Carbon-14 and Tritium Labelling of *m*-(1-Methyl-3-Propyl-Pyrrolidinyl)-Phenol Monohydrochloride (Profadol; CI-572)

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SUMMARY

Carbon-14 and tritium labelled preparations of m-*(1-methyl-3-propyl-3-pyrrolidinyl)-phenol monohydrochloride (CI-572) are described. CI-572-^{14}C, with the carbon-14 label in the C-2 position of the pyrrolidine moiety, was synthesized in five steps from $K^{14}CN$. CI-572-^{3}H was prepared by heterogeneous platinum-catalyzed exchange with tritiated water in acetic acid. Characterization of CI-572-^{3}H included an evaluation of the tritium label stability in water and in aqueous acidic and basic media.*

INTRODUCTION.

The pharmacologic and toxicologic studies of *m*-(1-methyl-3-propyl-3-pyrrolidinyl)-phenol, hereinafter referred to as CI-572, have recently been described by Winder *et al.* [1]. This analgetic agent has been shown to have antinociceptive activity in rats [1–3] and antitussive activity in dogs [1]. The need for radioisotopically labelled preparations of this compound for use in metabolic tracer studies prompted the carbon-14 and tritium labelling of CI-572.

I. SYNTHESIS OF CARBON-14 LABELLED CI-572.

Several synthetic routes to CI-572 have been previously reported [2–4]. The sequence of reactions used to synthesize CI-572-$^{14}C \cdot HCl$ are outlined in Figure 1.

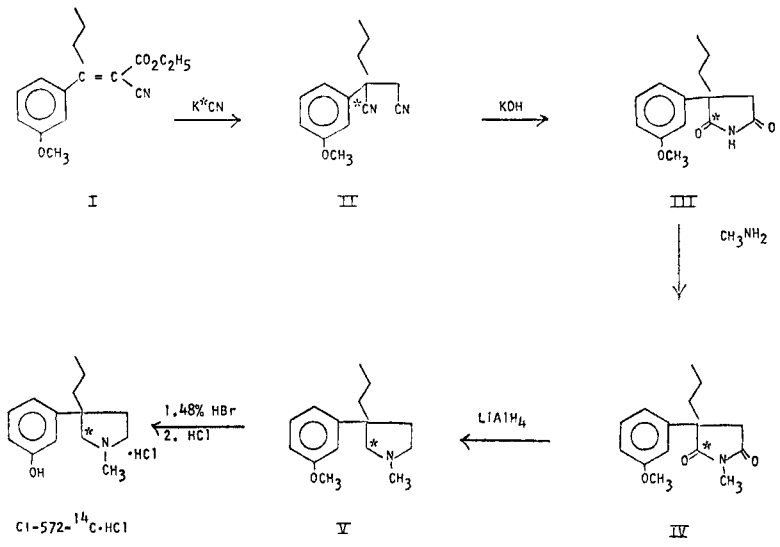


FIG. 1. Synthesis of CI-572-^{14}C · HCl.

*2-(*m*-methoxyphenol)-2-propylsuccinonitrile-1-^{14}C (II).*

A 88.6 mg portion of $K^{14}CN$ (New England Nuclear Corporation; 9.46 mCi/mmole) was diluted by the addition of 1,404.0 mg non-radioactive KCN to a calculated specific activity of 562 μCi/mmole and then dissolved in 2.2 ml H_2O. This aqueous solution of $K^{14}CN$ (22.9 mmoles) was added to a solution of 6.25 g (22.9 mmoles) ethyl-α-cyano-*m*-methoxy-β-propylcinnamate (I) dissolved in 20 ml ethanol. The reaction mixture was refluxed with stirring for 16.5 hours. After cooling to room temperature, insoluble materials were removed by filtration and washed with ethanol. The filtrate and ethanol washings were combined and vacuum distilled to remove the ethanol. The resultant residue was dissolved in 20 ml ether and washed three times with 10 ml portions H_2O, adding NaCl to each of the washes to obtain clean separation of the layers. The ether phase was dried with 0.3 g anhydrous $MgSO_4$, treated with charcoal, and filtered, washing the solids with anhydrous ether. The filtrate and washings were combined and stirred for 15 minutes in an ice-water bath. An unidentified white substance crystallized from the solution and was removed by filtration (50 mg; m. p. 175-180° C). The ethereal filtrate containing the product was vacuum distilled to remove the ether. The residual product was dissolved in 25 ml isopropyl alcohol and then crystallized by cooling with stirring in an acetone-dry-ice bath for 1/2 hour. After the product was crystallized, 75 ml *n*-pentane was added very slowly while stirring in the acetone-dry-ice bath. The product was stirred for an additional 1/2 hour in the cold bath, filtered through a pre-cooled suction funnel and washed with

