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THE METABOLISM OF NORTRIPTYLINE-N-METHYL-^{14}C IN RATS

ROBERT E. MCMAHON, FREDERICK J. MARSHALL, HILMAN W. CULP and WARREN M. MILLER

The Lilly Research Laboratories, Indianapolis, Ind., U.S.A.

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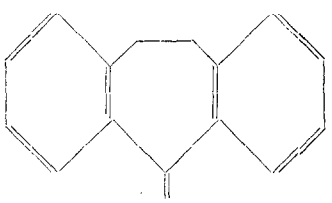
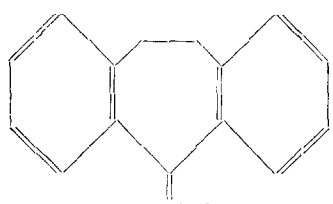
Abstract—Nortriptyline, labeled with radiocarbon in the N-methyl group, has been prepared and its metabolism, distribution, and excretion studied in the rat. About 25% of an administered dose undergoes N-demethylation in the whole animal. Another 40% of the dose is excreted in urine as conjugates of the *cis* and *trans* isomers of 10-hydroxy-nortriptyline. Distribution studies demonstrate that the drug undergoes wide distribution, with the highest levels found in lung and liver. Nortriptyline was identified in the brain, showing that it does pass the blood-brain barrier; it also was found to be slowly, but efficiently, absorbed from the intestinal tract.

NORTRIPTYLINE [5-(γ-methylaminopropylidene)dibenzo(a,d)cycloheptal(1,4)diene] possesses interesting antidepressant properties.[1] Although the metabolic fate of the related dimethylamino compound, amitriptyline, has been well investigated by Hucker,[2, 3] studies on nortriptyline have not as yet been reported.



$CHCH_2CH_2NHCH_3$

Nortriptyline



$CHCH_2CH_2N(CH_3)_2$

Amitriptyline

Hucker and Porter[2] found amitriptyline to be extensively metabolized *in vivo*. The two major metabolic reactions were N-demethylation and hydroxylation of the ethylene bridge.[3] It was thought that aromatic hydroxylation, rupture of the ethylene bridge, and oxidative deamination also occurred. Nortriptyline is not excreted in urine after administration of amitriptyline. It was also reported that administration of nortriptyline led to only negligible amounts of its excretion in the urine.

In order to facilitate metabolic studies, nortriptyline-N-methyl-^{14}C was synthesized. Its absorption, distribution, metabolism, and excretion in rats are described below.

MATERIALS AND METHODS

5-(γ-N-carbethoxy-^{14}C-aminopropylidene)dibenzo[a,d]cyclohepta-[1,4]diene

There was placed in a 50-ml, 2-neck flask connected to a vacuum system, 2·17 g of a solution of phosgene in toluene (41 mg/g of solution) containing 89 mg of phosgene. The flask was chilled in liquid nitrogen and was evacuated at 0·1 mm pressure. Twenty mg (1 mc) of phosgene-^{14}C was then introduced. This made a total of 109 mg (1·1mmoles) of phosgene. After 5 min the frozen solution was allowed to melt and then was refrozen and remelted.

One and two tenths ml of a dry toluene solution containing 133 mg (0·11 mmole, 0·145 ml) of purified collidine and 46 mg (0·1 mmole, 0·056 ml) of absolute ethanol (dried by means of sodium and diethyl phthalate) was added by drops to the phosgene solution, with stirring and cooling in ice water. The reaction was kept in the ice bath for 2 hr and was then stirred at room temperature for 18 hr.

A solution of 249 mg (1 mmole) of 5-(γ-aminopropylidene) dibenzo[a,d]cyclohepta[1,4]diene freshly prepared from its hydrochloride, and 0·5 ml of purified collidine in 1 ml toluene was added to this reaction mixture, and stirring was continued for 2 hr. The mixture was diluted with benzene and was washed three times each with water, 1 N hydrochloric acid, water, 1 N sodium hydroxide, and water. The organic phase was dried over magnesium sulfate and the solvent removed under reduced pressure. The residual noncrystalline radioactive carbamate was used without further purification.

