Metabolism of Triphenylmethane Colors (III)

Comparison of Tissue Distribution and Biliary Excretion for ^3H -Benzyl Violet 4B and ^3H -Fast Green FCF (Food Green No. 3) in Rats

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Distribution and biliary excretion of Benzyl Violet 4B and Fast Green FCF were compared in female Sprague-Dawley rats after an oral administration of the ^3H -labeled compounds.

The levels of Benzyl Violet 4B distributed in the plasma, ear, abdominal skin and abdominal muscle were higher than those of Fast Green FCF. The cumulative amounts of Benzyl Violet 4B and Fast Green FCF excreted in the bile were 4.26 and 3.81% of the dose by 24 hr, respectively. The biological half-lives for Benzyl Violet 4B in the above tissues were longer than those for Fast Green FCF.

1. Introduction

Since Hess and Fitzugh¹⁾ demonstrated that many triphenylmethane colors administered orally to rats were poorly absorbed and mostly excreted in feces, any special attention has not been paid for the metabolism of these colors except for the pharmacokinetical studies reported by Iga *et al.*²⁾

However, recently Uematsu and Miyaji³⁾ and Ikeda *et al.*⁴⁾ described that female Sprague-Dawley rats fed a diet containing 5% Benzyl Violet 4B for 12 months developed high incidences of mammary gland carcinoma and squamous cell carcinoma of the skin (mostly the ear-duct).

Therefore, in the previous paper,⁵⁾ we studied on the absorption, excretion and distribution of this color in adult male and female Wistar and Sprague-Dawley rats by the two-wavelength photometric method and showed that the half-lives for the color in brain, abdominal muscle, abdominal skin and ear of Sprague-Dawley rats were significantly longer than those of Wistar rats after intravenous injection of the color.

In the present study, two kinds of tritium-labeled triphenylmethane colors, Benzyl Violet 4B which was reported to be carcinogenic and Fast Green FCF which is not carcinogenic, were prepared and the distribution of these labeled

colors in the plasma, abdominal skin including mammary gland, ear and abdominal muscle of female Sprague-Dawley rats were compared after oral administration of the colors.

2. Materials and Methods

2.1 Preparation of labeled compound

Benzyl Violet 4B (Acid Violet 6B, Color Index No. 42640, former Food Violet No. 1), manufactured by Hodogaya Chemical Industries Co., Ltd. (Lot No. H0086), which contained 94.1% pure dye by spectrometry, and Fast Green FCF (Color Index No. 42053, FD and C Green No. 3), which was the National Institute of Hygienic Sciences Reference Standard (94% pure dye), were used.

Both ^3H -labeled colors were prepared by the platinum-catalyzed $^3\text{H}_2$ gas exposure technique in the Sinroihi Co., Ltd. In this technique, 698 mg of Benzyl Violet 4B were exposed to 10 Ci of ^3H gas for 6 days. The crude sample was dissolved in 5% sodium Bicarbonate solution and acidified immediately with 2N hydrochloric acid to eliminate exchangeable ^3H atoms. The sample was adjusted to pH 7-8 with 10% sodium hydroxide solution and extracted with *tert*-butanol. The *tert*-butanol layer was washed with distilled water five times. The sample solution was concentrated *in vacuo*, and the residue was redissolved in methanol. The solu-

tion was evaporated to dryness *in vacuo*. About 50 mg of the sample was passed through a column (2.5×35 cm) packed with Avicel (microcrystalline cellulose, 150–220 mesh, Asahi Kasei Co., Ltd) and eluted with a mixture of acetone-methanol-water (10 : 1 : 1). Each 2.5 ml of the eluate was collected by a fraction collector (Toyo Roshi Co., Ltd). The main color fractions were combined and the combined solution was evaporated to dryness *in vacuo*. The sample was applied on the cellulose spot film (Tokyo Kasei Co., Ltd) and developed with a mixture of acetone-isoamyl alcohol-water (6 : 5 : 5). ³H-Fast Green FCF was prepared by the same procedure as described above except for the extraction with *tert*-butanol from acidic medium. The specific activities of ³H-Benzyl Violet 4B and ³H-Fast Green FCF were 54.7 μ Ci/mg and 51.2 μ Ci/mg respectively, and the radiochemical purities of both colors were found to be greater than 97% by thin-layer chromatography. The purified ³H-colors were dissolved in methanol and then gently evaporated to eliminate volatile radioactivity under a stream of nitrogen at room temperature before use. Both purified colors were stable in an aqueous solution (0.2–0.4 μ g/ml) at room temperature for 21 days.