cold (—20° C) *n*-pentane. (The low temperatures were necessary to prevent the crystalline product from turning into an oil). The product was dried at 30° C under slightly reduced pressure to yield 3.89 g II (74.3 % yield), m. p. 45-50° C, with a specific activity of 494 μCi/mmole.

*2-(*m*-methoxyphenyl)-2-propylsuccinimide-1-^{14}C (III).*

A reaction mixture consisting of 3.85 g (16.85 mmoles) II (494 μCi/mmole), 10.6 ml ethanol, 8.5 ml ethylene glycol, 2.7 ml H_2O and 3.50 g (62.4 mmoles) KOH was heated gradually with stirring, distilling off ethanol and water, until the reaction temperature reached 110° C, and then refluxed with stirring for 20 hours. The reaction mixture was vacuum distilled to remove ethanol and water. The residual oil was mixed with 10 ml ice water, acidified with 6 ml concentrated HCl and extracted with three 10 ml portions of ether. The ether extracts were combined, washed with three 5 ml portions of water and distilled under reduced pressure to remove the ether, leaving a crude tan oil, III.

*2-(*m*-methoxyphenyl)-N-methyl-2-propylsuccinimide-1-^{14}C (IV).*

The yield of crude III was mixed with 25 ml 40 % aqueous monomethylamine and stirred gently at room temperature overnight. The mixture was heated with stirring to a gentle reflux for one hour, and then heated to 210° C, distilling off the excess methylamine. While stirring vigorously, the pot temperature was maintained at 210° C for one hour. The product was cooled, dissolved in 3 ml ether and transferred to a semi-micro vacuum distillation apparatus. The ether was removed by evaporation, and the product was distilled at 119° C (0.230 mm Hg)-111° C (0.100 mm Hg) to yield 3.74 g IV (84.9 % yield based on II) with a specific activity of 521 μCi/mmole.

*3-(*m*-methoxyphenyl)-1-methyl-3-propylpyrrolidine-2-^{14}C (V).*

A 100 ml flask was equipped with a dropping funnel and a reflux condenser protected with a drying tube, then baked in a heating mantle while flushing with nitrogen, and finally cooled under a stream of nitrogen to room temperature. In the flask was placed 1.26 g (33.2 mmoles) $LiAlH_4$ and 25 ml anhydrous ether. The dropping funnel was charged with 3.60 g (13.8 mmoles) IV dissolved in 25 ml anhydrous ether. The ethereal solution of IV was added dropwise over a period of 15 minutes, maintaining a gentle reflux. After refluxing with stirring overnight, the reaction mixture was stirred in an ice-water bath and hydrolyzed by the addition of 0.40 ml H_2O, then 1.20 ml 4 *N* NaOH and finally 1.60 ml H_2O. The hydrolyzed mixture was filtered, and the collected solids were washed with ether. The filtrate and ether washes were combined and vacuum distilled to remove the ether. The resultant oil

residue was dissolved in 10 ml benzene and extracted with three 5 ml portions of 2 *N* HCl. The aqueous layers were combined, washed with 5 ml benzene, then made basic with 2 ml 50 % NaOH and finally extracted with 10 ml and 5 ml portions benzene. The benzene extracts were combined, dried with 0.2 g anhydrous $MgSO_4$ and filtered. Benzene was removed from the filtrate by vacuum distillation leaving a clear white oil, V.

m-(1-methyl-3-propyl-3-pyrrolidinyl-2-^{14}C)-phenol monohydrochloride (CI-572-^{14}C · HCl).

