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DISPOSITION AND METABOLISM OF [^{3}H]PSEUDOMORPHINE IN THE RAT

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Abstract—[^{3}H]pseudomorphine (^{3}H-PM) was prepared by the oxidation of [^{3}H]morphine. A method for the estimation of ^{3}H-PM in biological materials was developed with a minimal sensitivity of 2·5 ng/ml of biologic material. Following the subcutaneous injection of ^{3}H-PM (10 mg/kg as free base) in rats, the levels of ^{3}H-PM in brain and spinal cord over a 2-hr period ranged between 4·6 to 17·9 ng/g and 24 to 76·5 ng/g, respectively. No detectable levels of ^{3}H-PM were present at 4 hr in the CNS. Peak plasma levels occurred at 30 min (501 ng/ml) and then fell rapidly with no detectable amounts present at 4 hr. About 78·2 per cent of the administered ^{3}H-PM was accounted for in the urine and feces after 48 hr. Conjugated pseudomorphine was not detected in biological material, and direct chromatography of urine provided evidence for the presence of only free ^{3}H-PM. The existence of a polar metabolite, however, cannot be precluded. Studies on rat brain extracts following the administration of [^{3}H]morphine (10 mg/kg as free base) provided no evidence for the formation of pseudomorphine as an oxidative metabolite of morphine *in vivo*.

PSEUDOMORPHINE (2:2′-bimorphine), as an oxidation product of morphine,[1–3] is of special interest because of a possible involvement in the agonistic action of morphine or the development of tolerance and/or physical dependence following chronic administration of morphine. This view has not received wide acceptance,[4,5] primarily because pseudomorphine has not been identified as a metabolite *in vivo* of morphine in biological materials, although Hosoya and Brody,[6] utilizing rat tissue homogenates in the presence of cytochrome c, apparently demonstrated the oxidation of morphine to pseudomorphine *in vitro*.

The known physicochemical characteristics of pseudomorphine,[7] i.e. very limited solubility in saline, biological fluids and common organic solvents, difficulty of separation from proteins and formation of an insoluble salt with trichloroacetic acid, have interfered with the development of a suitable, sensitive analytical method for the extraction and estimation of this compound from biological materials.

This communication describes the preparation, disposition and biotransformation of [^{3}H]pseudomorphine (^{3}H-PM) in the rat. The method described for the estimation of [^{3}H]pseudomorphine from biological materials has been used to ascertain whether this compound is a metabolite of [^{3}H]morphine in the CNS of rats.

MATERIALS AND METHODS

Preparation of tritium-labeled pseudomorphine (^{3}*H-PM*). [^{3}H]Pseudomorphine was prepared by the oxidation of [^{3}H]morphine (50 mg) by a microscale adaptation of the

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procedure of Bentley and Dykes.[1] The colorless powdery material (yield, 30 mg, m.p. over 300°) had a specific activity of 4·7 μc/mg. Paper chromatography with the solvent *n*-butanol–acetic acid–water (4:1:5, by vol.) and radioscanning of chromatogram showed a single peak of radioactivity coincidental to authentic pseudomorphine (R_f 0·1). No [^{3}H]morphine was present in the product (R_f 0·4).

Estimation of ^{3}H-PM from biological materials. A 2-ml sample of biological fluid or tissue (10% homogenate in 0·5 N HCl) was mixed with 2 ml of non-labeled pseudomorphine solution (200 μg/ml as free base) as carrier in a 40-ml centrifuge tube. The solution was adjusted to pH 10 with diluted NaOH and saturated with 1·5 g of NaCl. One ml of phosphate buffer* pH 10·4 and 15 ml of a 30% solution of *n*-amyl alcohol in ethylene dichloride were added and the mixture shaken for 40 min at 300 oscillations/min with an International shaker machine. The samples were centrifuged for 5 min at 1500 *g* and 10-ml aliquots of the upper organic phase were transferred to counting vials and evaporated at 65° on a Fisher slide warmer. The residues were dissolved in 0·8 ml of *n*-amyl alcohol and 10 ml of toluene phosphor solution.† All samples in duplicate were counted in a Tri-Carb liquid scintillation spectrometer. Background samples, known concentrations of ^{3}H-PM, and internal standards of [^{3}H]toluene were run concurrently with the unknown samples to provide a check on the extraction and counting efficiency.