5-(γ-Methyl-^{14}C-aminopropylidiene)dibenzo[a,d]cyclohepta[1,4]-diene.

The crude carbamate was dissolved in 10 ml dry ether, and the solution was added through a dropping funnel, with stirring, to 0·5 g (13·2 mmoles) of lithium aluminum hydride in 40 ml dry ether. The mixture was stirred and refluxed overnight and was then chilled in an ice bath. Hydrolysis was effected by dropwise addition of 0·5 ml ethanol, 0·5 ml water, 0·5 ml 20% sodium hydroxide solution, and 1·5 ml water. The ether was decanted, and the residue was extracted three times with 20-ml portions of boiling benzene. The combined ether and benzene solutions were extracted three times with 1 N hydrochloric acid (total volume about 30 ml) and were washed well with water. About 6 drops of concentrated hydrochloric acid was added to the combined aqueous solutions. The hydrochloride salt of radionortriptyline crystallized immediately. After chilling overnight the product was isolated by filtration, and dried first in air and then in a desiccator over Drierite. The yield was 132 mg (44 per cent based on the starting primary amine) of radionortriptyline HCl which melted at 212 to 214°.

Experiments in vitro

The preparation of rat liver microsomal N-demethylase activity and its use in substrate activity studies has been previously described.[4]

Respired radiocarbon dioxide studies

The rate of radiocarbon dioxide respiration was followed by use of the procedure described earlier.[5] In the present study 180g rats (Purdue Wistar) were used. In the inhibitor studies SKF 525 (β-diethylaminoethyl diphenylpropyl acetate HCl) and

DPEA (2, 4-dichloro-6-phenylphenoxylethylamine HCl) were given by the intraperitoneal route 10 min before administration of radionortriptyline (5 mg/kg, i.p.). Iproniazid and JB516 (α-methylphenethyl hydrazine) were given 1 hr before administration of labeled drug.

Tissue residue studies.

Radionortriptyline in saline solution was given by intraperitoneal injection to male rats (125 g) at a dose rate of 5 mg/kg. At given intervals the animals were decapitated, bled, and then opened. Duplicate samples of various tissues, including blood, were removed and their wet weight determined. The samples varied in weight between 150 and 450 mg. After drying for 16 hr at 60°, the radiocarbon content of each sample was determined by the Schöniger combustion method described by Kelly, *et al.*[6]

For the most part only one animal was used for each time period.

Urinary, biliary, and fecal excretion studies.

Male rats weighing 180 g were used for excretion studies. After dosing with 5 mg radionortriptyline/kg by intraperitoneal injection, the animals were held in stainless steel metabolism cages. Urine samples were collected and the radioactive content determined by liquid scintillation counting. Feces samples were dried, ground, and analyzed by the Schöniger combustion procedure.

For the bile studies 180-g male rats were sedated with secobarbital and a cannula placed in the common bile duct. In the oral studies the radionortriptyline was given prior to cannulation; in the intraperitoneal experiment the cannula was placed before injection. In the reabsorption experiments, bile from one rat which had received radionortriptyline (5 mg/kg, i.p.) was introduced into a bile cannula leading into the intestinal tract of a second rat. The common bile duct from the liver of the second rat was also cannulated, and the rate of appearance of radioactivity in bile from this cannula was followed.

Identification of urinary metabolites.

The radioactive metabolites present in urine were not extractable into ether, ethylene dichloride or isoamyl alcohol. However, after incubation (37°) at pH 5·0 with a mixture of β-glucuronidase and sulfatase (Glusulase, Endo Products, Inc.), 88% of the radioactivity was extractable into isoamyl alcohol at pH 11. A preliminary investigation of the crude radioactive metabolites was made by thin-layer chromatography on silica gel with a 1:1 mixture of ethanol and ethyl acetate for development. When the crude metabolites were co-chromatographed with cold nortriptyline and cold N-acetyl nortriptyline less than 0·3% of the radioactivity was associated with spot. Instead, the radioactivity was confined to a slower moving "streaked" area near the origin.

