Brit. J. Pharmacol. (1959), **14**, 137.

TRACER STUDIES OF THE DISTRIBUTION AND TRYPANOCIDAL ACTION OF STILBAMIDINE IN RATS

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(RECEIVED DECEMBER 19, 1958)

[^{14}C]Stilbamidine was used to study the distribution of the drug in the organs and tissues of rats following intravenous injection. The prophylactic action of stilbamidine was shown to depend upon the unchanged drug retained in tissues, especially the liver. Only the parent drug was extracted from trypanosomes when an infection was treated during the acute phase, as shown by the use of the fluorescent properties of stilbamidine in conjunction with scanning and chromatographic techniques. The action of stilbamidine on trypanosomes is therefore a direct one. A method for the synthesis of [^{14}C]stilbamidine in much improved yield is also described.

Reid and Weaver (1951) measured the distribution of radioactivity in tissues of mice at intervals after administration of [^{14}C]stilbamidine. In the experiments to be described similar measurements were made in rats and chromatographic methods were used to determine the chemical nature of the radioactive substances. The same methods enabled us to determine, more accurately than earlier methods allowed, the uptake of the drug by trypanosomes in the infected rat, and to indicate the nature of the compound responsible for prophylaxis.

Laveran and Mesnil (1904) observed that trypanosomes which had become sluggish or immobile in citrated blood recovered their motility on addition of fresh blood or serum. Biot, Biot and Richard (1911) found that glucose increased the survival time of *T. lewisi in vitro*. The need for glucose was reported for a number of other species of trypanosomes. This led to the search for trypanocidal agents with hypoglycaemic properties. Two groups of workers, von Jancsó and von Jancsó (1935) and Schern and Artagaveytia-Allende (1936), discovered that guanidine and certain derivatives possessed trypanocidal properties *in vivo*, the most active being decamethylenediguanidine (Synthalin). King, Lourie, and Yorke (1937) examined many compounds related to Synthalin, and discovered that certain diamidines had marked trypanocidal action *in vivo* and *in vitro*. The synthesis of these compounds was exploited by Ashley, Barber, Ewins, Newbery, and Self (1942). The most active substances they discovered were stilbamidine, propamidine, and pentamidine. The pharmacology, mode of action, and therapeutic uses of diamidines have been discussed by Heathcote (1946), Schoenbach and Greenspan (1948), Kirk (1957), and others. These substances have been shown to inhibit the growth of bacteria, protozoa, pathogenic fungi, and, to some extent, neoplastic cells. Stilbamidine and propamidine have proved somewhat toxic, but pentamidine is still widely used as a prophylactic against human trypanosomiasis, and an injection given every 6 months ensures protection from the disease. Kopac (1947) has observed that stilbamidine brings about a general denaturation of the nucleoproteins, but the mode of action of diamidines as a class has not been elucidated.

MATERIALS AND METHODS

Biological Methods

The strain of *Trypanosoma rhodesiense* used was isolated from a patient with sleeping sickness in 1923 and has since been maintained by syringe passage of infected blood in mice. White rats of weight 150 to 200 g. were used in the present experiments. Each animal was given 2 to 3 mg. of [^{14}C]stilbamidine (2.5 to 3.8 μC.) in distilled water by a tail vein or, more rarely, intraperitoneally. Specimens of blood, liver, kidney, lung, spleen, heart, intestine, and faeces were freeze-dried, then powdered and plated for measurement of radioactivity on polythene discs with an area of 1 cm.^{2} (Popják, 1950). Urine samples were directly evaporated on lens tissue on polythene discs. Samples were counted at "infinite thickness" by a thin-mica-window Geiger-Müller counter.

