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METABOLIC STUDIES OF SYNTHETIC ISOJURIPIDINE IN THE DOG AND RAT

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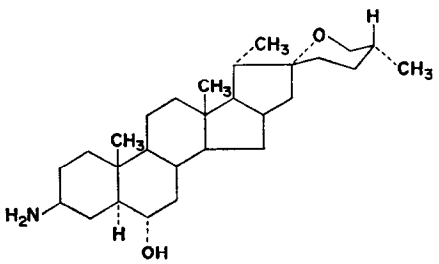
Abstract—Synthetic isojuripidine, an alkaloid of "Solanum Paniculatum", was labelled with ^3H by Wilzbach's method. In the dog, after single and repeated oral doses, the plasma levels were proportional to the amount administered and the drug seemed to be excreted very slowly. In the rat, after a single oral dose, the tritiated compound was absorbed and then retained by various tissues a long time, less than 1 per cent being eliminated in the urine in a 32 hr period. Over the same interval faecal excretion reached about 55 per cent of the dose. The metabolic pattern was studied at different times after dosing in the plasma of the two species and in the rat liver after lipophilic extraction. The major metabolite was the *N*-acetyl-derivative in both species. Contrary to what usually occurs, all these metabolites show less polarity than the original drug and this might explain their very slow disappearance from plasma and tissues.

OF THE eight steroid alkaloids with sapogeninic structure isolated by Cambiaghi *et al.*[1] from the roots of "Solanum Paniculatum L.", isojuripidine (I) presented particular interest for study, on account of its activity on the cardiovascular system, tested by Bergamaschi *et al.*[2] in the laboratory animal.

The natural product has been defined as (20βH, 22αO, 25R)-5α-spirostan-3β-amino-6α-ol, with the following structural formula:



Isojuripidine

Later, this compound and its isomers were synthesized by Gandolfi *et al.*,[3] starting from diosgenine and chlorogenone.

The present paper refers to some metabolic aspects of the compound and its tissue distribution in the dog and rat. The study was carried out to provide a better understanding of the duration of the pharmacological effect, and the toxicological implications of accumulation of the compound and its metabolites in the body, which was marked following repeated administration.

MATERIAL AND METHODS

Labelling of isojuripidine. 500 mg of isojuripidine were micronized in an agate mortar and tritiated with 7 Ci of tritium gas by the Wilzbach method at the Radiochemical Centre, Amersham. After tritium labile remotion, the compound was ground with hexane; the hexane was discarded by filtration, and the compound was dissolved in chloroform and washed with 2 N sodium hydroxide. The organic phase was evaporated to dryness and the residue was boiled with acetone for 1 hr. The isopropylidene-derivative was isolated by cooling. This, with acetic acid and the addition of ethyl ether, gave the acetate-derivative. The ^{3}H-isojuripidine was obtained as a free base from the acetate, dissolved in methanol, by alkalizing and slowly diluting with water. The compound was dried at 110° under reduced pressure. The yield of pure isojuripidine was 175 mg, sp. act. 620·8 μCi/mg.

This specific activity remained constant even after repeated treatment of the product with polar solvents to boiling. Radiochemical purity (98 per cent) was tested by thin layer gel and paper (Whatman No. 2) chromatography using the solvent system: benzene–acetone–diethylamine (50:45:5). Radioactivity distribution on the chromatograms was determined by direct reading with the Berthold flow counter scanner (model LB/2722) and the autoradiographic technique using Kodirex X-ray films. The sensitivity of the photographic response in thin layer chromatography was enhanced by mixing Silica gel with anthracene.[4] The cochromatographic presence of the synthesis standard was visualized by vanillin–H_2SO_4 spray reagent.[5]

Experiments in the dog. Three male beagle dogs, weighing 13, 12·7 and 13·6 kg were fasted overnight, then given orally micronized ^{3}H-isojuripidine in gelatine capsules. The first animal was treated with a single dose of 5 mg/kg, the second with a daily dose of 1 mg/kg for 5 days and the third with a daily dose of 0·1 mg/kg for 30 days. The dogs were kept in metabolic cages and fed normally throughout the experiment. Heparinized blood samples were taken from cephalic veins every 24 hr during the first month after administration and every 5 days during the following 2 months. The samples were centrifuged at 2000 *g* for 10 min and measurements of total radioactivity were carried out on plasma.