The yield of V was mixed with 10 ml 48 % hydrobromic acid and refluxed with stirring for 4 1/2 hours. Excess HBr was removed by vacuum distillation. The resultant clear brown oil was dissolved in 10 ml H_2O, made basic with 50 % NaOH to a pH $\geqslant$14 and then washed with 10 ml ether : toluene, 1 : 1, to remove any unreacted V. The aqueous phase was adjusted to pH 9-10 with concentrated HCl, precipitating the product, and then extracted with 20 ml and 10 ml portions of ether. The ether extracts were combined, washed with two 10 ml portions of H_2O, dried with 0.3 g anhydrous $MgSO_4$, treated with charcoal and filtered through a layer of Celite 545. The collected solids were washed with anhydrous ether. The filtrate and washings were combined, treated again with charcoal and filtered again through Celite 545, washing with anhydrous ether. The filtrate and washings were combined and evaporated to dryness with mild heat. The residual oil (CI-572-^{14}C free base) was dried at 60° C under vacuum for 1/2 hour, dissolved in 5 ml isopropyl alcohol and converted to the hydrochloride salt with the addition of 4 ml isopropyl alcohol saturated with HCl. This solution was concentrated to 6-8 ml with gentle heat and stirring, cooled to room temperature, seeded with non-radioactive CI-572 · HCl, and cooled to −25° C in the freezer overnight. While scratching occasionally with a glass rod, the product was immersed in an acetone-dry-ice bath for 1 1/2 hours and then returned to the freezer for 2 hours. A heavy mass of white crystals formed. The product was placed in a CCl_4-dry-ice bath and stirred vigorously while adding 60 ml cold anhydrous ether. After stirring in the cold bath for about 15 minutes, a fine white crystalline product was obtained which was collected by filtration, washed with anhydrous ether and dried overnight at 70° C under vacuum to yield 2,101 mg CI-572-^{14}C · HCl, m. p. 143-144.5° C, with a specific activity of 2.06 μCi/mg (526 μCi/mmole). The weight yield was 59.5 % based on IV and 35.9 % based on K^{14}CN.

The product, CI-572-^{14}C · HCl, was characterized to establish its chemical and radiochemical identity and purity. The chemical identity and purity, as indicated by infrared and ultraviolet analyses, appeared to be indistinguishable from an authentic reference standard, although the melting point was 4-5 centigrade degrees low. Radiochemically, the product behaved chromatographically the same as authentic CI-572 · HCl in four thin layer chromatography (T.L.C.) systems (Appendix A - systems A, B, C and D). However,

the radiochemical purity was estimated at only 97 %. In addition, a radiochemical impurity, amounting to approximately 2 %, was detected in all four T.L.C. systems and it behaved chromatographically the same as authentic N-demethylated CI-572, *m*-(3-propyl-3-pyrrolidinyl) phenol (VI). Because the radiochemical purity of CI-572-^{14}C · HCl was unsatisfactory for use as a tracer in human clinical trials, the product had to undergo further purification.

Purification and Characterization.

Because CI-572-^{14}C · HCl and VI-^{14}C, the probable 2 % impurity, are chemically very similar, the methods of purification considered were preparative thin layer chromatography and column adsorption chromatography (CAC). Since CAC was more adaptable to a 2 gram scale, pilot studies on small and large columns were initiated using small amounts of CI-572-^{14}C · HCl. The results indicated that the impurity, VI-^{14}C, would be eliminated by CAC, since it remained on the column while purified CI-572-^{14}C · HCl migrated down the column very rapidly.

