminals is not clear. In either case, the possibility must be considered that a metabolite of 4-chloroamphetamine rather than the drug itself is responsible for the reduction of brain 5-hydroxyindole levels *in vivo*. The present study compares the effects of two possible metabolites of 4-chloroamphetamine, the oxime and the hydroxylamine (Fig. 1).



4-Chloroamphetamine Hydroxylamine Oxime

FIG. 1. 4-Chloroamphetamine and two of its possible metabolites.

Both substances could be formed through oxidative attack on the amine nitrogen of 4-chloroamphetamine. The oxime has in fact been identified as a metabolite of 4-chloroamphetamine formed *in vitro* by liver microsomes.* *N*-hydroxy-amphetamine is a metabolite of amphetamine *in vitro*, and *N*-hydroxylation has been suggested to be a general metabolic pathway for amine metabolism.[4]

MATERIALS AND METHODS

4-Chloroamphetamine HCl was purchased from the Regis Chemical Co. *N*-hydroxy-4-chloroamphetamine HCl and 4-chlorophenylacetone oxime were synthesized in the Lilly Research Laboratories.

Male albino rats (Wistar, 150 g body weight) were obtained from a local supplier. The rats were given intraperitoneal injections of drugs in aqueous solutions and then were killed by cervical dislocation. Whole brains were removed, frozen immediately on dry ice, and stored at $-15°$ prior to analysis.

4-Chloroamphetamine levels in brain were measured by reaction with fluorescamine.[5] Tissue was homogenized in 4 vol. of 0·1 N HCl, then 1 vol. of 30% perchloric acid was added. After centrifugation, 1 ml of the supernatant fluid was added to screw-cap tubes containing 0·4 ml of 5 N NaOH and 4 ml toluene. The tubes were shaken for 10 min and centrifuged. A 3-ml aliquot of the toluene phase was washed by shaking with 1 ml of 0·2 M borate buffer for 10 min. After centrifugation, a 2-ml aliquot of the toluene phase was added to 2 ml of 0·1 N HCl. The tubes were shaken for 10 min and centrifuged. The toluene layer was aspirated and 1 ml of the acidic aqueous solution was added to 2 ml of 0·2 M borate buffer (pH 9). After addition of 1 ml fluorescamine solution (10 mg dissolved in 100 ml acetone), the fluorescence of the samples was measured (excitation 390 nm, emission 490 nm) and converted to nmoles of drugs by comparison to a standard curve for 4-chloroamphetamine. Recovery of 4-chloroamphetamine was greater than 95 per cent. Other toluene extracts prepared as above to the borate-wash step were dried under a stream of nitrogen, and the *p*-trifluoroacetyl derivatives of the amines were prepared according to the method of Anggard and Hankey.[6] The mass spectra were recorded on an LKB 9000 combined gas chromatograph–mass spectrometer. The column was a 4-ft 3% OV-17. The flow rate of helium carrier gas was 60 ml/min. The temperatures used were: flash heater, 240°; column, 140°; separator, 240°; and ion source (290°), 70 eV.

Brain serotonin and 5-hydroxyindoleacetic acid levels were determined spectrofluorometrically by condensation with *o*-phthalaldehyde according to the following modification of a previous procedure.[7] Whole brain tissue was homogenized in 10 vol of cold, acidified *n*-butanol (0·85 ml concn. HCl per l.). After centrifugation for 10 min at 1200 *g* (0°), 4·0 ml of the supernatant fluid was transferred to screw-cap tubes containing 1·0 ml of 0·1 N HCl and 4·0 ml *n*-heptane. The tubes were shaken on a mechanical shaker for 15 min and centrifuged for 5 min at room temperature. At this point, serotonin is in the aqueous phase and 5-hydroxyindoleacetic acid is in the organic phase. A 7-ml aliquot of the organic phase was transferred to screw-cap tubes containing 1·0 ml of pH 7·0 sodium phosphate buffer (0·1 M, containing 1 mg/ml of cysteine to protect 5-hydroxyindoleacetic acid from oxidation). After the tubes were shaken and centrifuged as above, the organic phase was aspirated and a 0·5-ml aliquot of the aqueous phase was taken for 5-hydroxyindoleacetic acid

* C. J. PARLI and N. LEE, unpublished observations.