Experiments in the rat. Male rats, fasted overnight (outbred CFE, SPF), weighing 130–140 g were used. The labelled compound, suspended in water containing 0·5% methocel 90 HG, was given by gavage to several groups of at least five animals at a single dose of 10 mg/kg in a first experiment and 1 mg/kg in a second experiment. The rats were housed in metabolic cages, providing for separate collection of urine and faeces, and kept fasted throughout the experiment. Water was supplied *ad lib.* Under ether anaesthesia, heparinized blood samples were taken from the abdominal aorta at different times after administration. Liver, kidneys, adrenals, aorta, heart, spleen and a sample of skeletal muscle were removed and weighed. Complete collection of urine and faeces was carried out at the end of the experiments.

The plasma samples were isolated after centrifuging the blood to take the radioactivity count; the organs and faeces were hydrolysed at 60–70° with a suitable amount of 0·5 M hyamine hydroxide in methanol, to complete dissolution.

Assay of radioactivity. All counts were performed on a Tri-carb liquid scintillation spectrometer, Packard model 3320. One-ml plasma samples were counted in 10 ml of the Packard scintillator system Insta-gel; aliquots of urine and of the hydrolysed solution of organs and faeces were counted in the same proportion in Bray's liquid

scintillator.[6] All samples were recounted after addition of ^{3}H-toluene internal standard. The number of impulses detected from each sample ensured a standard deviation of less than 1 per cent.

Metabolic pattern in dog and rat plasma and in rat liver. Dog plasma samples taken up at 1, 2 and 30 days after administration of a single dose of 5 mg/kg ^{3}H-isojuripidine and at 10 days after the first of five daily doses of 1 mg/kg, were deproteinized with 10 vol. of methanol. After centrifuging, the supernatant liquid was evaporated *in vacuo* and the residue, taken up with pH 9 phosphate–NaOH buffer, was extracted three times with 5 vol. of methylene chloride.

Recovery of the initial radioactivity in the organic phase was approx. 90 per cent. The methylene chloride extracts were evaporated to small volume and processed by thin layer chromatography in the presence of the following synthesis standards:[1,3] (20βH, 22αO, 25R)-5α-spirostan-3β-amino-6α-ol (isojuripidine), its 3β-(*N*-acetylamino)-derivative, (20βH, 22αO, 25R)-5α-spirostan-3β-amino-6-one, its 3β-(*N*-acetylamino)-derivative, (20βH, 22αO, 25R)-5α-spirostan-3-one-6α-ol and (20βH, 22αO, 25R)-5α-Δ^2-spirosten-6α-ol. Organic extracts were pre-chromatographed on the same Silica gel plates with ethyl ether in order to remove the lipidic impurities. Chromatographic separation was then carried out by the bi-dimensional technique, using these solvent systems: benzene–acetone–diethylamine (50:45:5); cyclohexane–ethyl acetate (50:50) and cyclohexane–ethyl acetate (70:30).

Radioactive spots and the co-chromatographic presence of the synthesis standards were detected by the counting equipment and spray reagent. To achieve better resolution of the single spots, these were refined by elution with methanol and further chromatography of the extracts in methylene chloride. The exact amount of radioactivity in these new chromatograms was detected by the scraping technique[7] with the liquid scintillation counter.

The same procedure was followed for pooled plasma samples taken from the rats 16 hr after treatment with 10 mg/kg of ^{3}H-isojuripidine. To separate the metabolites in the liver, the organs of the rats treated at the same dose and time were homogenized in 0·9% (w/v) saline, using Virtis apparatus (Virtis Co. Inc., N.Y.). An aliquot of the homogenate was analysed for radioactivity and another was adjusted to pH 9 with buffer solution and lyophilized. The powder was then extracted continuously with methylene chloride in a Soxhlet apparatus. The concentrated extract was processed by thin layer chromatography as already described.

