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Pharmacokinetic Study of Biliary Excretion. III. Comparison of Excretion Behavior in Xanthene Dyes, Fluorescein and Bromsulphthalein[1,2]

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In the previous papers, the authors reported the kinetic studies on the factors affecting the biliary excretion using Azo, Indigoid and Triphenylmethane dyes. The further study was tried using Xanthene dyes, Fluorescein and Bromsulphthalein, and the effect of halogenation was discussed. Four Xanthene dyes, *i.e.* Eosine Y, Phloxine B, Erythrosine B, Rose Bengal, and Fluorescein and Bromsulphthalein were used.

(1) The excretion behaviors were similar to those of Azo and Triphenylmethane dyes which had the dose dependency, but the excretion ratios showed common larger values than 75% except Fluorescein which had no halogen group.

(2) It was found that the halogen group had great influence on the biliary excretion as well as the sulfonate group. And it was also suggested that the effect of halogen group depended on the total electro negativity of substituents. And furthermore it was found that the increasing in molecular weight had not so much effect than the increasing of polarity.

(3) It was also suggested that the -I gave the delay of excretion in the early period and this was probably due to the larger uptake into liver cells.

(4) BSP showed markedly concentrical excretion pattern and large excretion ratio in a short period and it was found that these characteristic behavior was mainly ascribed to both halogen groups and sulfonate groups.

In the present serial papers,[4,5] the authors intended the kinetic studies on the factors affecting the biliary excretion with water soluble azo, indigoid and triphenylmethane dyes. It was concluded that sulfonate group beyond other groups had a remarkable effect on the biliary excretion ratio and pattern, and that the position of a sulfonate group as well as the number was important to determine its effect. Furthermore, the possibility of the compartmental analysis for the biliary excretion process was suggested in the excretion pattern of Brilliant Blue FCF.

In the present paper, the further studies have been tried with Fluorescein sodium, four Xanthene dyes which have been used for fed dyes and Bromsulphthalein which has been used for hepatic function test.[6] Xanthene dyes used are the halogenated derivatives of fluorescein by -Br, -Cl and -I. But Bromsulphthalein has both sulfonate and halogen groups on a phthalein structure. The effect of halogen groups on the biliary excretion ratio and pattern were discussed and were compared with that of sulfonate groups of azo dyes and triphenylmethane dyes in the previous papers.[4,5] It was found that the halogen group had a great influence on the biliary excretion as well as the sulfonate group, but the position effect was not seen in this study. And it was also found that Bromsulphthalein which has both sulfonate and halogen groups, showed the typical high dose type (Type A) biliary excretion

1) This forms Part III of "Pharmacokinetic Study of Biliary Excretion," by T. Iga; Part II: T. Iga, S. Awazu and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **18**, 243 (1970).
2) Partial fulfillment of Doctor of Pharmaceutical Science degree requirement of Tatsuji Iga to the Graduate school, University of Tokyo.
3) Location: *Hongo, Bunkyo-ku, Tokyo.*
4) T. Iga, S. Awazu, M. Hanano and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **18**, 2431 (1970).
5) T. Iga, S. Awazu and H. Nogami, *Chem. Pharm. Bull.* (Tokyo), **19**, 273 (1971).
6) The symbols for dyes in the present study are as follows: Eosine Y=EY, Phloxine B=PB, Erythrosine B=EB, Rose Bengal=RB, Fluorescein Sodium=FS, Bromsulphthalein=BSP.

and the most concentrical excretion pattern among dyes in this study. And its superiority as the drug for hepatic function test was also ascertained in both excretability and sensitivity in low dose range.

Experimental

Materials——Dyes used in this study were shown in Table I. All dyes except for Bromsulphthalein, were purchased from Wako pure chemical industries, LTD or Tokyo chemical industries, Co., LTD. Bromsulphthalein was used as the injective solution (Daiichi chemical industries, LTD). All other reagents were commercially available and of special grade.