Further studies directed toward the identification of the urinary metabolites were carried out as follows: 24-hr urine collections from six rats (200-g male), receiving 10 mg i.p. of radionortriptyline/kg, were combined with the urine collection from 20 rats receiving 10 mg cold nortriptyline/kg. After incubation with Glusulase the urine was extracted with isoamyl alcohol to yield about 1·g of a dark oil after solvent evaporation. The crude urine extract was now dissolved in 100 ml methanol and 10 ml

acetic anhydride. After the reaction mixture had stood overnight it was evaporated to dryness, and the residue was taken up in ether. The ether solution was washed with 5% HCl and with 5% NaOH, and after drying over anhydrous potassium carbonate the ether was removed under vacuum. This residue was dissolved in 50 ml of aqueous alcohol containing 0·5% NaOH and was refluxed 1 hr. Again the solvent was removed and the residue dissolved in ether and washed with aqueous acid and base. The ether solution was dried and the ether removed, to yield a brown radioactive gum (150 mg). This material was then subjected to chromatography on neutral alumina (activity grade 1) in a solvent sequence of benzene, ethyl acetate, ethanol for elution. Two major radioactive peaks were obtained. The first was eluted with a mixture of four parts benzene and six parts ethyl acetate, the second more polar material with ethyl acetate containing 5% ethanol. About 75% of the radioactivity was associated with the more polar metabolite and 25% with the less polar. The physical and chemical properties of these two metabolites are described below.

RESULTS AND DISCUSSION

Demethylation studies

Demethylation studies with liver microsome preparations *in vitro* can be used to forecast the *in vivo* behavior of N-methylamines with respect to demethylation.[7] For this reason the rate of demethylation of nortriptyline was compared with that of several related compounds. The results are summarized in Table 1.

TABLE 1. THE RATE OF DEMETHYLATION *IN VITRO* OF NORTRIPTYLINE AND RELATED COMPOUNDS*

Drug	HCHO formed (μmole)
Promazine	0·53
Chlorpromazine	0·42
Trifluoromazine	0·44
Imipramine	0·86
Amitriptyline	0·79
α-Methylamitriptyline	0·73
Nortriptyline	0·05

* Conditions: Each incubation flask contained 25 μmoles $MgCl_2$, 0·5 μmole $NADP^+$, 10 μmoles glucose-6-phosphate (sodium salt), 45 μmoles semicarbazide (neutralized), 0·5 ml of 0·5 M phosphate buffer (pH 7·4), supernatant fraction containing microsomes from 200 mg of rat liver, 1 μmole substrate and sufficient water to give a final volume of 3 ml. The flasks were incubated in air with shaking at 37° for 1 hr.

It was observed that the phenothiazines as a class were poorer substrates than were the compounds having an ethylene bridge in place of the heterocyclic sulfur of the phenothiazine drugs. It is possible that the phenothiazines are lost to the reaction through sulfoxide formation.[8] No information appears to be available on the demethylation of chlorpromazine sulfoxide or related sulfoxides. It was also observed that α-methyl branching has little effect upon rate, as would be predicted from our previous studies on hindered amines.[9]

The most interesting observation, however, was the immense difference in demethylation rate between amitriptyline and the corresponding secondary amine, nortriptyline. This is another example of well-recognized[10] differences between tertiary and secondary amines with respect to certain biochemical phenomena. The very slow rate of demethylation of nortriptyline *in vitro* observed in these studies led us to expect a correspondingly low demethylation rate *in vivo*. Interestingly enough, however, the *in vivo* studies described below showed that, although demethylation was not the major route of metabolism, it was indeed an important route.

By following the rate of radiocarbon dioxide expiration after administration of radionortriptyline, it was possible to estimate the rate and extent of N-demethylation *in vivo*. Male rats excreted about 23% of the radioactive dose as radiocarbon dioxide during a period of 8 hr (Fig. 1). As would be expected from the work of Quinn *et al.*,[11] female rats demethylate nortriptyline at a somewhat slower rate than do males. When nortriptyline was given orally the rate of N-demethylation was much slower, although the ultimate extent of demethylation was about the same as that obtained when the drug was given by intraperitoneal injection. It was concluded that nortriptyline is efficiently absorbed from the gastrointestinal tract but that absorption occurs over a time course of several hours. The rate of demethylation is probably controlled by the rate of absorption during the first hours after drug administration.