2.2 Animals

Adult female Sprague-Dawley rats weighing between 230 and 280 g were used. Separate groups of animals received a single oral administration of 10 μ moles (7.34 mg)/kg of Benzyl Violet 4B or 10 μ moles (8.09 mg)/kg of Fast Green FCF. Bile was collected from rats cannulated as described in the previous paper.⁵⁾

2.3 Radioactivity measurement

At the appropriate times, the animals were anaesthetised with pentobarbital (40 mg/kg, *i.p.*) and the blood was withdrawn by heart puncture. The animals were killed and the ear, abdominal skin, and abdominal muscle were removed and minced with scissors. An aliquot (80–100 mg or 0.1 ml) of the sample from tissue or plasma was transferred into a vial and then equal volume of methanol was added. To eliminate volatile radioactivity, the sample gently evaporated under a stream of nitrogen at room temperature and this procedure was repeated twice. After evaporation, the sample was made up to the original volume with water and then solubilized by the addition of 0.5 ml of soluene 350 (Packard) with

gentle shaking at 37°C overnight. To the solubilized sample, 10 ml of a toluene scintillator containing 0.4% 2,5-diphenyloxazole (DPO) and 0.01% 1,4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP) was added. As a scintillator for bile, 10 ml of a mixture constituted of 2 vol. toluene, 2 vol. dioxane, 1 vol. methanol with 8% naphthalene, 0.4% DPO and 0.01% POPOP was used. The radioactivity was determined by an LSC-651 scintillation counter (Aloka).

3. Results and Discussion

Hess and Fitzhugh¹⁾ indicated that many FD and C triphenylmethane colors administered

Table 1. Distribution of ³H-Benzyl Violet 4B and ³H-Fast Green FCF in Plasma, Ear, Abdominal Skin and Abdominal Muscle (*p.o.*)

Tissue	³ H-Benzyl Violet 4B	³ H-Fast Green FCF
	<i>p</i> moles dye equivalent/g or ml	
Plasma		
2 (hr)	532.8±39.00	148.5±11.22 ^c
7	195.9±37.50	87.2± 8.22 ^a
15	102.7±13.09	28.2± 7.03 ^c
24	68.7± 8.98	14.4± 1.44 ^c
Ear		
2 (hr)	271.3±15.11 ^a	96.0± 8.87 ^c
7	182.8±19.85 ^b	92.2± 9.18 ^c
15	153.4±10.77 ^a	60.6± 7.74 ^c
24	120.7± 7.30 ^b	31.4± 3.73 ^c
Abdominal skin		
2 (hr)	210.6±13.53	91.8± 8.83 ^c
7	168.4±16.40 ^b	72.4± 7.83 ^c
15	132.6±12.17 ^b	56.8± 6.59 ^c
24	113.3±12.27 ^b	25.6± 3.79 ^c
Abdominal muscle		
2 (hr)	184.4± 6.52	103.4±14.60 ^c
7	110.3± 6.35	72.6± 9.52 ^d
15	77.1±10.81	46.0±10.70
24	64.8± 9.72	23.2± 4.71 ^d

Five female Sprague-Dawley rats were used in each group and administered orally in a dose of 10 μ moles/kg of ³H-labeled colors (Mean values±S.E.)

Significantly different from abdominal muscle (^a*P*<0.01, ^b*P*<0.05)

Significantly different from Benzyl Violet 4B (^c*P*<0.01, ^d*P*<0.05)

Table 2. Biliary Excretion of ^3H -Benzyl Violet 4B and ^3H -Fast Green FCF in Rats (*p.o.*)

Time after administration (hr)	^3H -Benzyl Violet 4B		^3H -Fast Green FCF	
	% Dose excreted/hr	Cumulative % dose excreted	% Dose excreted/hr	Cumulative % dose excreted
1	0.15	0.15	0.11	0.11
2	0.37	0.52	0.36	0.47
3	0.37	0.89	0.29	0.76
4	0.37	1.26	0.26	1.02
5	0.34	1.60	0.19	1.21
6	0.26	1.86	0.18	1.39
7	0.20	2.06	0.17	1.56
24	0.17	4.26	0.13	3.81

Two female Sprague-Dawley rats were used in each group and administered orally in a dose of 10 $\mu\text{moles/kg}$ of ^3H -labeled colors.

orally to rats (200 mg/rat) were scarcely absorbed and recovered in excess of 90% from feces, except for Green No. 2 of which only 68% was recovered. From the biliary excretion studies of these colors in dogs and rats, they observed that less than 5% of the administered dose was absorbed and subsequently excreted to the intestine through the bile.