Table I. Dyes used in This Study

Dye	Name	Molecular formula	Molecular weight	Absorption max. (mμ)
Xanthene dyes	Eosine Y (FD Red No. 103)	$C_{20}H_6O_5Br_4Na_2$	691.88	517
	Phloxine B (FD Red No. 104)	$C_{20}H_2O_5Br_4Cl_4Na_2$	829.71	538
	Erythrosin B (FD Red No. 3)	$C_{20}H_6O_5I_4Na_2$	879.92	526
	Rose Bengal (FD Red No. 105)	$C_{20}H_2O_5I_4Cl_4Na_2$	1018.58	547
	Fluorescein Sodium	$C_{20}H_{10}O_5Na_2$	376.27	494
Sulfophthalein dye	Bromsulphthalein	$C_{20}H_8O_{10}S_2Br_4Na_2$	838.05	580

Drug Administration and Samplings——The procedure was carried out in the same way as described in the previous paper.[4] The excreted dye was determined as the equivalent amount to the authentic dye from the optical density at the wave length for each dye listed in Table I using Hitachi 124 spectro-photometer.

Analytical Methods——Bile sample was diluted in the same way as described in the previous paper[4] except for Bromsulphthalein (BSP) and Fluorescein (FS), and dyes were determined at each maximum wave length listed in Table I. As for BSP, the pH of diluted samples were adjusted to 9 with 0.05 ml of 1N NaOH solution and carried out in the same way. The fluorometrical measurement was used for FS and the relative fluorescein intensity was determined at 512 mμ excited at 435 mμ with Hitachi 203 Fluorospectro Meter using 0.4, 0.2 and 0.08 μmole/ml fluorescein sodium solution as standard.

Bile Flow——As reported previously,[4] the bile flow rate, under light ether anesthesia tended to fluctuate somewhat until 1 hr. But the effect of fluctuation on the excreted amount of a dye was found to be neglected. And the detailed data of bile flow and dye concentration in bile are available from the authors.

Result and Discussion

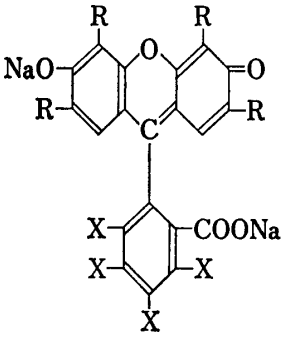
It has been said that in general,[7] the organic compound which has some halogen substituents tended to be excreted in bile and the larger the number of halogen groups, the larger was the excretion ratio, since the halogen groups, increased both the molecular weight and polarity. In the present paper the authors intended to discuss the halogenated effects of -Cl, -Br and -I on the excretion ratio and pattern for the number and the kind using four Xanthene dyes which are the halogenized derivatives of fluorescein and Fluorescein itself. And furthermore the effect of both halogen groups and sulfonate groups was also discussed in Bromsulphthalein (BSP).

Xanthene Dyes and Fluorescein

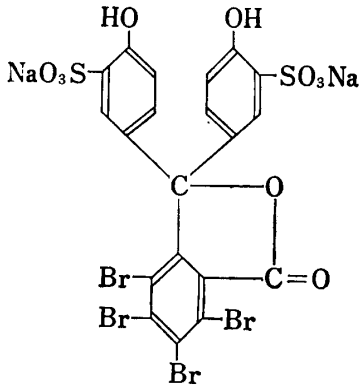
Xanthene dyes have been used as the popular red fed dyes and some cosmetric dyes up to this time and on the other, Fluorescein itself has been used as the drug for clinical function test in the medical fields. In this paper, four Xanthene dyes, *i.e.* Eosine Y (EY), Phloxine B (PB), Erythrosine B (EB), Rose Bengal (RB) were used. As the standard compound

7) P.T. Williams, P. Millbrun and R.L. Smith, *Ann. N.Y. Acad. Sci.*, **123**, 110 (1965).

which has no halogen group, Fluorescein Sodium (FS) was used. Their molecular weights are between about 700 and 1000 except FS (Table I) and all these values are much more larger than those of azo dyes and triphenylmethane dyes in the previous papers.[4,5] But FS has 376 molecular weight and this value is the lowest one in this series of study. As shown in Chart 1, all these dyes have the common phthalein structure and differentiated only with the number and kind of halogen group.