RESULTS AND DISCUSSION

Figure 1 shows the plasma levels of isojuripidine and its metabolites in the dog, following single or repeated oral doses of the labelled compound. After absorption, the product persisted in the body for a long time, giving almost constant plasma radioactivity levels, which fell only slowly. Plasma radioactivity was still evident, 3 months after administration. The slow release of the product is also shown by the fact that the plasma radioactivity curve for the dog treated with 1 mg/kg daily for 5 days overlaps that for the dog treated with a single dose of 5 mg/kg. In both cases, 6 days after administration, values are around 140–150 μg equivalent of isojuripidine per millilitre of plasma. The third dog, which was treated with a daily dose of 0·1 mg/kg for 30 consecutive days, showed a very similar pattern. In all three animals, plasma radioactivity decline showed a half-life of about 25 days.

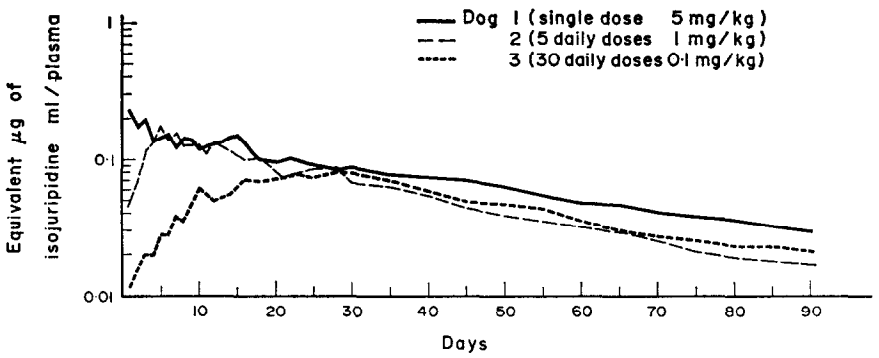


FIG. 1. Radioactivity plasma levels in dogs after oral administration of a single or repeated dose of ^{3}H-isojuripidine.

The volume of distribution of the compound and its metabolites in the dog, calculated graphically by extrapolation of the plasma radioactivity levels at time 0, and assuming complete absorption of the drug, amounts to about three times body weight. This gives an idea of how widely the compound spreads through tissues. Experiments in the rat (Table 1) show how much of the product and its metabolites are taken up by the various organs. The liver and kidneys in the rat reach peak radioactivity concentration 8 hr after administration, giving levels about ten times higher than in plasma. From 16 to 32 hr after oral administration of 10 mg/kg, plasma levels agree closely with those found in the dog at 24 hr, taking into account the different doses used. High radioactivity concentrations have also been found in the large blood vessels and myocardium in the rat, with even higher levels in the adrenals, liver, kidneys and spleen. In the spleen, the concentration is 40-times higher than in plasma. This high uptake and the fact that the product and its metabolites are not released rapidly from the organs involved, may explain the generalized toxic effects[2] mentioned at the outset.

A second investigation in the rat, treating the animals with single doses of isojuripidine 10-times smaller (Table 2), shows that even at lower doses the body is still unable to excrete the compound rapidly. The amounts of radioactivity in plasma and liver 16 hr after administration are still fairly proportionate to those noted with the 10 mg/kg dose.

Table 3 shows the radioactivity excreted at the end of the two experiments in the rat. Excretion of the product and its metabolites in urine is practically nil, but a fairly large amount appears in the faeces. Nevertheless, almost half the radioactivity administered is still present in the body 32 hr after the dose was given.

Figure 2 shows the metabolic pathway of the compound; the percentage distribution of unchanged product and its metabolites in plasma in the dog and rat, and in the liver in the rat, are shown in Table 4. Metabolization of the compound is very similar in the two species examined, both from the qualitative and quantitative points of view. This occurs mainly through *N*-acetylation, oxidation of the steroid structure in position 6, and deamination of the product. In both animals a good proportion of the unchanged product remains in plasma for a long time after administration, and in rat liver the metabolic picture is similar.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN RATS AFTER ORAL ADMINISTRATION OF ^{3}H-ISOJURIPIDINE AT THE DOSE OF 10 mg/kg*