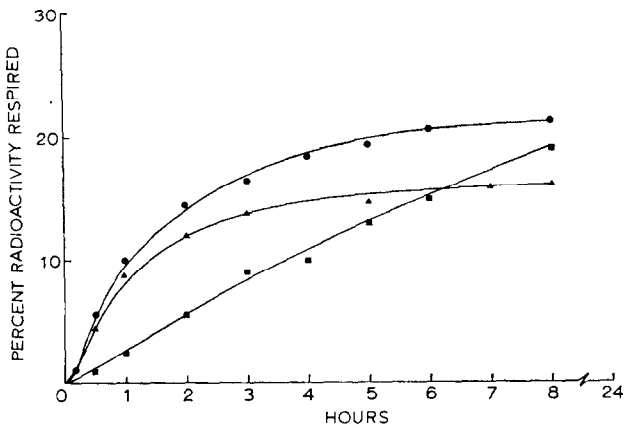


FIG. 1. Rate of radiocarbon dioxide respiration after administration of radionortriptyline; ● male rats (5 mg/kg, i.p.); ■ male rats (5 mg/kg oral); and ▲ female rats (5 mg/kg, i.p.).

It seemed likely that the demethylation *in vivo* of nortriptyline was effected by the microsomal demethylase activity of liver. There was, however, the possibility that monoamine oxidase might be involved. The effect of a few selected inhibitors upon the rate of demethylation of nortriptyline *in vivo* was investigated in order to resolve this uncertainty. The results are summarized in Fig. 2. SKF-525, an active inhibitor of oxidative microsomal enzyme activity,[12] reduced the rate of demethylation by about one half, while *dl*-phenylisopropyl hydrazine, a potent MAO inhibitor,[13] had little effect. DPEA, a potent inhibitor of the N-demethylation of tertiary amines but not of secondary amines,[14] did not affect the demethylation rate. Finally, iproniazid, which has been reported to be both an MAO inhibitor and a potent inhibitor *in vivo*

of microsomal oxidations[15, 16] was also an effective inhibitor of the demethylation of nortriptyline *in vivo*. It can be concluded from these results that the liver microsomal N-demethylase is the most likely enzyme system responsible for the demethylation of nortriptyline *in vivo*.

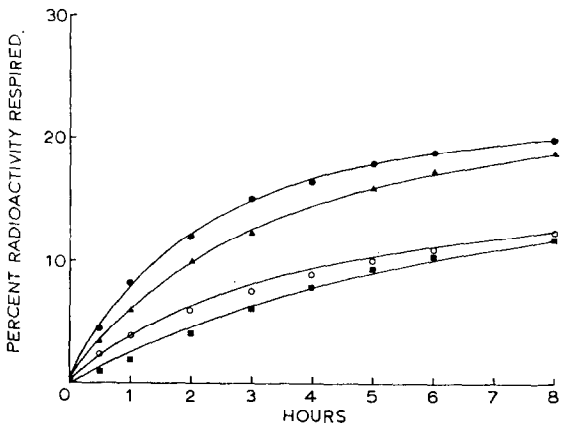


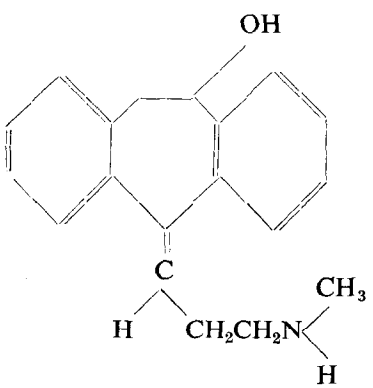
FIG. 2. Effect of various inhibitors upon the rate of radiocarbon dioxide respiration after administration of radionortriptyline by intraperitoneal injection (5 mg/kg). The inhibitors and dosages are: ● JB516 (15 mg/kg); ▲ DPEA (24 mg/kg); ■ SKF 525A (39 mg/kg); ○ iproniazid (100 mg/kg).