Conditions for the purification of CI-572-^{14}C · HCl by CAC were as follows : 270 g Aluminum Oxide (neutral, activity 1; Woelm) was added slowly to a 60 cm × 3 cm column equipped with a sintered glass base and a needle valve stopcock, and containing 270 ml of chloroform : methanol, 3 : 1, developing solvent. The adsorbent was allowed to settle overnight. Sand was added to the top of the column to a depth of 2 cm. Excess developing solvent drained from the column amounted to 15 ml, leaving a hold-up volume of 255 ml. A 2.03 g portion of CI-572-^{14}C · HCl (2.06 μCi/mg; 4,182 μCi ^{14}C) was dissolved in 4 ml of chloroform : methanol, 3 : 1, and placed on the column. The column was developed with 675 ml of chloroform : methanol, 3 : 1, and then eluted with 385 ml absolute methanol at the rate of 1.5 ml per minute. Eluate fractions were collected from the time that CI-572-^{14}C · HCl was placed on the column.

Each of the column eluate fractions was analyzed for carbon-14. The results, illustrated in a radiochromatogram in Figure 2, show that the recovery of carbon-14 was approximately 100 % and that the major carbon-14 peak appeared immediately after the collection of the hold-up volume; a second minor peak appeared at the methanol front. T.L.C. analysis (Appendix A—system D) of the last fraction grouped in Part A detected CI-572-^{14}C with a radiochemical purity of >99 %; no VI-^{14}C was detected (sensitivity permitted the detection of 0.07 %). Since the earlier pilot studies established that the impurity, VI-^{14}C, migrated down the column more slowly than CI-572-^{14}C, it was assumed that all of the eluate fractions in Part A contained purified CI-572-^{14}C.

The eluate fractions in Part A were pooled together in an evaporating dish, as were the fractions in Parts B and C, and evaporated to dryness. The residues were dissolved in methanol and analyzed for carbon-14 yield and

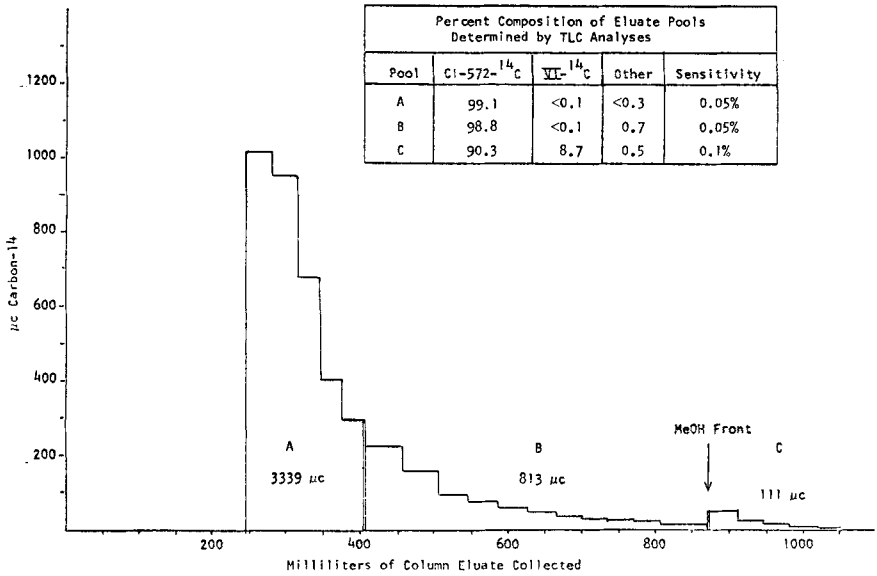


FIG. 2. Radiochromatogram of Purification of CI-572-^{14}C · HCl by Column Adsorption Chromatography.

for purity by T.L.C. (Appendix A—system D). The results, summarized in a Table in Figure 2, indicated that pools A and B contain CI-572-^{14}C in acceptable yield and purity. The impurity, VI-^{14}C, appeared in pool C.