Time (hr)	Plasma (μg/ml)	Liver (μg/g)†	Kidneys (μg/g)	Adrenals (μg/g)	Aorta (thoracic arch) (μg/g)	Heart (μg/g)	Skeletal muscle (μg/g)	Spleen (μg/g)
1	1·69 ± 0·21	8·17 ± 1·34	3·08 ± 0·57	3·62 ± 0·67	0·83 ± 0·09	1·72 ± 0·26	0·57 ± 0·09	
2	1·81 ± 0·10	12·02 ± 0·73	5·33 ± 0·33	6·67 ± 0·70	0·92 ± 0·05	2·35 ± 0·17	0·86 ± 0·07	
4	1·52 ± 0·08	12·32 ± 1·63	7·23 ± 0·93	8·95 ± 1·51	1·28 ± 0·19	3·16 ± 0·38	0·96 ± 0·11	
8	1·39 ± 0·08	16·02 ± 0·63	12·65 ± 0·46	15·34 ± 0·88	1·80 ± 0·25	4·75 ± 0·17	2·13 ± 0·15	
16	0·55 ± 0·06	13·41 ± 11·41	10·93 ± 1·20	17·26 ± 3·25	2·52 ± 0·58	4·85 ± 0·71	2·49 ± 0·38	2·43 ± 8·31
32	0·45 ± 0·01	11·41 ± 0·77	10·51 ± 0·40	14·58 ± 0·68	2·27 ± 0·12	4·61 ± 0·15	2·79 ± 0·24	

* Mean values and S. E. are expressed as equivalent micrograms of compound and referred to five animals for each treatment.
† Intended as wet weight for all the organs.

TABLE 2. PLASMA AND LIVER RADIOACTIVITY IN RATS AFTER ORAL ADMINISTRATION OF ^{3}H-ISOJURIPIDINE AT THE DOSE OF 1 mg/kg*

Time (hr)	Plasma (μg/ml)	Liver	
		μg/g†	%
1	0·174 ± 0·024		
2	0·201 ± 0·007		
4	0·151 ± 0·008		
8	0·108 ± 0·005		
16	0·081 ± 0·003	0·93 ± 0·03	2·67 ± 0·07
32	0·070 ± 0·003		

* Mean values and S. E. are expressed as equivalent micrograms of isojuripidine and as per cent dose, and referred to six animals for each treatment.

† Intended as wet weight.

TABLE 3. RADIOACTIVITY EXCRETION IN URINE AND FAECES OF RATS 32 hr AFTER ORAL ADMINISTRATION OF ^{3}H-ISOJURIPIDINE*

Dose	Urine	Faeces
10 mg/kg	0·83 ± 0·02	58·58 ± 1·93
1 mg/kg	1·66 ± 0·27	54·82 ± 6·36

* Mean values and S. E. are expressed as per cent dose and referred to five and six animals respectively, for the dose of 10 mg and 1 mg/kg.

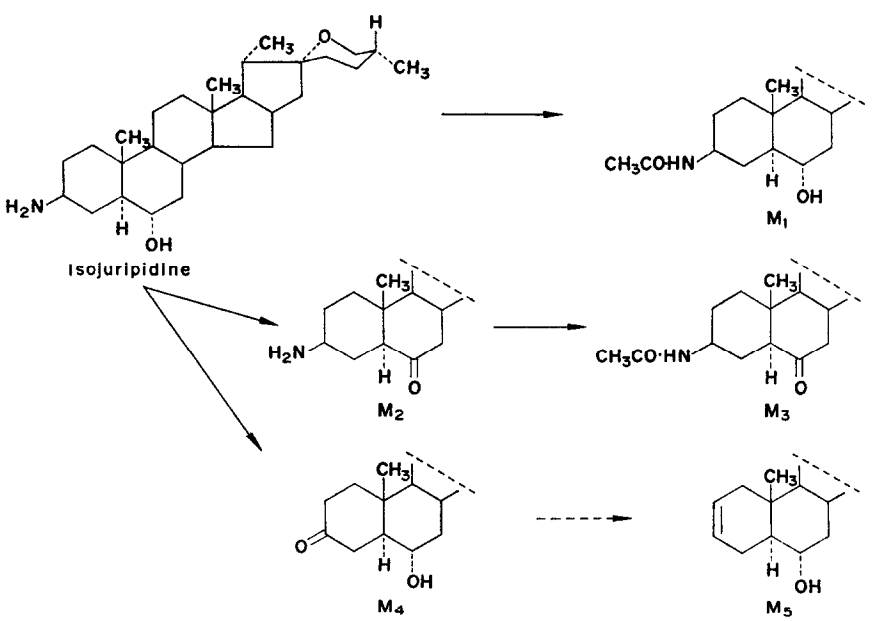


FIG. 2. Metabolic pathways of isojuripidine in the dog and rat.