For the study of the uptake of stilbamidine by trypanosomes *in vivo*, infected rats were treated with 2 mg. of the radioactive material (0.69 μC./mg.). After 3 hr. the animals were anaesthetized and bled by cardiac puncture into citrated saline and the parasites isolated as described by Fulton and Spooner (1956). They were washed by resuspension in 10% horse serum in buffered saline (Krebs and Eggleston, 1940) and then centrifuged. After a final wash in saline they were isolated as a white mass free from white cells, then freeze-dried and plated as above. Correction was made for sodium chloride in the suspending liquid. For chromatographic examination the trypanosomes were extracted by grinding with sand mixed with 80% aqueous ethanol containing 1 to 2% acetic acid. In another series of experiments 6 rats were given a dose of 2.0 mg. of [^{14}C]stilbamidine (1.27 μC./mg.), and after certain intervals varying from 1 to 21 days an animal was heavily infected with trypanosomes. 24 hr. later trypanosomes had multiplied, the animal was killed, and the parasites were isolated from the blood. On freeze-drying, the radioactivity of the sample was measured.

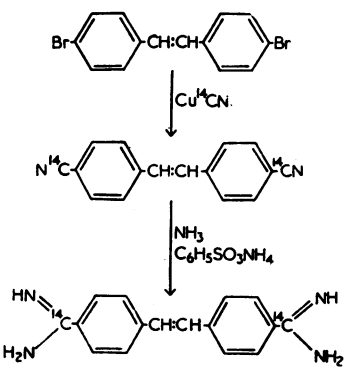
To find whether stilbamidine was metabolized by rat tissue *in vitro*, 10 g. of fresh liver was obtained aseptically and ground up in approximately 32 ml. saline containing 0.2% glucose. The suspension was divided into 3 portions of 10 ml. To one portion was added 1.3 ml. of a [^{14}C]stilbamidine solution containing 1 mg./ml. (activity 1.27 μC./mg.). All 3 portions were incubated for 4 hr. at 37°. A similar amount of stilbamidine was added to the second portion at the end of incubation. To the third portion the same volume of saline was added. Each portion was then centrifuged at 15,000 *g* for 30 min. The supernatant was separated from the deposit and its radioactivity measured. The supernatant portion and extracts from the deposit were also examined chromatographically.

A typical extraction of a tissue was carried out as follows. Wet liver (1.5 g.) was ground with clean sand and stirred with 50 ml. of 1% acetic acid (Lieben and Snapper, 1950) for 20 min. The suspension was centrifuged at 3,000 rev./min. for 30 min. and the clear solution decanted. The residue was re-extracted with 30 ml. of 1% acetic acid as before. The solid residue was separated as far as possible from sand and when dried gave 37.5 counts/min. as against 430 in the original sample. The combined extracts were centrifuged again to remove a further small amount of precipitate and concentrated under reduced pressure to a small volume.

For determination of radioactivity due to stilbamidine in urine, carrier technique was employed. To 20 ml. of urine was added 70 mg. of non-radioactive stilbamidine hydrochloride in 20 ml. water. Stilbamidine sulphate was precipitated by addition of 5 ml. 2N-H_2SO_4. The sulphate was collected by centrifugation and stirred with 5 ml. of N-NaOH; the base was collected by centrifugation and converted by dilute HCl to the hydrochloride which was crystallized three times in the presence of charcoal from dilute HCl. The purified hydrochloride (17.6 mg.) was powdered and plated.

Chemical Methods

A synthesis of [^{14}C]stilbamidine was carried out by Reid and Weaver (1951), but the overall yield based on cyanide (the form in which ^{14}C was introduced) was only 19%. To improve yields, the reaction of 4:4′-dibromostilbene with cuprous cyanide and the preparation of the amidine from the dinitrile were studied in detail with non-radioactive materials. By using a very pure specimen of the dibromo-compound and a 7% excess of cuprous cyanide, an 81% yield based on cyanide was obtained. The conversion to amidine was effected by fusing the dinitrile with ammonium benzenesulphonate (Oxley and Short, 1946; Oxley, Partridge and Short, 1948) in a stream of ammonia to give a 75% yield. The synthesis may be represented thus:



4 : 4′-Dibromostilbene.—This was prepared from dibenzyl according to Bance, Barber, and Woolman (1943) and formed pearly white crystals after four recrystallizations from glacial acetic acid; m.p. 210 to 212°.