Pools A and B were combined, vacuum distilled to dryness to remove the methanol, dissolved in 12 ml 2 *N* NaOH and washed with 10 ml toluene : ether, 1 : 1. The aqueous phase was adjusted to pH 9-10 with concentrated HCl and extracted with two 20 ml portions of ether. The ether phases were combined, washed with 10 ml and 5 ml portions of water, dried with 0.3 g anhydrous $MgSO_4$, treated with charcoal and filtered through a layer of Celite 545, washing the solids with anhydrous ether. The filtrate and wash were combined and evaporated to dryness with gentle heat. The residual CI-572-^{14}C free base was dried for one hour at 60° C under vacuum and converted to the hydrochloride salt as described previously in this paper to yield 1,496 mg CI-572-^{14}C · HCl, m. p. 146-146.5° C, with a specific activity of 2.06 μCi/mg (527 μCi/mmole) and an estimated chemical and radiochemical purity of >99 %; no VI-^{14}C was detected (T.L.C. sensitivity permitted the detection of 0.06 %). All of the characterization data are summarized in Table 1.

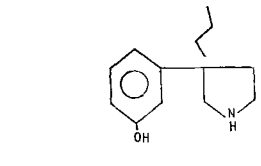
II. Preparation of tritium-labelled CI-572.

CI-572 · HCl was labelled directly with tritium by a heterogeneous platinum-catalyzed exchange reaction with tritiated water in acetic acid as

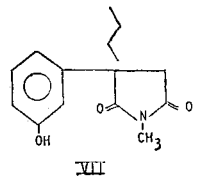
TABLE 1. Summary of characterization for Cl-572-^{14}C . HCl and Cl-572-^{3}H . HCl.

	Cl-572-^{14}C·HCl	Cl-572-^{3}H · HCl	Authentic Cl-572 · HCl
Color	White	White	White
Melting Point, °C	146-146.5	145.5-147	147-148
Specific Activity			
μCi/mmole	527 (measured)	5.170 (measured)	—
μCi/mg HCl salt	2.06 (measured)	20.21 (measured)	—
μCi/mg (free base)	2.40 (calculated)	23.57 (calculated)	—
Ultraviolet Analysis			
In MeOH	λ275 ∈2220	λ274 ∈2190	λ275 ∈2250
In MeOH-KOH	λ291 ∈3010	λ291 ∈2970	λ291 ∈3040
Infrared Analysis	The infrared spectrum of Cl-572-^{14}C·HCl is identical to the infrared spectrum of authentic Cl-572·HCl.		
Tritium Label Stability	The tritium activity of Cl-572-^{3}H·HCl is non-labile at room temperature in water and aqueous solutions of 1*N* NaOH and 1*N* HCl; as much as 40.1 % of the tritium activity can be removed by heating in a steam bath in 4*N* HCl for 4 hours. See Table 2.		
Chromatography			
Cl-572-^{14}C·HCl	A major carbon-14 spot from Cl-572-^{14}C·HCl having the same mobility as authentic Cl-572·HCl was detected in four T.L.C. systems (Appendix A — systems A, B, C and D). Analogues [a] II, IV, V, VI and VII were resolved from Cl-572-^{14}C·HCl in at least one T.L.C. system and were not detected as radiochemical impurities (sensitivity ≥0.06 %). An unidentified radiochemical impurity of 0.8 % was detected in only one system. No other radiochemical impurities were detected. The radiochemical purity of Cl-572-^{14}C·HCl was estimated as ≥99 %.		
Cl-572-^{3}H·HCl	A major tritium spot from Cl-572-^{3}H·HCl having the same mobility as authentic Cl-572·HCl was detected in five T.L.C. systems (Appendix A — systems B, C, E, F and G). Analogues [a] VI, VIII and IX were resolved from Cl-572-^{3}H·HCl in at least one T.L.C. system and were not detected as radiochemical impurities (<0.1 %); X may be present in the amount of <0.2 %. Some unidentified radiochemical impurity (<0.1 % to <1.0 %) was detected in all the T.L.C. systems but one. The radiochemical purity of Cl-572-^{3}H·HCl is estimated as ≥99 %.		