Urinary metabolites

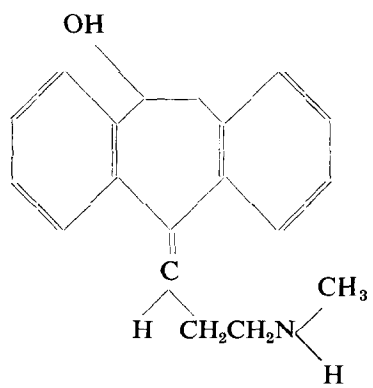
Since only about one fourth of the nortriptyline was metabolized by demethylation, the urinary excretion of metabolites was next investigated in order to determine the fate of the rest of the drug. When nortriptyline was given by intraperitoneal injection, 30 to 40% of the radioactivity appeared in urine in 24 hr. Attempts to extract the radioactivity from urine at pH 10 with organic solvents were largely unsuccessful. However, after incubation of the urine with a mixture of β-glucuronidase and sulfatase, most (~85%) of the radioactivity was extractable. This result suggests strongly that the metabolites are hydroxylated derivatives. The hydroxyl group could be either an aromatic hydroxyl (phenol) or an aliphatic (alcohol).

Co-chromatography of the extracted radioactive metabolites on thin-layer silica gel plates with nortriptyline and N-acetyl nortriptyline showed that these were minor components (<0·3% of dose), if present at all. In order to identify the unknown metabolites, partial purification by column chromatography was undertaken. Since amines are difficult to separate by absorption chromatography, it was decided to work with the metabolites as amides. To this end the crude metabolites were exhaustingly acetylated with acetic anhydride. Any O-acetyl groups that may have formed were cleaved by mild hydrolysis, and the radioactive N-acetyl metabolites were then chromatographed on alumina. Two major radioactive peaks were obtained, one eluted with 4:6 benzene:ethyl acetate and the second with 20:1 ethyl acetate:ethanol. There was about three times as much of the more polar metabolite. The radioactive fractions near the radioactive peaks were now combined to yield in both cases a small amount of a radioactive brown gum. The infrared spectra of these two materials were

nearly identical and showed the presence of the amide carbonyl and a hydroxyl group. The ultraviolet spectra were also the same, each showing an absorption maximum at 239 mμ. This peak did not shift in alkali, showing that the hydroxyl is not phenolic. The 239-mμ peak is characteristic of nortriptyline itself.[17] Acetylation of the two metabolite derivatives with acetic anhydride yielded derivatives which were shown by infrared analysis to contain both an ester carbonyl and an amide carbonyl. The hydroxyl band was now absent, as would be expected. Two different chemical reactions were carried out on each of these O,N-diacetates. First a small amount of each was heated with phosphoric acid at 160°. The products, which were radioactive, still contained the amide carbonyl but not the ester carbonyl. The ultraviolet spectra of the two products were the same and had maxima at 255, 332, 348, 366, and 386·5 mμ. In addition, there were shoulders at 248, 301, and 315 mμ. Qualitatively this UV spectrum is identical with that of 9-methylanthracene and shows that under the influence of strong acid the nortriptyline metabolite undergoes a rearrangement to the aromatic system. The nature of this rearrangement is at present unknown. Treatment with milder acid (6 N HCl at reflux) resulted in an elimination reaction to yield an amide which showed an ultraviolet maximum at 286 mμ, characteristic of the molecule in which the dimethylene bridge of nortriptyline has been replaced by an unsaturated double bond.[17] The same product was obtained from each metabolite derivative. These results demonstrate that the two metabolites must be isomers of each other and indeed must be the *cis* and *trans* isomers of 10-hydroxynortriptyline.



cis



trans

At present, of course, there is no way to tell which of the two isomers found in urine is the *cis* and which is the *trans*. It should be further noted that since 10-hydroxynortriptyline contains an asymmetric carbon atom, the metabolites may be optically active. Until they are obtained in pure form, however, this cannot be determined.