Xanthene dyes



Sulfophthalein dye

Bromsulphthalein

Eosine Y : R=Br, X=H
Phloxine B : R=Br, X=Cl
Erythrosin B : R=I , X=H
Rose Bengal : R=I , X=Cl
Fluorescein (Sodium) : R=H , X=H

Chart 1. Chemical Structure of Xanthene Dyes and Sulfophthalein Dye

About the metabolism and biliary excretion of Xanthene dyes, Webb, *et al.*[8] reported that the dyes which had small number of halogen groups tended to conjugate with glucuronic acid or to be dehalogenized, but that the larger the number of halogen groups, the more hardly occurred the conjugation and dehalogenation. It was also reported that the halogenation of fluorescein markedly increased the biliary excretion with a concomitant decrease in urinary excretion as shown in Table II, and they attributed these results to the increased acidity and polarity with halogen groups of which number were above three. The total recovery into bile (feces) and Urine were about 90 or 100% after administration of Eosine, Erythrosine B and Phloxine in rats. And furthermore, Hansen, *et al.*[9] reported that the halogen groups increased the excretion ratio and decreased the toxicity of drugs. As for the biliary excretion of Fluorescein (FS), Hanzon[10] reported that Fluorescein itself had some affinity for hepatic elimination in rats.

Table II. Excretion Ratio in 2 Hr (%)[a]

Dye	Bile	Urine
Fluorescein	14	30
4,5-Dibromofluorescein	29	5
4,5-Diiodofluorescein	32	5
4,5-Dibromo-2,7-diiodofluorescein	52	3
2,4,5,7-Tetraiodofluorescein (Erythrosin B)	55	1
2,4,5,7-Tetrabromofluorescein (Eosine Y)	63	4
2,4,5,7-Tetraiodo-12,13,14,15-tetrachlorofluorescein (Rose Bengal)	66	0

a) data from Webb, *et al.* *b*) dose : 3 mg/kg *i.v.*

8) J.N. Webb, M. Fonda and E.A. Brouwer, *J. Pharmacol.*, **137**, 141 (1962).
9) W.H. Hansen, O.G. Fitzhugh and M.W. Williams, *J. Pharmacol.*, **122**, 29A (1958).
10) V. Hanzon, *Acta. Physiol. Scand.*, **28**, *suppl.*, **101**, 1 (1952).

Although Webb, *et al.*[8] reported the excretion ratio for Xanthene dyes, the experimental period was relatively short and did not make any comment on the time course of the excretion. In order to know the substituents effects on the biliary excretion patterns, the authors obtained the cumulative excretion data for Xanthene dyes and Fluorescein for 4 hr and the time courses were analyzed. The result and excretion ratios and patterns were given as the follwoing.

(1) Excretion Ratio——Their cumulative excretion curves were shown in Fig. 1—3 and the biliary excretion ratio of 3, 15 and 30 μ mole doses for each dye were listed in Table III.

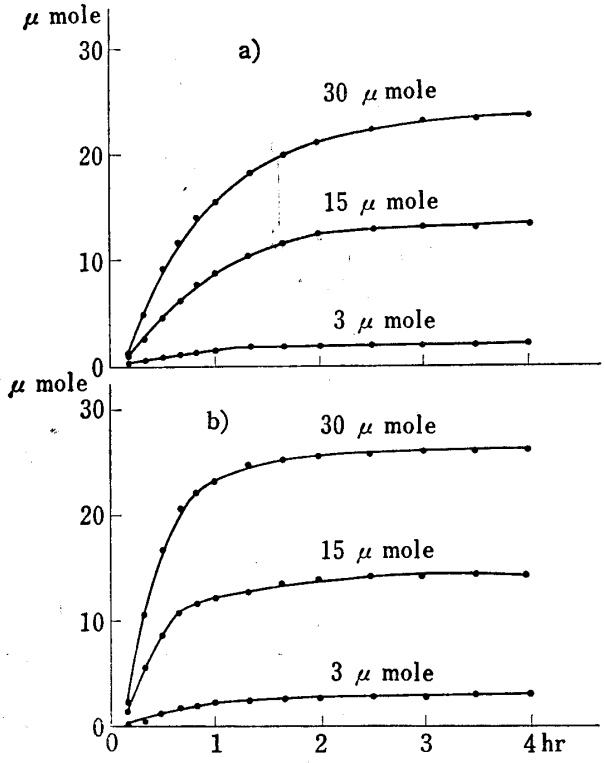


Fig. 1. a) Cumulative Eosine Y (EY) Excretion Curves in Bile; b) Cumulative Phloxine (PB) Excretion Curves in Biles

