Double Isotopic Labeling in Toxicological Analysis I. Strychnine in Blood—A Preliminary Report

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Initial results in the development of toxicological assays based on double isotopic labeling methodology are presented. It has been found that strychnine can be analyzed more accurately by this method than by more conventional procedures.

Among accepted analytical procedures for strychnine in biological fluids and tissues, probably the most commonly used is the spectrophotometric method, which ordinarily follows ethylene dichloride extraction (1). This method appeared to be the best available, but suffered from the serious disadvantage that recovery of strychnine from samples containing known amounts was never higher than 75%, and was most commonly in the 40–60% range. Since very sensitive radiochemical assays using double isotopic labeling have been reported (2) for aldosterone and other steroids, the authors were prompted to investigate the applicability of these methods to strychnine analysis.

In the steroid procedures, it is usual to add a quantity of the ^{14}C-labeled steroid to be assayed, allow the total sample to react with a tritium labeled reagent, purify the steroid derivative of interest chromatographically, and count the ^{14}C and tritium. Using ^{14}C recovery to evaluate losses during purification, it is then possible to calculate the total tritium activity, thus the concentration of the desired steroid, in the sample.

Since ^{14}C-labeled strychnine is not readily available, it was necessary to prepare a suitable ^{14}C-labeled derivative of strychnine. The planned assay envisioned allowing strychnine present in the sample to react with methyl iodide, so it was obvious that the derivative of choice would be ^{14}C-labeled strychnine methiodide. This would be added to the sample at the outset, following which the total sample would be allowed to react with ^{3}H-labeled methyl iodide. The resulting double-labeled strychnine methiodide would be purified chromatographically, and strychnine estimated by calculating the total tritium content of the sample as in the steroid case.

Thin-layer chromatography seemed well suited to the planned assay, due to its speed and simplicity. Numerous reports of recovery of radioactive substances from thin-layer plates can be found, but in this case strychnine methiodide was so firmly bound to the adsorbent that effective elution and counting were not possible. Paper chromatography was next investigated, and a number of experiments indicated that, although strychnine methiodide could not be eluted from the paper, immersion of small pieces of paper containing the radioactive material into standard fluor solutions enabled ^{14}C and ^{3}H to be counted with efficiencies of 60.3 and 21.4%, respectively. Counting efficiency was reasonably reproducible with all samples of paper used. In counting double-labeled samples, the authors found that it is a simple matter to insure that all activity due to tritium is confined to the tritium channel. However, it is never possible completely to eliminate activity due to ^{14}C from the tritium channel, so that a correction for this factor must be applied. It was found that the ratio ^{14}C c.p.m. in ^{3}H channel/^{14}C c.p.m. in ^{14}C channel exhibited sufficient variation with different pieces of filter paper that it was not possible to calculate a single correction factor for use in all assays. Instead, it was necessary to apply a spot of ^{14}C-labeled strychnine methiodide to each chromatogram and to calculate this factor independently for each run. Observed radioactivity appeared to be independent of the positioning of filters in the counting vials.

Suitable chromatographic procedures having been found, it was necessary to establish conditions ensuring 100% reaction between strychnine and methyl iodide-^{3}H. Initial attempts, using a 100:1 *M* excess of methyl iodide-^{3}H and making no particular effort to remove the water from the reaction mixture, indicated that the reaction had proceeded to about 30% completion. On increasing the molar ratio of methyl iodide to about 1000:1 and removing most of the water initially present by evaporation in the presence of a large excess of ethanol, quantitative reaction was achieved. Results, including complete calculations, for a typical assay of strychnine in aqueous solution are presented in Table I, where it is seen that recovery is essentially quantitative.

Table I.—Assay Results for Strychnine in Water

		Sample		
		Blank	1	2
^{14}C	c.p.m. (obs.)	1116	500	360
	dpm[a]	1850	829	600
	recovery, %[b]	19.3	8.64	6.22
^{3}H c.p.m. (obs.)		619	1120	775
^{14}C in ^{3}H, c.p.m.[c]		513	210	151
^{3}H c.p.m. (corr.)		106	910	624
^{3}H dpm[a]	obs.	495	4250	2920
	total	2560	49,200	46,900
^{3}H dpm due to strychnine			46,600	44,300
Strychnine, mg.[d]			0.34	0.32
Strychnine recovery, %[e]			104	100

[a] Counting efficiency: ^{14}C, 60.3%; ^{3}H, 21.4%. [b] ^{14}C added: 9600 dpm. [c] ^{14}C c.p.m. ratio: ^{3}H channel/^{14}C channel = 0.42 (this run). [d] Strychnine specific activity: 140,000 dpm/mg. [e] Strychnine added: 0.32 mg.