The finding that 10-hydroxylation is a major route of metabolism of nortriptyline is not surprising. Indeed, Hucker[3] has reported that 10-hydroxylation is an important

pathway for the metabolism of amitriptyline. Enzymatic hydroxylation on a carbon atom adjacent to an aromatic ring is a well-known metabolic pathway.[18–20]

Bile excretion of metabolites

Preliminary excretion experiments showed that about 30% of the radioactive dose was excreted in feces. This suggested that it might be profitable to study the bile excretion of labeled metabolites. Some of the results obtained are summarized in Fig. 3. It was first observed that when nortriptyline-^{14}C was given by the intraperitoneal

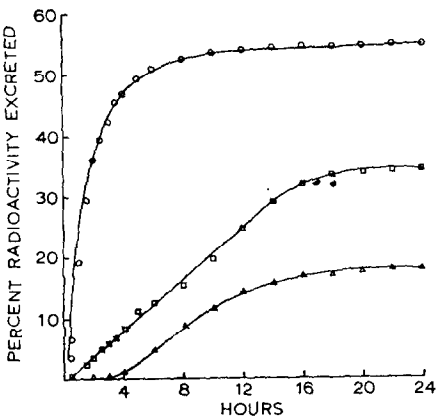


FIG. 3. Biliary excretion of radioactive metabolites by rats receiving: (1), ○ radionortriptyline (5 mg/kg, i.p.); (2), ■ radionortriptyline (5 mg/kg, oral); and (3), ▲ radioactive bile from Rat 1.

route, radioactive metabolites rapidly appeared in bile. The nature of the biliary metabolites was not investigated other than to show that they were, for the most part, conjugated materials. Since the amount of radioactivity secreted in bile (about 55% of the dose) was considerably more than was ultimately excreted in feces, it seemed possible that reabsorption was occurring. To establish this, radioactive bile obtained from Rat 1 of Fig. 3 was introduced into the intestine of a second rat through a cannula placed in the bile duct and directed toward the intestine. Bile coming from the liver and urine was now collected from this second rat. From Fig. 3 it is seen that about 18% of the original radioactive dose appears in the bile of this rat. An additional 11% of the radioactivity appeared in urine over a 15-hr period. These data show that some of the radioactivity excreted in bile is indeed reabsorbed into the body from the gut.

The rate of excretion of radioactive metabolites in bile after oral administration of radionortriptyline was also investigated. It is interesting to note that over a period of 14 hr the excretion rate is nearly constant. Since the rate of excretion of radioactivity as carbon dioxide after oral administration of radionortriptyline is also constant over a considerable time period, it suggests that both rates are being controlled by the same factor. This factor is most likely the rate of absorption of drug from the gut into

the blood stream. In this connection it was found (Fig. 4) that blood levels after oral administration of radionortriptyline approached a plateau at about 2 hr and then stayed at a constant level for at least 12 hr.

Zehnder *et al.*[21] observed a similar constant rate of bile excretion of metabolites after administration of radiosulfur-labeled ET (3-methylthio-10[β(1′-methyl-2-pyrrolidyl)-ethyl]phenothiazine). They proposed that the rather slow uptake of ET from gut occurred because the intestinal wall itself retained the drug and only slowly released it to circulation. A similar situation may exist with nortriptyline.

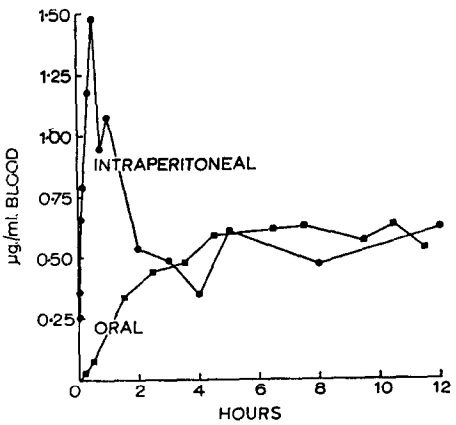


FIG. 4. Levels of radioactivity in blood after administration of 5 mg radionortriptyline/kg. Radioactivity levels are given as radionortriptyline equivalent.

