[Chem. Pharm. Bull. 22(9)2042—2047(1974)]

UDC 547.632.09 : 615.31.033.076.9

Metabolism of Triphenylmethane Colors. I. Absorption, Excretion, and Distribution of Guinea Green B (FD and C Green No. 1) in Rats[1]

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(Received November 30, 1973)

Absorption, excretion, and distribution of Guinea Green B were investigated in rats. This color was hardly absorbed when given orally; only 2.28% of the dose of the color was recovered in the bile after 24 hr. The cumulative recovery of biliary excretion amounted to 86.5% at 4 hr and 96.5% at 24 hr after intravenous injection. The color was not metabolized except the formation of the leuco form in the large intestine. In the case of intravenous injection, the color disappeared rapidly from various tissues except in the liver and kidney.

Hess and Fitzhugh[3] have indicated that many FD and C triphenylmethane colors administered orally to rats were not absorbed and almost completely excreted in the feces, and that the colors were found in small amounts in the bile of dogs after oral administration.

Iga, *et al.*[4] have pharmacokinetically compared the biliary excretion of several triphenylmethane dyes in rats, and observed a remarkable effect of sulfonate group in the structures and of the binding ratio with plasma protein.

In his chronic study, Hansen[5] found that a long-term feeding of Guinea Green B to rats induced a small incidence of hepatic tumors after 2 years; 9 of 50 animals fed on the diet containing 5% of the color had primary hepatic tumors, among which two cases were apparently malignant.

We have been reinvestigating the metabolic fate of various food additives in order to ascertain their safety. As the first step in the metabolic study on some triphenylmethane food colors, we are interested in the detailed metabolic fate of Guinea Green B which has already been prohibited from use as a food additive because of such a dangerous effect on the rat liver as described above.

This paper deals with the absorption, excretion, and distribution of Guinea Green B in rats, and an improved assay method for the color in tissues according to the two-wavelength photometric technique.

Experimental

Animals and Treatment——Adult male Wistar rats weighing 300 to 350 g were maintained on a standard diet and tap water given freely. The animals were divided into six groups of 5 animals each, except in the experiments on the plasma level and biliary excretion.

Guinea Green B dissolved in water was administered orally to rats in a single dose of 50 mg/kg by a stomach tube unless otherwise noted.

Chemicals——National Institute of Hygienic Sciences standard for Guinea Green B (97.5% pure) was used. Chloranil, benzethonium chloride (Hyamine 1622), and *tert*-butanol were purchased from Wako Pure Chemical Industries, Ltd., Tokyo.

1) This work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 6, 1973.
2) Location: *18-1 Kamiyoga 1-Chome, Setagaya-ku, Tokyo, 158, Japan.*
3) S.M. Hess and O.G. Fitzhugh, *J. Pharmacol. Exp. Therap.*, **114**, 38 (1955).
4) *a*) T. Iga, S. Awazu, and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 273 (1971); *b*) T. Iga, S. Awazu, M. Hanano, and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 2609 (1971).
5) W.H. Hansen, "Chronic Studies on FD & C Colors," Food and Drug Administration, 1962.

Extraction of the Color from Various Tissues and Serum——After administration of the color, each animal was placed in an individual metabolism cage to collect the urine and feces.

Various tissues and blood samples were obtained at regular intervals from separate groups of animals. The tissues were removed, minced with scissors, and homogenized with 3 volumes of distilled water in a Polytron. Serum was obtained from blood samples by centrifugation at 4300×*g* for 10 min. The color was extracted from 0.5—1 g of tissue or 0.5—1 ml of serum with *tert*-butanol in acidic medium. The details of the extraction procedure are given in Fig. 1.