4 : 4′-Di[^{14}C]*cyanostilbene.* — Radioactive cuprous cyanide from the Radiochemical Centre, Amersham (27.8 mg., 500 μC.), was mixed with ordinary cuprous cyanide (166 mg.) and pure 4:4′-dibromostilbene (359 mg.). Dry pyridine (0.25 ml.) was added and the mixture heated under reflux for 1 hr. at 220° in an atmosphere of nitrogen. The cooled product was extracted with 12 ml. of hot pyridine, and the solution poured into excess concentrated HCl (20 ml.). The precipitated dicyanostilbene was collected on sintered glass, washed with concentrated HCl, then water, and dried. Crystallization from 7 ml. of pyridine gave a very pale yellow product (202 mg.), m.p. 283 to 286°.

The mother liquid was mixed with ordinary dicyanostilbene (100 mg.), concentrated a little, and cooled. The crystals (107 mg.) were collected and used to prepare stilbamidine of lower radioactivity.

4 : 4′-Stilbenedi[^{14}C]*amidine* (*Stilbamidine*). — The radioactive dicyanostilbene (202 mg.) was mixed with dry ammonium benezenesulphonate (760 mg.) in a small tube and heated to 275° in a metal bath. A slow stream of dry ammonia was bubbled through the melt for 1½ hr.; after the first 15 min. a solid began to separate from the melt. The cooled product was warmed with water, broken up, and treated, while cooling, with 2N-KOH solution (5 ml.). The crude solid amidine was collected and washed with water on a sintered glass filter. It was dissolved on the filter in 0.1N-HCl (20 to 25 ml.). The small residue (mostly unchanged dicyanostilbene) was added after washing with water to dicyanostilbene of lower radioactivity. The pale yellow solution of the hydrochloride (filtrate and washings) was concentrated to 7 ml. under low pressure and treated with 1 ml. of concentrated HCl. After cooling, the crystals of stilbamidine hydrochloride were collected, washed with a small quantity of 3N-HCl, and dried. The product (242.6 mg., 74%) was crystallized twice from water (3 to 4 ml.) with addition of charcoal to give nearly colourless crystals (160 mg.).

The same product from a similar run of non-radioactive material was analysed after drying in air for 24 hr. (Found: C, 51.66; H, 5.86; N, 14.97; Cl, 18.4; required for $C_{16}H_{16}N_4$, 2HCl, $2H_2O$: C, 51.47; H, 5.89; N, 15.02; Cl, 19.01%.)

The dicyanostilbene of lower radioactivity (107 mg.) was converted to stilbamidine by heating with ammonium benzenesulphonate (500 mg.) as before. The solution of the hydrochloride was mixed with the mother liquors of the previous run, then concentrated and crystallized. Colourless material (75 mg.) was obtained on recrystallization from water with addition of charcoal. Counting was done after dilution of each sample with several hundred times its weight of non-radioactive stilbamidine hydrochloride followed by recrystallization. The specific activities found for the two (undiluted) samples were: Sample I, 1.27 μC./mg., and sample II, 0.70 μC./mg.

Chromatographic Methods

Paper chromatographic methods have not previously been reported for the separation of stilbamidine and the amides derived from it. Ascending chromatography in pyridine-*iso*amyl alcohol-water (7:7:6 by volume) was the most satisfactory system of several tried. The chromatography was run for 18 to 24 hr. and the spots were detected on the dried paper by ultraviolet light.

The R_F values for some of the compounds were: stilbamidine, 0.28; 4-amidino-4′-carbamoylstilbene, 0.51; 4:4′-dicarbamoylstilbene, 0.71.