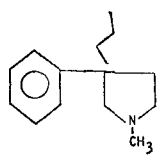
[a] The structures of II, III, IV and V are given in Figure 1. The structures of VI, VII, VIII, IX and X are given below.



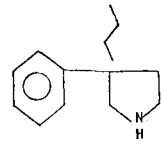
VI



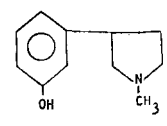
VII



VIII



IX



X

follows * : 25 mg CI-572 · HCl was dissolved in 0.3 ml glacial acetic acid containing 10 curies tritiated water and 25 mg pre-reduced platinum catalyst. The reaction mixture was heated at 100° C overnight while being stirred magnetically. The reaction mixture was cooled and the reaction solvent was removed by vacuum distillation. The residue was mixed with 10 ml methanol and then vacuum distilled to dryness to remove labile tritium. The product, containing 520 mCi ^3H, was dissolved in 10 ml methanol.

Chromatographic studies (Appendix A—systems B, C, E and I) revealed that the crude product was CI-572-^3H with a radiochemical purity of approximately 75 %. Furthermore, the studies indicated that not all of the radiochemical impurities could be separated from CI-572-^3H in a single T.L.C. system. Thus, to purify the product by preparative T.L.C., it was necessary to use two different T.L.C. systems, one followed by the other. A concentrated methanol solution of the crude product was streaked along a line 1 cm × 15 cm at the origin of the thin layer plate (0.25 mm) and then chromatographed (Appendix A—system E). The purified product was eluted with methanol to yield 212 mCi CI-572-^3H with a radiochemical purity of 91 %. The methanol eluate of the product was concentrated to a volume of 1 ml and then chromatographed again in a different T.L.C. system (Appendix A—system C). The product was eluted with methanol to yield 98.2 mCi CI-572-^3H with a radiochemical purity of >99 %. At this stage, 68.7 mCi CI-572-^3H, with an estimated specific radioactivity of 20 mCi/mg (4.4 Ci/mmole), was stored in methanol at −25° C for future use in metabolic studies requiring a high specific activity.

* The exchange labelling procedure was performed by New England Nuclear Corporation, Boston, Massachusetts, U. S. A.

To be sure that the final product was homogeneous in the form of the hydrochloride salt and to lower its specific activity, the remaining 29.5 mCi was treated as follows : 1.40 g non-radioactive CI-572 · HCl was mixed with the remaining 29.5 mCi high specific activity product in 4 ml methanol and then vacuum distilled and dried at 50° C (under vacuum). The resultant residue was dissolved in 15 ml H_2O, adjusted to pH 10 with 1 *N* NaOH, and extracted with 30 ml and 15 ml portions of diethyl ether. The ether extracts containing the product were combined, washed with two 10 ml portions of water, dried with 2 g anhydrous magnesium sulfate, evaporated to dryness, and dried for 30 minutes at 50° C at reduced pressure. The resultant oil, CI-572-^{3}H (free base), was converted to the hydrochloride salt as previously described in this paper to yield 1.292 g CI-572-^{3}H · HCl, m. p. = 145.5-147° C, with a specific activity of 20.21 μCi/mg and an estimated chemical and radiochemical purity of >99 %. The characterization data for this product are summarized in Table 1.

Tritium label stability.

The tritium labelled preparation was intended for use in metabolic experiments where CI-572 and its metabolic products could be detected by means of the tritium activity. To serve this function, the tritium to carbon bond must be stable under conditions existing in biological systems. As a preliminary step to the use of tritium-labelled compounds in metabolic experiments, it has been found useful to evaluate the stability of the tritium label in water and in aqueous acidic and basic conditions [5, 6].