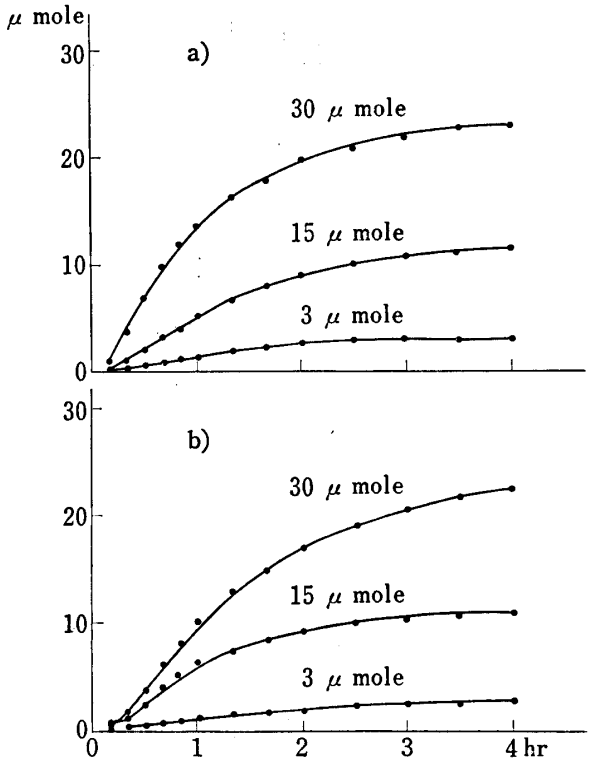


Fig. 2. a) Cumulative Erythrosin B (EB) Excretion Curves in Bile; b) Cumulative Rose Bengal (RB) Excretion Curves in Bile

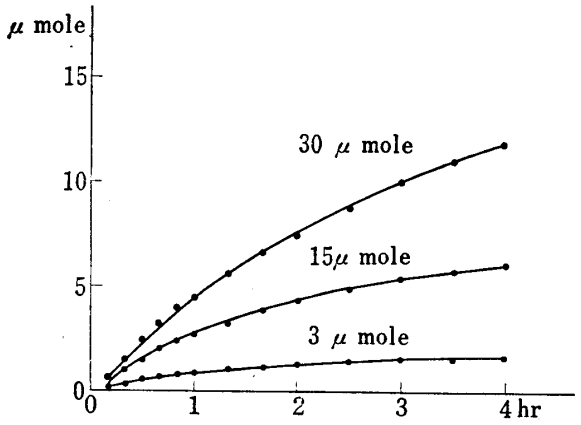


Fig. 3. Cumulative Fluorescein Sodium (FS) Excretion Curvess in Bile

It is of interest that all dyes except FS which has no halogen groups, showed common larger excretion ratios than 75% and this results was different from those of azo dyes and triphenylmethane dyes which showed significant difference in excretion ratios depending on the number and position as shown in the comparison between Amaranth (AM) and New Coccine (NC) in azo dyes, or Brilliant Blue FCF (BB) and Light Green SF (LG) in spite of the same substituent groups.