In the previous paper, we improved the two-wavelength photometric method to determine Guinea Green B³⁾ and Benzyl Violet 4B³⁾ in tissues, and investigated the absorption, excretion and distribution of these colors in rats. Because of a poor absorption of these colors, the distribution in various tissues was investigated by intravenous administration of a large dose (100 $\mu\text{moles/kg}$).

In this study, the distribution of ^3H -labeled colors was studied by oral administration of a small dose (10 $\mu\text{moles/kg}$). The levels of colors in the ear and the portion of abdominal skin near the site of tumor development,^{3), 4)} and those in the plasma and abdominal muscle apart from tumor developing site were compared.

The results are shown in Table 1. In the case of Benzyl Violet 4B, the levels of color in the ear and abdominal skin (except at 2 hr) were significantly higher than those of the abdominal muscle apart from the site of tumor development. These results were essentially similar to the patterns of distribution after intravenous administration in the previous paper.⁵⁾ On the other hand, in the case of Fast Green FCF, for which there was no gross evidence of

Table 3. Half-lives^{a)} of ^3H -Benzyl Violet 4B and ^3H -Fast Green FCF in Some Tissues in Female Sprague-Dawley Rats after Oral Administration of the Labeled Colors

Tissue	Half-lives in tissue (hr)	
	^3H -Benzyl Violet 4B	^3H -Fast Green FCF
Plasma	12.38 $\pm$ 1.71	6.59 $\pm$ 0.12 ^c
Ear	36.15 $\pm$ 3.96 ^a	11.44 $\pm$ 0.67 ^b
Abdominal skin	38.05 $\pm$ 6.33	12.05 $\pm$ 0.39 ^b
Abdominal muscle	21.37 $\pm$ 1.87	10.15 $\pm$ 0.90 ^b

The conditions were the same as in Table 1.

^{a)} Half-lives were calculated from the data of 7, 15 and 24 hr in Table 1.

Significantly different from abdominal muscle (^a $P < 0.05$)

Significantly different from Benzyl Violet 4B (^b $P < 0.01$, ^c $P < 0.05$)

tumor development in rats by oral administration⁷⁾ except the production of fibrosarcomas at the site of subcutaneous injection of the color,^{3), 9)} there was no significant difference between these tissues. Furthermore, the levels of Benzyl Violet 4B in the tissues were higher than those of Fast Green FCF at any time tested.

Table 2 shows the biliary excretion of ^3H -Benzyl Violet 4B and ^3H -Fast Green FCF in the rats administered orally with the labeled colors (10 $\mu\text{moles/kg}$). The cumulative amounts were 4.26 and 3.81% of the dose by 24 hr respectively. The maximum excretion was observed

between 2 and 4 hr for Benzyl Violet 4B and at 2 hr for Fast Green FCF, and followed by a gradual decrease. In the case of a large dose of non-labeled Benzyl Violet 4B (68.1 μ moles/kg or 50 mg/kg), the cumulative amount was only 0.89% of the dose at 24 hr.⁵⁾ Therefore, it was suggested that the ratio of absorption of Benzyl Violet 4B in a small dosage was higher than that in a large dosage. There was no significant difference between both colors in the biliary excretion.

Table 3 shows the biological half-lives for Benzyl Violet 4B and Fast Green FCF in the tissues of female Sprague-Dawley rats after oral administration of the colors.

The biological half-lives of Benzyl Violet 4B in plasma and other tissues were significantly longer than that of Fast Green FCF. It was noticeable that the biological half-lives in the oral administration was longer than in the intravenous administration.⁶⁾

From the results of this study and the previous paper,⁶⁾ the accumulating amount and retaining period of Benzyl Violet 4B in tissues might well be taken in consideration as a possible factor

of tumor development, although tumor formation in rats by long-term feeding of the color seems to be influenced by other complicating factors.

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