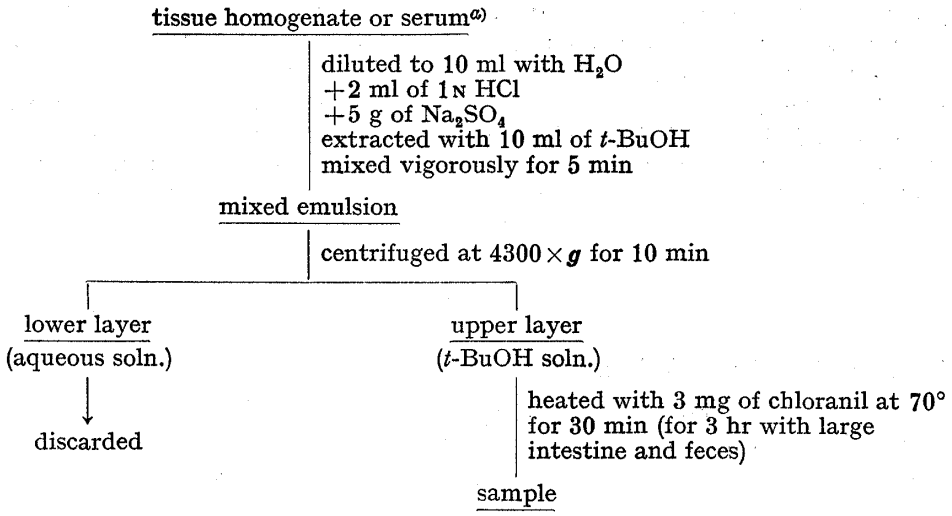

Fig. 1. Extraction of Guinea Green B from Various Tissues and Serum

a) added 2 ml of 10% Hyamine (benzethonium chloride) and diluted to 10 ml with H_2O

Biliary Excretion——The animals were anesthetized with ether. The bile duct was surgically exposed by a mid-line incision and cannulated with a fine polyethylene tubing. The cannula was then tied up and the abdomen was closed. After recovery from anesthesia, each animal received the color by a single intravenous injection (5 mg/kg) through a femoral vein or by a single oral administration (50 mg/kg).

Bile was collected in a 10-ml graduated tube every 30 min or 1 hr. After an aliquot (0.1—1.0 ml) of the bile collected was diluted to 10 ml with distilled water, the color was extracted by the same procedure as from tissues and serum.

Determination of Guinea Green B in Tissues——As shown in Fig. 1, the *tert*-butanol extracts from the samples were usually heated at 70° with chloranil for 30 min in order to reconvert the leuco form to the original color according to the modified method of Hess and Fitzhugh.[3)] In the case of large intestine and feces, the leuco form was present so much that the heating time had to be prolonged up to 3 hr. The color solution treated with chloranil was eventually determined according by the two-wavelength photometric technique with a Hitachi Model 356 Two-wavelength/Double-beam Spectrophotometer.

The color recovered from the leuco form by the above procedure was identified as Guinea Green B by the following experiment. The feces, in which most of Guinea Green B was converted to the leuco form (see Table III), was extracted with *tert*-butanol and treated with chloranil under the condition described above.

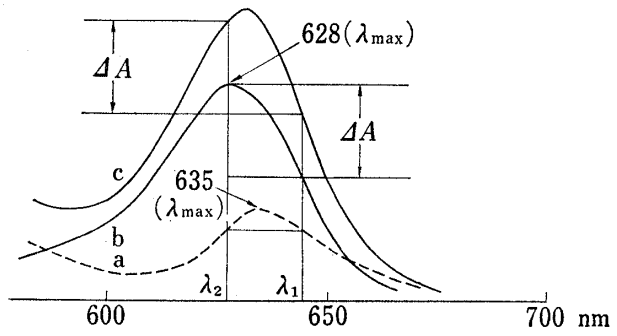

Fig. 2. Typical Absorption Spectra of Liver Samples

a: liver
b: Guinea Green B
c: liver+Guinea Green B
λ_1: 645 nm
λ_2: 629.5 nm

TABLE I. Two-wavelengths used in Various Tissues

Tissue	λ_1 (nm)	λ_2 (nm)
Stomach Small intestine Large intestine Feces	700	628
Liver Kidney	645	629.5
Serum	649.5	629.5
Abdominal muscle	600	625
Brain	594	625
Bile	700	628

The colored extract was spotted on thin-layer plates 1 and 2, and developed with the solvent systems a and b (see below).

The *Rf* values agreed with those of authentic Guinea Green B. The spot was extracted with *tert*-butanol followed by measurement of the absorbance spectrum, the curve of which was also the same as that of the authentic sample ($E_{max}=628$ nm).