The chromatograms were scanned for radioactivity by an instrument designed to count every square cm. for 1 min. and record the results automatically (Piper and Arnstein, 1956). Fluorescent non-radioactive spots were frequently observed, but no definite indication of non-fluorescent radioactive spots was found. At the start of this work confusion was caused by the presence of a well-defined radioactive spot a little in front of that due to stilbamidine which developed fluorescence gradually under ultraviolet illumination, and was due to the *cis*-form of stilbamidine which appears when stilbamidine is exposed to light but not otherwise. This was confirmed by using an authentic specimen of the *cis*-form kindly supplied by Dr. J. N. Ashley of May and Baker Ltd. Subsequently light was excluded during chromatography and no *cis*-stilbamidine was then found.

RESULTS

Distribution and Excretion of Drug.—The distribution of radioactivity in different organs and blood of the white rat is shown in Table I.

TABLE I

DISTRIBUTION OF [^{14}C]STILBAMIDINE IN RATS

The percentage of total injected radioactivity in the whole organ is given in the first column under each organ. The second numeral is the number of counts recorded/min. by a dried sample at infinite thickness on a 1 cm.^{2} disc and is a measure of the specific activity of the organ.

Rat	Dose; Route; Radioactivity	Interval After Dosing	Liver		Kidneys		Lungs		Spleen		Heart		Intestines		Serum	
			%	Counts	%	Counts	%	Counts	%	Counts	%	Counts	%	Counts	%	Counts
1	2 mg.; i.v. (2·55 μC.)	2 hr.	24·6	423	7·59	594	1·41	75	0·79	130	0·12	24	2·59	33	0·04	4·0
2	3 mg.; i.v. (3·8 μC.)	24 ,,	18·8	272	8·5	795	0·51	36·5	0·49	79	0·05	11·0	1·25	21·4	—	—
3	,, ,,	3 days	19·3	285	5·03	327	0·96	81·7	0·32	48	0·13	24·6	1·28	20·0	—	—
4	2·7 mg.; i.p. (3·4 μC.)	3 ,,	11·0	191	4·8	415	0·97	82·7	0·41	59·6	0·10	13·1	—	—	—	—
5	3 mg.; i.v. (3·8 μC.)	7 ,,	4·75	64·6	0·87	70	0·34	41·2	0·21	23·0	0·07	12·0	0·69	6·9	—	—
6	,, ,,	14 ,,	6·61	132	0·76	80	0·36	60·5	0·19	24·2	0·10	16·3	1·61	25·5	—	—
7	,, ,,	21 ,,	1·18	19·4	0·56	56·7	0·37	48·0	0·18	24·0	0·07	15	0·55	8·8	—	—
8	,, ,,	28 ,,	2·78	48·5	0·71	71·7	0·28	39·4	0·066	9·6	0·08	15·2	1·23	18·4	—	—
9	,, ,,	50 ,,	0·39	6·2	0·44	43·2	0·17	22·0	0·11	12·7	0·06	9·1	0·51	5·6	—	—
10	,, ,,	165 ,,	0·16	2·3	0·30	25·9	0·12	9·4	0·02	2·4	0·10	18·0	—	—	—	—
11	,, ,,	230 ,,	0·16	2·2	0·18	13·8	0·17	13·0	—	—	0·05	10·0	—	—	—	—
12	,, ,,	280 ,,	—	—	0·18	14·4	0·07	5·3	—	—	0·04	7·7	—	—	—	—