TABLE 1. BRAIN 5-HYDROXYINDOLE LEVELS IN RATS 6 hr AFTER THE I.P. INJECTION OF 4-CHLOROAMPHETAMINE OR ITS DERIVATIVES

Drug injected (0·1 m-mole/kg)	Brain 5-hydroxyindoles* (μg/g) Serotonin	5-Hydroxyindole-acetic acid
Control (saline)	0·60 ± 0·02	0·54 ± 0·02
Amine	0·24 ± 0·02 (P < 0·01)	0·32 ± 0·02 (P < 0·01)
Hydroxylamine	0·20 ± 0·01 (P < 0·01)	0·28 ± 0·02 (P < 0·01)
Oxime	0·48 ± 0·01 (P < 0·01)	0·49 ± 0·02 (NS)

* Mean values with standard errors for five rats per group are shown. NS = not significant.

analysis. The remainder of the organic phase was aspirated from the original screw-cap tube along with the tissue disc separating the aqueous phase. Then a 0·5-ml aliquot of the aqueous phase was taken for serotonin determination. For the determination of both 5-hydroxyindoles, the 0·5-ml aliquots were mixed with 1·0 ml of 0·01% *o*-phthalaldehyde solution (10 mg/100 ml of 10 N HCl). After the tubes were heated at 90° for 15 min, they were cooled, and the fluorescence was measured in an Aminco–Bowman spectrophotofluorometer (excitation 360 nm, emission 490 nm). Internal standard curves containing 0·125, 0·25 or 0·5 μg serotonin or 5-hydroxyindoleacetic acid per 0·5 g of tissue were used for converting fluorescence readings to 5-hydroxyindole concentrations. Recoveries of serotonin and 5-hydroxyindoleacetic acid were 77 and 84 per cent respectively.

RESULTS AND DISCUSSION

As shown by the data in Table 1, both serotonin and 5-hydroxyindoleacetic acid levels were greatly reduced by injection of the *N*-hydroxy derivative as well as by 4-chloroamphetamine itself. The differences between the amine and the hydroxylamine were not statistically significant. The oxime caused only a slight decrease in ser-

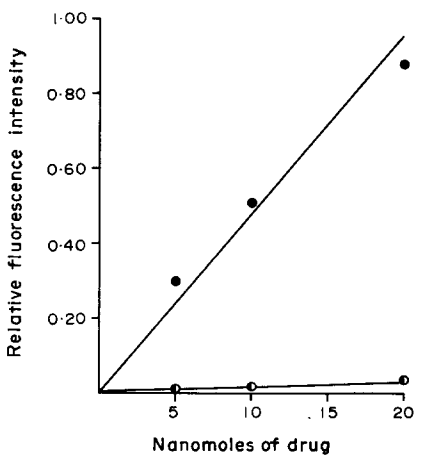


FIG. 2. Discrimination between 4-chloroamphetamine (●) and its hydroxylamine (◐) by reaction with fluorescamine.

TABLE 2. 4-CHLOROAMPHETAMINE LEVELS AND 5-HYDROXYINDOLE LEVELS IN RAT BRAIN 6 hr AFTER INJECTION OF 4-CHLOROAMPHETAMINE OR ITS HYDROXYLAMINE*

Treatment group	Dose (m-moles/kg)	4-Chloro-amphetamine level (nmoles/g)	Brain 5-hydroxyindoles (μg/g) Serotonin	5-Hydroxyindole-acetic acid
Control			0·62 ± 0·03	0·31 ± 0·02
4-Chloroamphetamine	0·025	25 ± 4	0·32 ± 0·02†	0·21 ± 0·01†
	0·05	42 ± 2	0·24 ± 0·02†	0·20 ± 0·01†
	0·1	105 ± 6	0·23 ± 0·02†	0·24 ± 0·03†
N-Hydroxy-4-chloroamphetamine	0·025	24 ± 2	0·26 ± 0·02†	0·16 ± 0·01†
	0·05	52 ± 2	0·36 ± 0·03†	0·19 ± 0·02†
	0·1	94 ± 4	0·32 ± 0·03†	0·23 ± 0·02†

* Drugs were injected i.p. 6 hr before the rats were killed. Mean values with standard errors for five rats per group are shown.

† Significantly different from control group, $P < 0·01$.

otonin levels and had no significant effect on 5-hydroxyindoleacetic acid levels. A possible reason for the ineffectiveness of the oxime might be that it does not cross the blood–brain barrier readily. (We have no data on that point.) The data in Table 1 suggest that neither the oxime nor any further metabolite from it plays a role in the reduction of brain 5-hydroxyindoles by 4-chloroamphetamine, assuming that any formation of the oxime from 4-chloroamphetamine would occur mainly in extracerebral tissues such as the liver. The findings leave open the possibility that the action of the amine and the hydroxylamine *in vivo* occurs after metabolic conversion of one to the other.