Fig. 2 shows the typical absorption spectra of the extracts from rat liver homogenate (curve a), Guinea Green B (curve b), and their mixture (curve c), and the graphic explanation of how to select the two-wavelengths, λ_1 (645 nm) and λ_2 (629.5 nm). The interference of other colored materials in the liver could be abolished when the differential absorbance of the mixture, $\Delta A=\lambda_1-\lambda_2$ was measured (*cf.* curve c). The values of λ_1 and λ_2 in other tissues were determined by the same procedure, and their wavelengths are listed in Table I. The calibration curve was separately obtained for each tissue.

Thin-Layer Chromatography——Thin-layer chromatography was carried out by using (1) Avicel SF and (2) a mixture of Avicel SF and silica gel HF (7: 3) as stationary phases. The chromatograms were developed with two solvent systems: (a) acetone–isoamyl alcohol–water (6: 5: 5), (b) *n*-propanol–ethyl acetate–water (6: 1: 3). *Rf* values of authentic Guinea Green B in these systems were 0.61 in (1)-(a) and 0.72 in (2)-(b).

Result

Concentration of Guinea Green B in Serum

Guinea Green B dissolved in water was administered orally in doses of 50, 100, or 200 mg/kg. As the dose was increased, a gradual increase of the color in serum was observed at 3 hr after administration (Fig. 3).

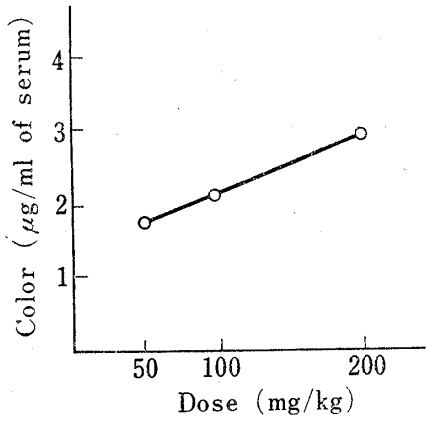

Fig. 3. Dose-response Curve of Guinea Green B in Serum

The color was orally administered to rats in doses of 50, 100, or 200 mg/kg. Serum samples were obtained from separate groups of rats 3 hr after administration.

In the case of 50 mg/kg dose, as shown in Table II, the maximum concentration was reached in the first hour, but no color was detected at 6 hr after the administration.

Leuco Form of Guinea Green B in the Large Intestine and Feces

Table III shows the percentage of the leuco form in the large intestine and feces of rats. Within 3 hr after the administration, 31.7% and 11.0% of the color were converted to its leuco form in the large intestine and feces, respectively. The leuco form increased gradually to 95.4% in the large intestine and to 93.1% in the feces at 24 hr after the administration.

Distribution of Guinea Green B in Gastrointestines

Hess and Fitzhugh[3] have indicated that, with the exception of Light Green SF, 91—98% of 200 mg doses of triphenylmethane colors could be recovered in the feces at 36 hr. In the present study, the removal of Guinea Green B from the gastrointestinal tract was examined in detail. As shown in Table IV, most of the color disappeared from the stomach and small intestine in 12 hr. The maximum recovery of the color from the large intestine was 74.8% at 12 hr, and that from feces was 58.3% at 24 hr, but the total recovery at each period was 89.6—98.5% of the dose.

Biliary Excretion

As Guinea Green B absorbed by rats was thought to be primarily eliminated through the bile, the biliary excretion was investigated by two different routes of administration (*i.v.* and oral). The color was given intravenously through a femoral vein (5 mg/kg or 7.25 μmoles/kg) or orally (50 mg/kg or 72.5 μmoles/kg). The results are shown in Tables V and VI, respectively.

In the case of intravenous injection, the color appeared in the biliary cannula within 3—5 min after the injection and the maximum excretion was observed within the first hour. Total recoveries of biliary excretion were 86.5% at 4 hr and 96.1% at 24 hr.