TABLE 4. ISOJURIPIDINE AND ITS METABOLITES IN PLASMA AND LIVER AFTER ORAL ADMINISTRATION OF TRITIUM-LABELLED COMPOUND TO DOGS AND RATS*

	Dog plasma				Rat plasma†	Rat liver†
	Dose: 10 mg/kg			5 Doses: 1 mg/kg	Dose: 10 mg/kg	Dose: 10 mg/kg
	24 hr	48 hr	30 days	10 days	16 hr	16 hr
Total radioactivity as equiv. ng/ml	230	175	88	130	550	13·410‡
Unchanged isojuripidine	46·7	45·1	35·3	40·0	46·0	30·2
3-*N*-acetylamino-derivative (M_1)	18·8	25·6	32·7	28·4	13·2	20·4
(20βH, 22αO, 25R)-5α-spirostan-3β-amino-6-one (M_2)	8·1	4·4	1·9	3·6	7·1	7·4
3-*N*-acetylamino-6-one-derivative (M_3)	4·5	5·3	12·0	11·5	7·9	4·9
(20βH, 22αO, 25R)-5α-spirostan-3-one-6α-ol (M_4)	5·4	4·4	2·5	2·7	9·7	15·6
(20βH, 22αO, 25R)-5α-Δ^2-spirosten-6α-ol (M_5)	5·3	3·5	1·8	2·6	4·5	3·2
Unidentified metabolites	5·7	3·7	1·7	2·8	6·6	9·2
Not extractable radioactivity	5·5	7·9	12·1	8·4	5·0	9·1

* The amounts of unchanged drug and its metabolites are expressed as percentage distribution of the radioactivity present in the sample.
† Pooled samples of five animals.
‡ Equivalent nanogram per gram of wet organ.

Overall distribution of the metabolites in plasma in the dog changes over time, the deamination products declining and *N*-acetyl derivatives increasing. It is interesting that the dog, considered to have poor acetylating capacity,[8] primarily metabolized isojuripidine by this route. The radioactivity corresponding to unidentified metabolites which probably form in the deamination process, could not be identified because of the lack of corresponding synthesis standards.

TABLE 5. R_f VALUES OF ISOJURIPIDINE AND ITS METABOLITES IN THIN LAYER CHROMATOGRAPHY

	Solvent systems		
	A	B	C
Isojuripidine	0·20	0·00	0·00
3-*N*-acetylamino-derivative (M_1)	0·29	0·07	0·00
(20βH, 22αO, 25R)-5α-spirostan-3β-amino-6-one(M_2)	0·42	0·00	0·00
3-*N*-acetylamino-6-one-derivative (M_3)	0·66	0·00	0·00
(20βH, 22αO, 25R)-5α-spirostan-3-one-6α-ol (M_4)	0·71	0·29	0·16
(20βH, 22αO, 25R)-5α-Δ^2-spirosten-6α-ol (M_5)	0·84	0·61	0·41

A: Benzene, acetone, diethylamine (50:45:5).
B: Cyclohexane, ethyl acetate (50:50).
C: Cyclohexane, ethyl acetate (70:30).

In Table 5 the R_f values of isojuripidine and its identified metabolites are presented. All these metabolization compounds show lower polarity than original drug. On the other hand it is known that drug metabolism is commonly a process of polarization and metabolites are almost invariably more polar (less lipid soluble) than the parent compound and generally less toxic for their more rapid excretion by the kidney. There is considerable evidence that in both species examined, isojuripidine does not follow the usual metabolization processes and its metabolic pattern helps reach a plausible explanation of why it is released so slowly from tissues, and why the body has such difficulty excreting it.

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