In order to assay differentially the amine and the hydroxylamine, we used fluorescamine, a newly described reagent that reacts specifically with primary amines to yield an intensely fluorescing derivative.[5] Figure 2 shows the ability of fluorescamine to discriminate between 4-chloroamphetamine and the *N*-hydroxyl derivative. The latter substance gave fluorescence readings only slightly above blank values (4 per cent of the 4-chloroamphetamine readings), and this slight reaction might be accounted for by the presence of small amounts of 4-chloroamphetamine as an impurity.

We next found that after injection of *N*-hydroxy-4-chloroamphetamine into rats, levels of 4-chloroamphetamine in brain were equivalent to those found after injection

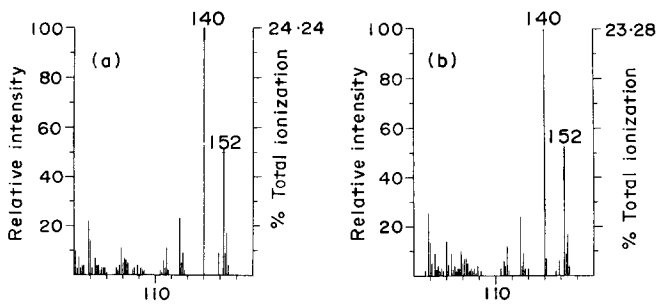
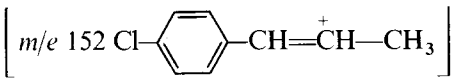
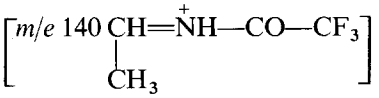


FIG. 3. Gas–liquid chromatography–mass spectrometry identification of 4-chloroamphetamine in brains from rats treated with (a) 4-chloroamphetamine or (b) *N*-hydroxy-4-chloroamphetamine. The relative intensity plots are obtained from the mass fragmentation patterns of the *N*-trifluoroacetyl derivative of 4-chloroamphetamine (M^+ 265).

of 4-chloroamphetamine itself (Table 2). The identity of the fluorescamine-reacting material was established as 4-chloroamphetamine by gas chromatography–mass spectrometry (Fig. 3). The mass spectra of the chloroamphetamine isolated from the brains of rats treated with either 4-chloroamphetamine (Fig. 3a) or *N*-hydroxy-4-chloroamphetamine (Fig. 3b) were essentially the same and identical to those of standard *N*-trifluoroacetyl-4-chloroamphetamine (not shown). The spectra showed that two main cleavages occurred, resulting in the formation of a 4-chloropropenyl ion



and the most abundant fragment



The fragmentation patterns are in agreement with the fragmentation data obtained with *N*-trifluoroacetyl-amphetamine.[6]

Quantitation of 4-chloroamphetamine levels by gas chromatography confirmed that 4-chloroamphetamine levels in brain were identical regardless of whether the hydroxylamine or 4-chloroamphetamine was injected. These results indicate that the *N*-hydroxy derivative is rapidly and almost quantitatively reduced to 4-chloroamphetamine when it is injected into rats. It is not surprising then that the effects of various doses of the two agents on serotonin and 5-hydroxyindoleacetic acid levels are virtually identical (Table 2). A marked and statistically significant reduction of both serotonin and 5-hydroxyindoleacetic acid levels occurred at all three doses of both drugs.

These results do not completely exclude the possibility that the actions of 4-chloroamphetamine on brain 5-hydroxyindoles are mediated by the hydroxylamine (if only small amounts of the substance were active), but that possibility seems unlikely. If a rapid equilibrium heavily in favor of the amine were established when either compound was injected, then the hydroxylamine could be responsible for the effects on 5-hydroxyindole levels, however, To rule out that possibility, we would like to be able to show that blocking the conversion of the hydroxylamine to 4-chloroamphetamine abolishes the ability of the hydroxylamine to lower brain 5-hydroxyindole levels. An attempt to block the conversion with β-diethylaminoethyl diphenylpropyl acetate (SKF 525A), a known inhibitor of microsomal drug-metabolizing enzymes in liver, was unsuccessful. Four hr after injection of 0·1 m-mole/kg i.p. of the *N*-hydroxy-4-chloroamphetamine into rats, drug levels of 4-chloroamphetamine in brain averaged 123 ± 5 nmoles/g in five rats. In rats pretreated 1 hr earlier with SKF 525A (10 mg/kg, i.p.), the average was 124 ± 5. Thus the conversion of the hydroxylamine to 4-chloroamphetamine is not sensitive to inhibition by this classic microsomal inhibitor.

The rapid reduction of *N*-hydroxy-4-chloroamphetamine to 4-chloroamphetamine and of *N*-hydroxyamphetamine to amphetamine* may account for the inability to demonstrate formation *in vivo* of *N*-hydroxy metabolites of these and other amines.

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