In the case of oral administration, the color appeared in the biliary cannula within 15—20 min after the administration and the maximum excretion was observed at 2 hr, followed by

Table II. Concentration of Guinea Green B in Serum

Time after administration (hr)	Concn. of color (μg/ml)
0.5	0.90
1	7.65
3	1.35
6	0
12	0
24	0

The color was orally administered to rats in a dose of 50 mg/kg. Serum samples were obtained from separate groups of rats at 0.5, 1, 3, 6, 12, and 24 hr after administration of the color.

Table III. Leuco Form of Guinea Green B in Large Intestine and Feces of Rats

Time after administration (hr)	Leuco form of color (%)	
	Large intestine	Feces
3	31.7±15.41(3)	11.0 (1)
6	74.8± 6.68(3)	64.0 (1)
12	83.6± 2.87(5)	80.9±3.46(5)
24	95.4± 1.08(6)	93.1±0.51(6)

The number of rats used in parentheses.
mean values ± S.E.
dose: 50 mg/kg oral

Table IV. Distribution of Guinea Green B in Gastrointestinal Tract of Rats

Time after administration (hr)	Stomach and its contents % ± S.E.	Small intestine and its contents % ± S.E.	Large intestine and its contents % ± S.E.	Feces % ± S.E.	Total recovery % ± S.E.
0.5	64.7±10.43	33.8±10.09	0	0	98.5±0.82
1	65.0± 5.22	28.9± 7.54	0.5±0.25	0	94.5±3.42
3	31.6± 3.43	33.5± 4.64	28.2±5.96	0.3±0.34	93.5±1.09
6	22.7± 6.96	19.1± 4.11	48.0±5.53	2.8±1.78	92.6±2.36
12	1.3± 0.63	5.2± 2.32	74.8±6.86	8.4±3.06	89.6±2.22
24	0.1± 0.04	0.4± 0.09	32.4±5.59	58.3±5.41	91.3±2.88

Five rats were used in each group and the color was given orally in a single dose of 50 mg/kg.

Table V. Biliary Excretion of Guinea Green B in Rats (*i.v.*)

Time after administration (hr)	Cumulative % dose excreted	% dose excreted/hr
0.5	21.7±3.87	
1	43.6±4.57	43.6±4.57
1.5	61.4±4.54	
2	72.1±3.26	28.5±1.87
3	82.1±2.75	10.0±0.79
4	86.5±2.30	4.4±0.57
5	88.9±2.05	2.4±0.31
6	90.4±1.80	1.6±0.29
7	91.5±1.58	1.1±0.25
8	92.4±1.39	0.9±0.24
9	93.1±1.23	0.7±0.21
24	96.1±1.60	0.1±0.03

Five animals were used and the color was given intravenously in a dose of 5 mg (7.25 μmoles)/kg through a femoral vein. mean values ± S.E.

Table VI. Biliary Excretion of Guinea Green B in Rats (oral)

Time after administration (hr)	Cumulative % dose excreted	% dose excreted/hr
1	0.27±0.112	0.27±0.112
2	0.82±0.367	0.56±0.276
3	1.19±0.530	0.36±0.164
4	1.42±0.618	0.23±0.095
5	1.58±0.673	0.16±0.069
6	1.70±0.715	0.12±0.046
7	1.78±0.741	0.08±0.033
8	1.85±0.762	0.07±0.025
9	1.90±0.776	0.05±0.017
24	2.28±0.820	0.02±0.005

Ten animals were used and the color was administered orally in a dose of 50 mg (72.5 μmoles)/kg by a stomach tube. mean values ± S.E.

a gradual decrease in the level until the total recovery was only 2.28% at 24 hr.

Distribution of Guinea Green B in Various Tissues

Table VII shows the levels of Guinea Green B in serum, liver, kidney, abdominal muscle, brain, stomach, small intestine, large intestine, and feces of rats at different periods after the intravenous injection of the color. With the exception of the gastrointestinal tract, the maximum distribution of the color in each tissue was reached immediately after the injection. The color was rapidly distributed in the liver and its level was higher than that in serum at 30 min after the injection. Disappearance of the color from the liver occured at an early period, from 30 min to 3 hr after the injection, first rapidly and then gradually. However, in the case of the kidney, disappearance was rather slow.