Conjugated ^{3}H-PM was determined by autoclaving each sample with 0·1 volume of conc. HCl for 25 min at 18–20 lb pressure, adjusting the pH to 10 with diluted NaOH and extracting the samples as described above.

Recoveries of 50–1000 ng of ^{3}H-PM added to rat brain and plasma and carried through the described extraction procedure were 95 ± 5% (S.E.M.). The minimal sensitivity of detection of ^{3}H-PM by this method was 5 ng (2·5 ng/ml).

Disposition studies. ^{3}H-PM was injected subcutaneously at a dose of 10 mg/kg‡ to male Sprague–Dawley rats weighing 200–250 g. Prior to sacrifice, the animals were lightly anesthetized with ether and blood obtained by cardiac puncture. The blood was centrifuged (in 15-ml potassium-oxalated centrifuge tubes) for 40 min at 1500 *g* to obtain plasma which was suitably diluted and analyzed as described. At intervals of 30 min to 4 hr after administration of drug, the animals were sacrificed by a light blow on the head and immediate incision of the heart. The internal carotids were catheterized and the brain perfused with 8–10 ml of physiological saline. Brain and spinal cord were removed, weighed and homogenized in sufficient 0·5 N HCl to provide a final volume of 5 ml. Two-ml samples in duplicate were then analyzed as described previously.

Urinary and fecal studies. ^{3}H-PM was injected subcutaneously at a dose of 10 mg/kg to male Sprague–Dawley rats. Urine and feces were collected for 48 hr in metabolism cages. The feces were homogenized with a known volume of 0·5 N HCl and analyzed for free and conjugated ^{3}H-PM as described.

Chromatographic experiments. Descending paper chromatography of aliquots of urine after addition of non-radioactive pseudomorphine as carrier was performed on

* Potassium phosphate buffer was prepared by dissolving 350 g anhydrous potassium dibasic phosphate (K_2HPO_4) and 50 g potassium tribasic phosphate (K_3PO_4) in 1 l. of double distilled water.

† Toluene phosphor solution was prepared by dissolving 3 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) in 1 l. of analytical grade toluene.

‡ ^{3}H-PM injection solution was prepared as the tartrate salt with given dose calculated as the free base.

Whatman 3 MM paper using *n*-butanol–acetic acid–water (4:1:2) as the solvent system. The chromatogram was observed with u.v. light and sprayed with the iodoplatinate reagent, and the remaining section scanned by elution of strips with diluted hydrochloric acid and counting the radioactivity in the liquid scintillation counter.

RESULTS

Distribution of ^{3}H-PM in the CNS and plasma of rats. It is evident from Table 1 that the levels of free ^{3}H-PM in the CNS at various time intervals were quite low with the maximum value occurring in brain at 30 min and no detectable amounts (< 5 ng) at 4 hr. In the spinal cord, the maximum concentration of ^{3}H-PM occurred at 2 hr (76·5 ng/g) with no detectable amounts (< 5 ng) at 30 min and 4 hr.

TABLE 1. LEVELS OF FREE [^{3}H]PSEUDOMORPHINE (^{3}H-PM) IN THE BRAIN, SPINAL CORD AND PLASMA OF RATS AT INTERVALS AFTER THE SUBCUTANEOUS INJECTION OF 10 mg/kg (AS FREE BASE)

Time after injection	Concentration of ^{3}H-PM* (ng/g or ml)			
	Brain	Spinal cord	Plasma	B/P†
30 min	17·9 ± 0·3	ND‡	501 ± 21	0·034
1 hr	4·6 ± 1·5	24·0 ± 9·0	288 ± 18	0·016
2 hr	5·9 ± 0·4	76·5 ± 14	203 ± 35	0·029
4 hr	ND	ND	ND	

* Duplicate determinations for free ^{3}H-PM were performed on three to four rats per time interval. The values expressed as ± S.E.M.

† B/P, represents the ratio obtained by dividing the concentration in brain by the concentration in plasma at each time interval.

‡ ND, the drug was not detected at the stated time interval.

Plasma levels of ^{3}H-PM indicated rapid absorption of ^{3}H-PM with a maximum level of 501 ng/ml at 30 min. The level of drug fell rapidly to 288 ng/ml at 1 hr, then remained relatively stable through 2 hr, with no detectable quantity (< 5 ng) at 4 hr. Brain to plasma ratios at 30 min, 1 and 2 hr were 0·034, 0·016 and 0·029 respectively.