The stability of the tritium activity in CI-572-^{3}H · HCl was studied in aqueous media according to the following : CI-572-^{3}H · HCl dissolved in H_2O, 1 *N* NaOH and 1 *N* HCl was allowed to stand at room temperature for two or three days, then neutralized where necessary, and finally lyophilized. In one experiment, CI-572-^{3}H · HCl was heated on a steam bath in 4 *N* HCl for 4 hours, neutralized and then lyophilized. The total tritium content of the resulting residues and lyophilizates (condensate) was measured. If the tritium activity in CI-572-^{3}H was readily removed under these conditions, then if would be exchanged from CI-572-^{3}H for non-radioactive hydrogen in the test medium, and ultimately would be detected as ^{3}HHO in the lyophilizate; if the tritium activity were stable, no significant exchange or loss of tritium from CI-572-^{3}H would occur and no ^{3}HHO would be detected in the lyophilizate. A sketch of the high vacuum lyophilization apparatus used in these experiments is seen in Figure 3, the experimental details are described in Appendix B and the results are presented in Table 2.

The results of these tritium stability experiments showed that the tritium activity in CI-572-^{3}H was stable for at least two days at room temperature in water and in aqueous solutions of 1 *N* NaOH and 1 *N* HCl (experiments 1, 2 and 3); in these experiments, ⩽0.7 % of the tritium activity was found in

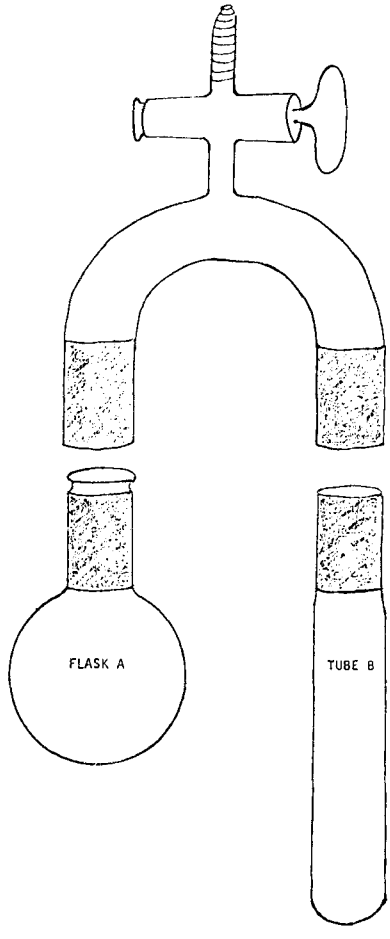


FIG. 3. High Vacuum Lyophilization Apparatus.

the lyophilizates. However, the tritium activity in CI-572-^{3}H was labile when heated on a steam bath in 4 *N* HCl for 4 hours (experiment 4); in this experiment, 40.1 % of the tritium activity was found in the lyophilizate. A T.L.C. study (Appendix A—system E) of this lyophilizate detected < 2 % of the total tritium spotted; the tritium that was detected was uniformly distributed along the chromatographic lane from the origin to the solvent front. These observations suggest that the tritium in the lyophilizate was in a volatile form such as ^{3}HHO. The results of these experiments indicated that tritium activity was removed from CI-572-^{3}H · HCl by heating in 4 *N* HCl.

While it has been shown that tritium activity can be removed from CI-572-^{3}H in hot 4 *N* HCl, it has also been established that the tritium activity

TABLE 2. Tritium label stability experiments on CI-572-^{3}H·HCl.

Experiment	Test Conditions [a]	% ^{3}H in Residue [b]	% ^{3}H in Lyophilizate [b]
1	H_2O, 3 days	101.4	0.1
2	1 *N* NaOH, 2 days	95.0	0.5
3	1 *N* HCl, 2 days	91.1	0.7
4[c]	4 *N* HCl, steam bath 4 hrs.	39.7	40.1

[a] Experiments 1, 2 and 3 were conducted at room temperature (approximately 25° C). See Appendix B.