In this paper, in order to know the effect of the halogen groups on the biliary excretion depending on the number and kind, FS which had no halogen group was selected as the standard. The excretion ratio showed much smaller values (40—50%) than other four Xanthene dyes. The effect of the halogen substituent was remarkably found and this agreed well with the results of Webb, *et al.*[8] as

Table III. Averaged Excretion Ratio in 4 Hr (%)[a]

Dye	Dose (μmole)		
	3	15	30
Eosine Y (EY)	73.9 (3)[b]	79.3 (3)[b]	75.5 (4)[b]
Phloxine B (PB)	92.7 (2)	93.4 (3)	92.5 (3)
Erythrosine B (EB)	72.8 (2)	75.7 (3)	76.5 (3)
Rose Bengal (RB)	78.2 (3)	78.9 (3)	79.4 (3)
Fluorescein (FS)	47.5 (2)	45.9 (3)	41.5 (4)

Dye	Dose (μmole)		
	0.6	3	6
Bromsulphthalein (BSP)	85.0 (2)[b]	88.3 (3)[b]	84.5 (3)[b]

a) $\text{ratio} = \frac{\text{excreted amount in 4 hr}}{\text{dose}} \times 100(\%)$

b) number of experiments

shown in Table II. The ratio from their results showed only 14% until 2 hr, but the authors obtained much larger values (30—35%) until the same periods. The discrepancy may depend on the strains or the methods of study or both.

It was found that there was no difference in the comparison of excretion ratios between EY (2,4,5,7-Tetrabromofluorescein) and EB (2,4,5,7-Tetraiodofluorescein) as shown in Table II. But considering that the latter has 200 molecular weight larger than the former and that the molecular weight is one of the large factors affecting the biliary excretion, it was suggested that -Br had much effect than -I in spite of the latter had 1.5 times larger molecular weight than that of the former.

The similar tendency that -Br had much increasing effects on the excretion ratio than -I was also found in the comparison between PB (2,4,5,7-Tetrabromo-12,13,14,15-tetrachlorofluorescein) and RB (2,4,5,7-Tetraiodo-12,13,14,15-tetrachlorofluorescein). The former showed above 90% excretion ratios and furthermore the initial rapid excretion pattern which was designated as Type A in the latter section in all dose range (3—30 μ mole), but the latter showed about 80% excretion ratio, though its molecular weight was larger than 1000 and the largest one in this series of tar dyes. On the other hand, the effect of -Cl was remarkably observed in the comparison between EY and PB, but not so remarkably between EB and RB.

Summarizing these findings, Table IV was given. The Table shows that -Cl shows the largest excretion ratio and the second is RB which has both -I and -Cl, and both EY which has only -Br and EB which has only -I show smaller ratios than PB and RB, but between EY and EB, no significant difference is found in the excretion ratio.

Table IV

Dye	Molecular weight	Number of harogen groups			Biliary excretion ratio in 4 hr (%)[a]	Excretion type		
		Cl	Br	I		3[b]	15[b]	30[b]
Phloxine B (PB)	829.71	4	4	—	93 (8)[c]	A	A	A
Rose Bengal (RB)	1018.58	4	—	4	79 (9)	B	C	C
Eosine Y (EY)	691.88	—	4	—	76 (10)	B	B	B
Erythrosine B (EB)	879.92	—	—	4	75 (8)	B	C	C
Fluorescein (FS)	376.27	—	—	—	44 (9)	B	B	B

a) averaged value of all experiments b) dose (μmole) c) number of experiments

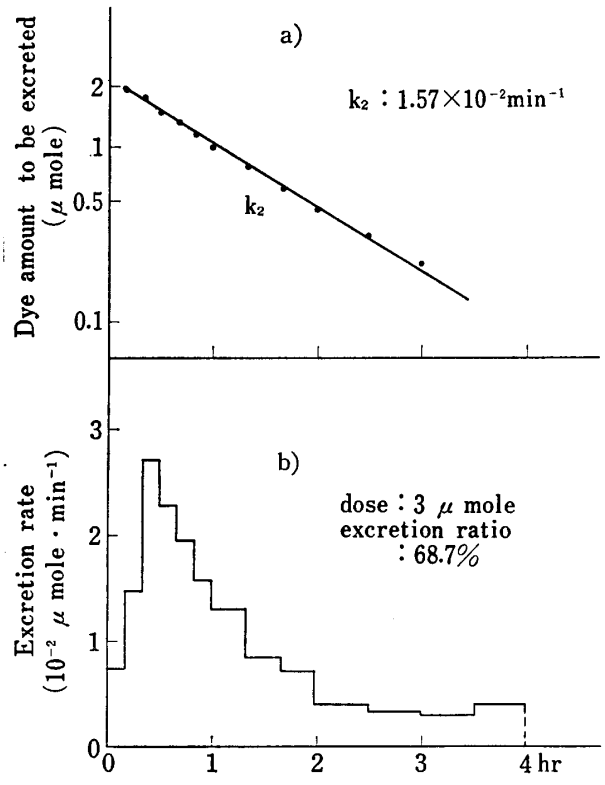


Fig. 4. a) Semilogarithmic Plots of Eosine Y (EY) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Eosine Y (EY)