Conjugated ^{3}H-PM was not detected in the CNS or plasma at the various time intervals studied.

Urinary and fecal excretion of ^{3}H-PM. The data on percentage recovery of ^{3}H-PM and total radioactivity in the urine and feces are summarized in Table 2. Conjugated ^{3}H-PM was not detected in the urine or feces.

Metabolic studies. Direct chromatography in *n*-butanol–acetic acid–water (4:1:2) of a small aliquot of urine collected over a 48-hr period following the administration of 10 mg/kg of ^{3}H-PM showed the presence of a single radioactive spot with R_f 0·17 coincidental to authentic non-labeled pseudomorphine (R_f 0·18). The presence of some radioactivity at the origin might suggest the existence of a polar metabolite of pseudomorphine.

In another series of experiments, the brains of five male rats removed 60 min after a subcutaneous injection of tritium-labeled morphine (10mg/kg as free base) were

TABLE 2. URINARY, FECAL EXCRETION OF FREE ^{3}H-PM AND TOTAL RADIOACTIVITY 48 hr AFTER THE SUBCUTANEOUS ADMINISTRATION OF 10 mg/kg (AS FREE BASE) TO RATS

	Mean percentage recovery of ^{3}H-PM*		
	Urine	Feces	Total
Male rats	72·5	5·7	78·2
Total radioactivity†	84·2	21·2	105·4

* Figures represent the mean percentage of the administered dose of ^{3}H-PM recovered from the urine and feces of five male rats.

† This was determined in duplicate by adding 0·2 ml of suitably diluted urine or feces to a scintillation counting vial and mixing with 3 ml of absolute ethanol, and 6·8 ml of toluene phosphor solution. All samples were corrected for quenching.

pooled, homogenized in 1 N HCl containing non-labeled pseudomorphine, morphine and normorphine as carriers. The homogenates were extracted at pH 9 and as described and the pooled organic phase evaporated to dryness. Paper chromatography of the residue with *n*-butanol–acetic acid–water (4:1:5, by vol.) and radioscanning showed the presence of free morphine but no evidence for the formation of pseudomorphine as a metabolite of morphine in the brain of rats. A conversion of 1% of morphine-^{3}H present in rat brain 1 hr after injection to pseudomorphine-^{3}H would give rise to a concentration of about 3·0 ng/g (about 20–30 ng in the pooled brain sample) of pseudomorphine-^{3}H in rat brain and would have been easily detected by our procedure.

DISCUSSION

This study demonstrates that pseudomorphine, unlike morphine, does not penetrate the blood–brain barrier to provide sufficient concentrations in the central nervous system of rats. The concentrations of morphine-*N*-methyl^{14}C in rat brain at the same dose level and by the same route of injection have been reported[8] to be 324 and 285 ng/g at 30 and 60 min respectively. Although, the method of anesthesia, technique of sacrifice of animals and perfusion of cerebral vascular system might alter the distribution of ^{3}H-PM in brain tissue, our observation would agree with previous studies[9,10] which indicated that pseudomorphine given orally or subcutaneously produced neither narcosis nor respiratory depression. Acute circulatory depression resulting chiefly from peripheral vasodilatation characterized by muscular weakness and some general depression have been observed[10] with intravenous administration of pseudomorphine.

Approximately 78·2 per cent of the administered dose of ^{3}H-PM could be accounted for in urine and feces collected over a 48-hr period by our extraction procedure. Since almost the entire dose of ^{3}H-PM was accounted for on the basis of data on total radioactivity, it would appear that a polar metabolite not extracted by our procedure might be present. Although acid hydrolysis of urine and chromatography studies indicated the absence of a glucuronide or ethereal sulfate conjugate of ^{3}H-PM, this does not preclude the possibility of the presence of some other polar metabolite which may escape detection by methods used in this study.

The present study provided no evidence for the formation of ^{3}H-PM as a metabolite of morphine in the brain of rats *in vivo* and furnishes suggestive evidence that ^{3}H-PM is probably not responsible for or associated with the agonistic actions of morphine, the phenomena of tolerance and/or physical dependence.

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