No particular difficulty was encountered in applying the method to analysis of strychnine in blood, for which results from one run are shown in Table II. Blood was selected as the biological specimen to be used because in our experience it is the most difficult fluid from which to obtain good analytical results, since it contains many compounds of all chemical types. As can be seen in Table II, blood yielded

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TABLE II.—ASSAY RESULTS FOR STRYCHNINE IN BLOOD

		Sample		
		Blank	1	2
^{14}C	c.p.m. (obs.)	350	290	109
	dpm[a]	580	481	181
	recovery, %[b]	6.04	5.01	1.89
^{3}H c.p.m. (obs.)		1330	1561	569
^{14}C in ^{3}H, c.p.m.[c]		161	133	50
^{3}H c.p.m. (corr.)		1179	1428	519
^{3}H dpm[a]	obs.	5509	6670	2425
	total	91,200	133,200	128,300
^{3}H dpm due to strychnine			42,000	37,100
Strychnine, mg.[d]			0.30	0.27
Strychnine recovery, %[e]			94	84

[a] Counting efficiency: ^{14}C, 60.3%; ^{3}H, 21.4%. [b] ^{14}C added, 9600 dpm. [c] ^{14}C c.p.m. ratio: ^{3}H channel/^{14}C channel = 0.46 (this run). [d] Strychnine specific activity: 140,000 dpm/mg. [e] Strychnine added: 0.32 mg.

quite high blank values, but the analytical results show that the method is capable of yielding much better recoveries than the spectrophotometric procedure, despite this complication.

It is believed that the results presented here are sufficient to establish the feasibility of the method. Although an insufficient number of assays has been run to present the usual estimates of accuracy and precision over a range of concentrations, results obtained in several duplicate assays lead the authors to believe recoveries of at least 80% may be routinely achieved. In principle, the sensitivity of the method is limited only by the specific activity of the reagents. As strychnine concentrations decrease, it will probably be necessary to perform two or more chromatographic separations on each sample in order adequately to purify the strychnine methiodide for counting. In these initial studies, relatively substantial quantities of strychnine were employed in order to minimize the required specific activity of the reagents. The authors will continue to explore the method with respect to accuracy, precision, sensitivity, and applicability to other substances of toxicological interest which are assayed only with difficulty by other methods.

EXPERIMENTAL

Preparation of Strychnine Methiodide-^{14}C.—Strychnine (430 mg.) was dissolved in 50 ml. of absolute ethanol, and a twofold molar excess of methyl iodide-^{14}C in absolute ethanol was added. The mixture was heated under reflux for 2 hr., at which time a thin-layer chromatogram indicated the reaction to be complete. On cooling the solution in dry ice, a precipitate appeared, which was collected, washed, and dried, affording 170 mg. of strychnine methiodide. Evaporation of the solvent yielded an additional 270 mg. of product. Both fractions were again checked by TLC and found to be homogeneous. The specific activity of the strychnine methiodide was found to be 20.9 μc./mmole.

Preparation of Strychnine Methiodide-^{3}H.—In order accurately to determine the specific activity of the methyl iodide-^{3}H to be used, strychnine methiodide-^{3}H was prepared in a similar fashion from 105 mg. of strychnine. Its specific activity was found to be 12.6 μc./mmole.

Preparation of Strychnine Stock Solution.—Strychnine (8 mg.) was added to 5 ml. water, and 5 drops of 0.1 *N* HCl was added to dissolve the alkaloid. Five drops of 0.1 *N* NaOH was then added, and the solution made to a volume of 25 ml. Its pH was found to be 8.2, and the solution was stored in a refrigerator for not more than 1 week.

Assay Procedure.—Strychnine stock solution and/or water was added to 1 ml. human citrated blood to make a total volume of 2 ml., followed by addition of 0.2 ml. (9600 dpm) of a solution of strychnine methiodide-^{14}C in absolute ethanol. After addition of 2 ml. of absolute ethanol, the mixture was evaporated at 60° on a rotary evaporator under water pump vacuum nearly to dryness. After addition of about 20 ml. of ethanol, the mixture was again evaporated nearly to dryness. One milliliter of a solution of methyl iodide-^{3}H in absolute ethanol (0.1 Gm. or about 30 μc./ml.) was then added, and the mixture was sealed in a glass tube. After heating at 60° for 4 hr., the tube was opened, and excess methyl iodide and ethanol evaporated in an efficient hood, until the final volume of solution was approximately 0.1 ml. This was transferred to Whatman No. 1 chromatography paper. Two control spots were also applied to each chromatogram. First, in order to be sure of the location of strychnine methiodide, "cold" strychnine methiodide was spotted. After development of the chromatogram, the edge on which this spot had run was cut off and sprayed with potassium iodoplatinate reagent to locate the strychnine methiodide. A band of the chromatogram corresponding to the R_f displayed by this strychnine methiodide was used for counting. Second, a spot containing a total of 2400 dpm of strychnine methiodide-^{14}C was applied in order to be sure of quenching corrections, as previously detailed. The chromatogram was developed by the descending technique with ethyl acetate–absolute methanol–28% NH_4OH (100:10:5). The band of the chromatogram found to contain the strychnine methiodide from each spot was then cut out and inserted in a counting solution consisting of 10 ml. of a toluene fluor solution containing 0.5% PPO and 0.04% dimethyl POPOP and 5 ml. of toluene. The amount of ^{14}C and ^{3}H contained in each spot was then determined, using a Beckman liquid scintillation spectrometer. Using the percentage recovery of ^{14}C to correct for losses during workup, the amount of strychnine methiodide-^{3}H, thus the amount of strychnine, in the original sample was calculated.

REFERENCES

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