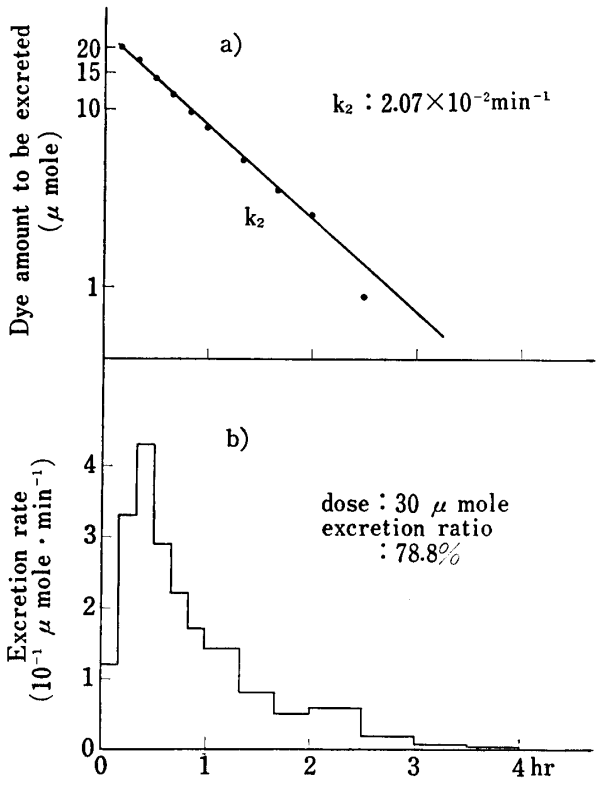


Fig. 5. a) Semilogarithmic Plots of Eosine Y (EY) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Eosine Y (EY)

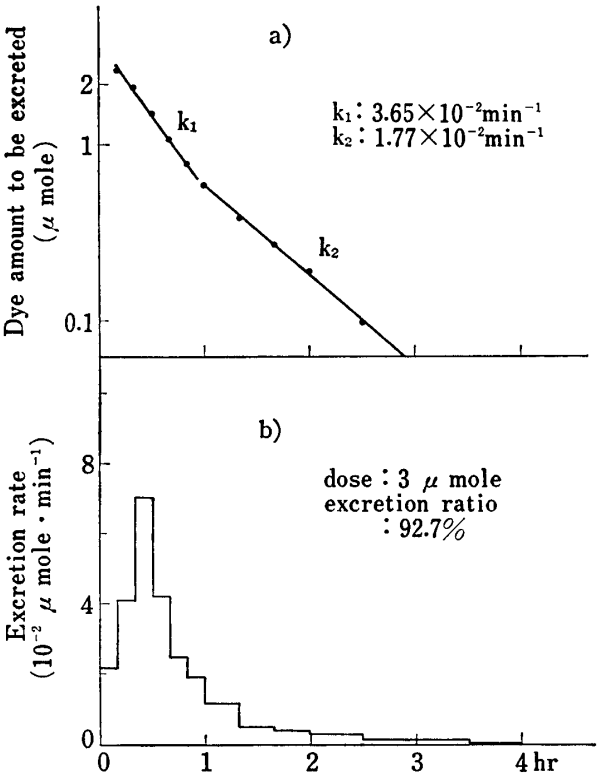


Fig. 6. a) Semilogarithmic Plots of Phloxine B (PB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Phloxine B (PB)