Previously Reid and Weaver (1951) had studied the same problem in mice while investigating the possibility of selective irradiation of plasma cells by administration of the drug to patients with multiple myeloma, and our results agree qualitatively with theirs. Probably because of the fact that only one animal was sacrificed at the end of each interval after administration of drug in these experiments the fall in radioactivity was not strictly correlated with the time of examination. It will be noted that the liver and kidney took up respectively 25 and 7.5% of the injected dose within the first 2 hr., the highest concentration (specific activity) being in the kidneys. In about 50 days most of the drug had been eliminated as indicated by the fact that radioactivity was below 0.5% of that originally present in liver and kidneys. Although lung, spleen, and heart took up relatively little drug they retained activity longer than some other organs. The radioactivity of serum corresponded to about 0.25 μg. of the drug/ml. 2 hr. after its administration and thereafter activity fell to zero. The red blood cells were also found to have no appreciable activity after 4 hr., and this confirms the earlier work of Fulton and Goodwin (1945). The radioactivity excreted in urine and faeces during the first 24 hr. following drug administration was 2.5 and 6.1% respectively of the injected dose.

Chemical Nature of Retained and Excreted Radioactivity.—Urine collected over 8 days from 12 rats each of which received 3 mg. (3.8 μC.) of stilbamidine was assayed for radioactivity, and the proportion of radioactivity present as stilbamidine was found by the carrier technique (see Methods) to be 68%. Chromatography of the urine showed minor proportions of the two amides, 4-amidino-4′-carbamoylstilbene and 4:4′-dicarbamoylstilbene, known to be formed from stilbamidine in solution by hydrolysis (Henry, 1948; Fulton and Goodwin, 1949). Substantially all the radioactivity in urine was extractable by chloroform or *iso*amyl alcohol after addition of alkali, but extractions of acidified urine by the same solvents removed only traces of radioactivity. Thus almost all the radioactivity was present in basic substances.

Extracts were made of liver and kidney from the series of injected animals and chromatograms of the extracts were scanned for fluorescence and for radioactivity. This study showed that even after long periods the radioactivity remaining in these tissues was due largely to unaltered stilbamidine; traces of the related amides were also present. Since extraction of radioactivity from tissue was virtually complete, and no non-fluorescent radioactive spots were detected, the possibility is slight that significant amounts of another metabolite were overlooked.

Chromatography of extracts of faeces showed stilbamidine accompanied by rather higher proportions of the amides than were found in tissues.

TABLE II

ABSORPTION OF PROPHYLACTIC DOSES OF STILBAMIDINE BY TRYPANOSOMES

Interval between Drug Administration and Infection (Days)	Radioactivity of Isolated Trypanosomes (Counts/Min. at Infinite Thickness)
1	13·5
2	16·0
4	8·1
7	6·4
14	3·0
21	Slightly above background

Prophylactic Experiments and Uptake of Drug by Trypanosomes.—The results of experiments designed to show the mode of action of stilbamidine in prophylactic experiments are given in Table II. It had been found previously that the prophylactic activity of the drug lasted about three weeks in mice treated with the maximum tolerated dose of non-radioactive material (Fulton, 1944). It is clear from the present results that appreciable quantities of drug are taken up by the parasites during the first few days after administration, but not after 21 days when the amount of drug stored in the liver was only 1 to 2% of the initial dose. This fact taken in conjunction with the absence of metabolites suggests that the prophylactic action of stilbamidine is the result of a direct action by the unchanged drug stored in the body. Fulton and Grant (1955, 1956) came to the same conclusion when they used non-radioactive stilbamidine. The incubation of stilbamidine with rat liver in the present series of experiments also failed to indicate conversion of the drug to a metabolite. In the same way, when a rat infected with trypanosomes was treated with the radioactive drug, and the parasites, which rapidly absorb stilbamidine, were harvested after 4 hr. and extracted with a suitable solvent, no substance other than the parent drug was detected by chromatographic and scanning techniques. The results in the present experiments having been obtained with more accurate techniques indicate a somewhat higher uptake of drug by the trypanosomes than was previously reported by the above workers.

It is a pleasure to acknowledge the generous help of Dr. J. W. Cornforth. One of us (K. K. M.) wishes to express his gratitude to the British Council and the Government of India for a scholarship under the Colombo Plan, which enabled him to take part in this work.

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