[b] The % ^{3}H in the residues and lyophilizates is based on the measured tritium content of 3 ml of stock solution prior to any pH adjustments or lyophilization. The sensitivity of these experiments permits the detection of ≥ 0.1 % ^{3}H.

[c] No explanation for the low recovery of tritium is apparent.

is stable at room temperature in water and in aqueous 1 *N* NaOH and 1 *N* HCl. Thus, from these observations, it would appear that the tritium activity should serve as a satisfactory tracer for the carbon skeleton of CI-572-^{3}H.

ACKNOWLEDGEMENTS.

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APPENDIX A : *Chromatographic methods*

CI-572-^{14}C · HCl or CI-572-^{3}H · HCl and the authentic reference compounds (Fig. 1 and Table 1) were chromatographed together by the ascending technique on thin layers of Silica Gel G_{F254} until the solvent front was 15 cm from the origin. Using liquid scintillation spectrometry, the radiochromatograms were scanned for carbon-14 or tritium by measuring the dpm ^{14}C or

dpm ^3H on each of the 1 cm and/or 0.5 cm sections along the chromatographic lane from the origin to the solvent front. The T.L.C. developing solvent systems are listed below.

System	Composition	
A	EtOAc : MeOH : Et_3N,	75 : 5 : 1
B	EtOH : Et_2NH,	10 : 1
C	$CHCl_3$: MeOH : H_2O,	3 : 2 : 1 (lower phase)
D	MeOH : CH_3NH_2 (40 %, aqueous),	50 : 1
E	MeOH : NH_4OH (conc.),	100 : 1
F	$CHCl_3$: MeOH : Et_2NH,	45 : 5 : 1
G	$CHCl_3$: MeOH : 3 % NH_4OH,	3 : 2 : 1 (lower phase)
H	Benzene : MeOH : HOAc,	45 : 8 : 2
I	MeOH	

APPENDIX B : *Tritium stability tests on CI-572-^3H · HCl*

A stock solution of CI-572-^3H · HCl in water having a concentration of approximately 100 μg/ml was prepared and analyzed for tritium content per 3 ml aliquots. *Experiment 1* : A 3 ml aliquot of the stock solution was allowed to stand at room temperature for 3 days and then lyophilized. *Experiment 2* : A 3 ml aliquot of the stock solution was adjusted to 1 *N* NaOH with 1 ml 4 *N* NaOH and allowed to stand 2 days at room temperature. This solution was neutralized with 1 ml 4 *N* HCl and then lyophilized. *Experiment 3* : A 3 ml aliquot of the stock solution was adjusted to 1 *N* HCl with 1 ml 4 *N* HCl and allowed to stand 2 days at room temperature. This solution was neutralized with 1 ml 4 *N* NaOH and then lyophilized. *Experiment 4* : A 3 ml aliquot of the stock solution was adjusted to 4 *N* HCl by the addition of 1.47 ml 12.13 *N* HCl (concentrated) and heated on a steam bath for 4 hours. This solution was neutralized with 0.92 ml 19.3 *N* NaOH and a few drops of 4 *N* NaOH and then lyophilized.

The lyophilization procedure for all of the experiments was as follows (See Fig. 3) : A 50 ml flask (A) containing the test solution to be lyophilized was fitted with a nylon mesh screen over the open end of the flask and then fixed on one end of a small U-shaped high vacuum lyophilization apparatus.

The contents of the flask were frozen in liquid nitrogen. The system was evacuated to a pressure of $\leqslant$0.025 mm Hg and then closed at the stopcock. The liquid nitrogen bath was removed from under flask A and placed under tube B. Lyophilization was allowed to proceed with the lyophilizate (water condensate) being trapped by liquid nitrogen in tube B.

In all of the experiments, the resulting residues were dissolved in 3 ml 0.01 *N* HCl; these solutions and the corresponding lyophilizates were analyzed for total tritium content using liquid scintillation spectrometry.

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