In both the liver and kidney, certain amount of the color was still detected at the concentrations of 23 nmoles/g and 29 nmoles/g of respective tissues even after 24 hr. A small amount of the color was found in the brain, but it disappeared completely within 3 hr. Similarly, the color in abdominal muscle disappeared within 12 hr.

A small amount of the color was detected in the stomach at an early period, from 30 min to 6 hr. The maximum distribution of the color in the small intestine appeared at 3 hr. Total recovery of the color in the large intestine and feces was 87.8% of the dose at 24 hr. The color was excreted in urine in the range of 0—0.53% of the dose at 24 hr.

Table VII. Distribution of Guinea Green B in Various Tissues of Rats

Time after administration (hr)	nmoles/g or ml				
	Liver	Kidney	Serum	Abdominal muscle	Brain
0.5	948.4±76.38	179.6± 8.05	475.7±13.70	11.5±0.53	2.9±1.43
1	810.7±81.47	164.6±16.41	215.3±41.07	9.1±1.23	1.0±0.83
3	120.4±12.57	103.9±13.57	29.9± 4.04	5.7±1.50	0
6	68.1± 3.06	91.3±13.15	9.1± 0.63	3.2±1.16	0
12	33.8± 5.12	47.3± 3.77	2.8± 0.63	0	0
24	23.4± 3.77	29.1± 5.12	0.9± 0.31	0	0

Time after administration (hr)	% dose			
	Stomach and its contents	Small intestine and its contents	Large intestine and its contents	Feces
0.5	0.6±0.24	7.2±1.00	1.2± 0.59	0
1	3.8±1.79	13.8±2.30	0.6± 0.12	0
3	4.6±2.55	40.4±9.82	10.8± 4.38	0
6	3.5±3.16	8.9±3.63	40.5±10.60	1.4±1.26
12	0.1±0.02	1.3±0.27	40.4±10.50	23.8±7.75
24	0.1±0.02	0.3±0.07	13.4± 4.95	74.4±6.57

Five animals were used and the color was given intravenously in a dose of 100 μmoles (69.1 mg)/kg. mean values ± S.E.

Detection of Metabolites

Bile, feces, and urine of rats receiving the color intravenously were tested by thin-layer chromatography, but no detectable metabolites could be found other than the unchanged color.

Discussion

The present data of blood level and biliary excretion of Guinea Green B indicate that only a small amount of the color is absorbed from the gastrointestinal tract after its oral

administration, in agreement with the findings of Hess and Fitzhugh.[3] The biliary excretion rate of the color was approximately similar to the results of Iga, *et al.*[4a]

It is well known that triphenylmethane colors are readily converted to colorless triarylmethylcarbinols in alkaline medium, while the colors are reversibly restored to the original colors in acidic medium. However, when the large intestine or feces were extracted with *tert*-butanol in acidic medium, most of Guinea Green B extracted was in the colorless form which was converted to the original color only by the oxidative procedure by heating with chloranil. Therefore, this colorless leuco form might be formed by the reductive process of duct microflora and should be distinguished from the colorless form in alkaline medium. In this paper, the term leuco form was used only for the reductive one in order to avoid the confusion of two kinds of colorless forms.

The amount of the leuco form in the large intestine and feces of rats increased gradually with time (Table III). This fact suggests the participation of biological reduction by gut bacteria in the large intestine, because such a case was not found in other tissues. Detailed experiments to support this idea are under way.

Since Hess and Fitzhugh reported that 91% of the administered color was excreted in feces within 36 hr,[3] we also examined in detail the removal of this color from gastrointestinal tract in 24 hr period. There was no significant difference between intravenous and oral administration of the color in the removal pattern of the color from gastrointestinal tract except the stomach (Tables IV and VII). This color was hardly absorbed from the gastrointestinal tract and a considerable amount of the color was rapidly excreted in the leuco form in feces, while the absorbed color was rapidly excreted in bile and poorly in urine.

As to the distribution in various tissues after intravenous injection, a larger amount of the color was detected in the liver than in serum at 30 min after injection, and it still remained in the liver and kidney even after 24 hr. On the other hand, the color disappeared rapidly from the brain and abdominal muscle.