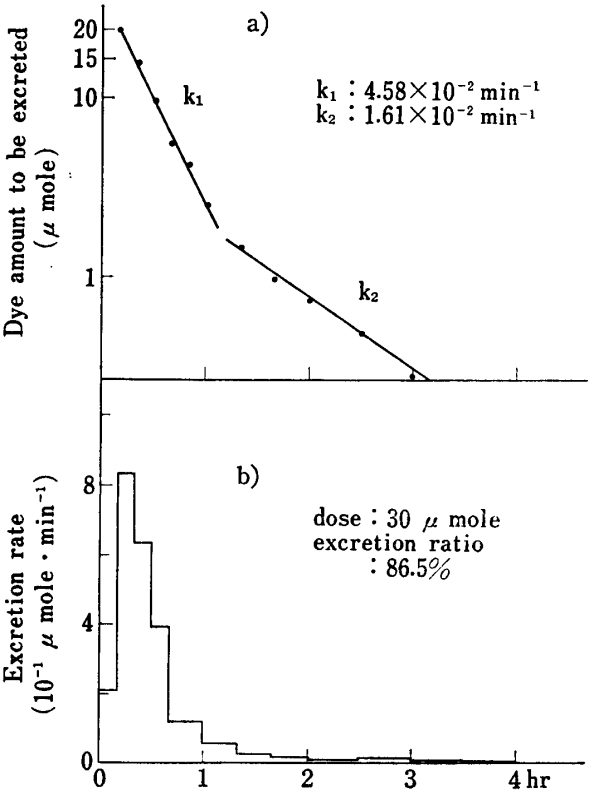


Fig. 7. a) Semilogarithmic Plots of Phloxine B (PB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Phloxine B (PB)

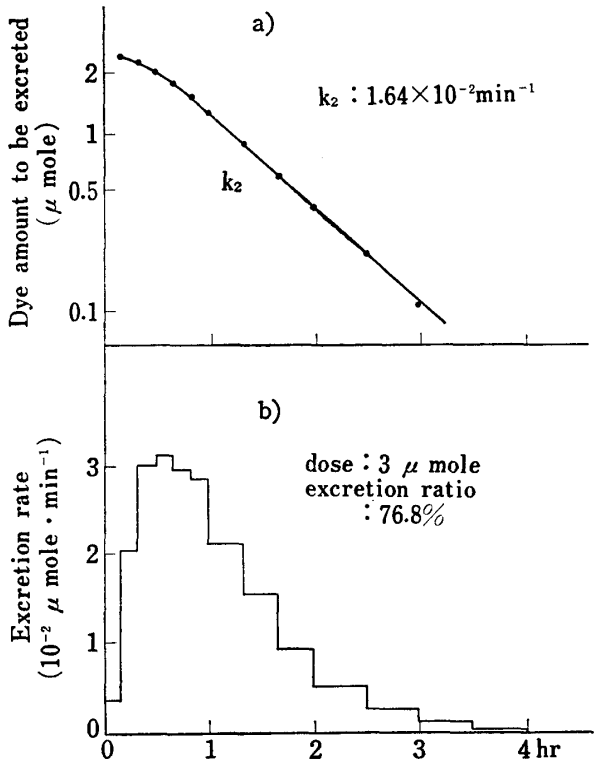


Fig. 8. a) Semilogarithmic Plots of Erythrosin B (EB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Erythrosin B (EB)