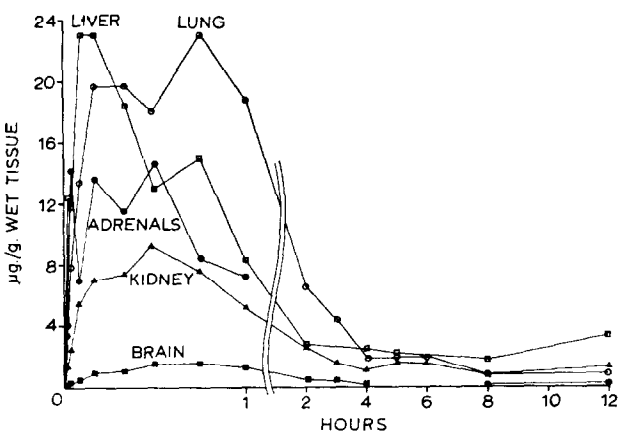


FIG. 5. Levels of radiocarbon in various tissues after i.p. injection of radionortriptyline. Radioactivity levels are given as radionortriptyline equivalents (µg/g wet tissue).

Tissue residue studies

In order to understand the dynamics of the drug in the body, a short study was made of the distribution of radioactivity in various tissues after intraperitoneal injection of radionortriptyline. It was observed that whole-blood levels (Fig. 4) rose rapidly to reach a peak in 30 min. It then fell sharply, leveling off at about 1·5 hr at a

level which then remained constant for the next several hours. With the exception of the liver, the levels of radioactivity in those tissues studied (Fig. 5) showed a time course which was qualitatively similar to the blood levels. Apparently, equilibrium between blood and tissue is rapid and, based on the relative levels observed, it lies greatly in favor of the tissues, except in the case of brain tissue. Although the brain levels were low, they were nevertheless greater than blood levels over much of the time period under study. The fact that liver levels reached a maximum earlier than other tissues probably means that, after intraperitoneal injection, the drug was first transported to the liver and then entered the general circulation.

High levels of radionortriptyline (and metabolites) found in lung are not surprising since it has been observed previously that many lipid-soluble amines have a special affinity for lung tissue.[21–24] The reason for this is not understood at present. In connection with these tissue-level studies it should be realized that radiocarbon levels are being measured, and it is not possible to say whether drug or metabolite level is being measured. It seems likely that the levels measured in the first few minutes represent the drug. Later, however, more and more of the radioactivity detected is probably in the form of metabolites. Since the identity of the radioactivity present in brain was of some importance, this matter was explored further. Brains from three rats which had received 10 mg i.p. radionortriptyline/kg 0·5 hr before sacrifice were homogenized, and radioactive amines were extracted into benzene at basic pH. Thin-layer chromatographic studies were employed to demonstrate that the brain radioactivity was indeed identical with nortriptyline. Thus it was demonstrated that nortriptyline can cross the blood–brain barrier, and brain levels of the order of 1 to 2 μg/g wet tissue can be obtained.

The fact that appreciable tissue levels existed at 12 hr prompted a study of the tissue levels after longer intervals. Radiocarbon levels for several different tissues, 0·5 day, 1 day, and 14 days after drug administration, are presented in Table 2.

TABLE 2. TISSUE LEVELS OF RADIOCARBON AT VARIOUS TIMES AFTER ADMINISTRATION OF RADIONORTRIPTYLINE (5 MG/KG, I.P.)

Tissue	Wet tissue (μg/g)* 0·5 day	1 day	14 days
Blood	0·63	0·24	0·10
Plasma	0·90	0·27	0·01
Brain	0·22	0·09	0·10
Liver	3·42	1·14	0·19
Kidney	1·32	0·60	0·24
Adrenal		0·77	0·14
Lung	0·94	0·40	0·20

* Radioactivity levels are calculated in terms of radionortriptyline equivalent.

The most significant observation is that even after 14 days measurable amounts of radioactivity remain in the tissues. It is felt that this radioactivity does not represent nortriptyline or metabolites but is most likely a consequence of the radioformaldehyde from the demethylation reaction becoming involved in the 1-carbon metabolism of the body. Once this radiocarbon becomes incorporated into nucleic acids, we could

expect it to be eliminated only very slowly from the body.[25] It is noted that the radioactivity present in circulating blood is associated with the RNA containing red cells and not with the plasma.

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