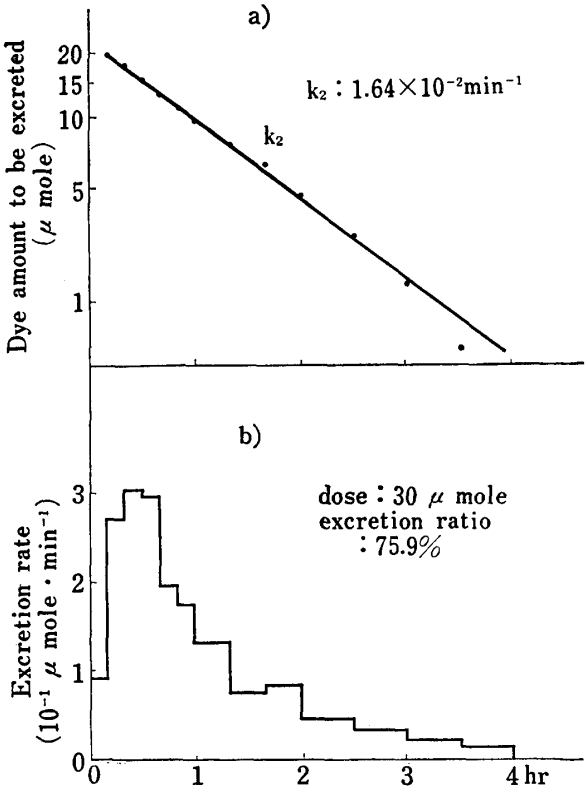


Fig. 9. a) Semilogarithmic plots of Erythrosin B (EB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Erythrosin B (EB)

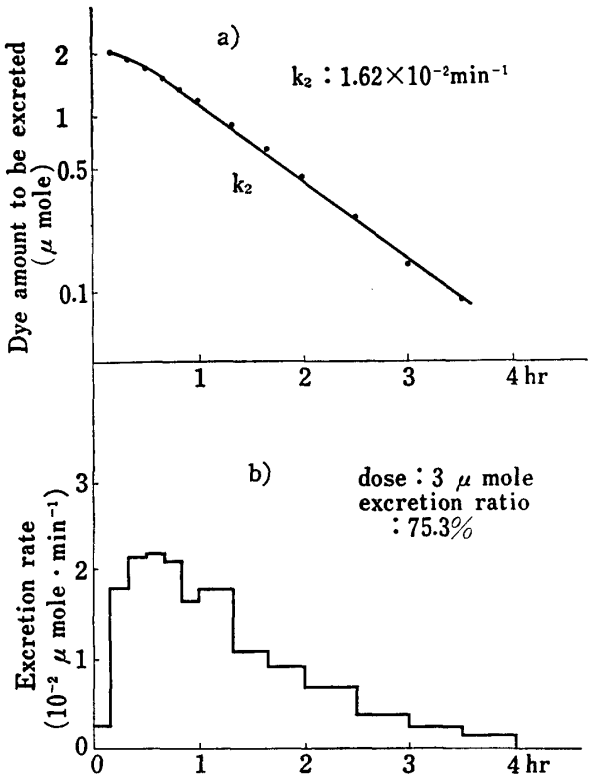


Fig. 10. a) Semilogarithmic Plots of Rose Bengal (RB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Rose Bengal (RB)

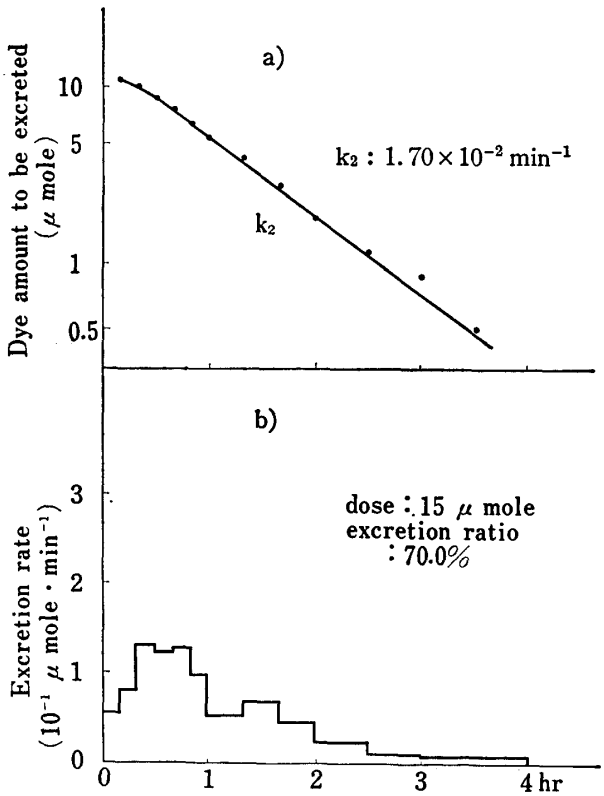


Fig. 11. a) Semilogarithmic Plots of Rose Bengal (RB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Rose Bengal (RB)

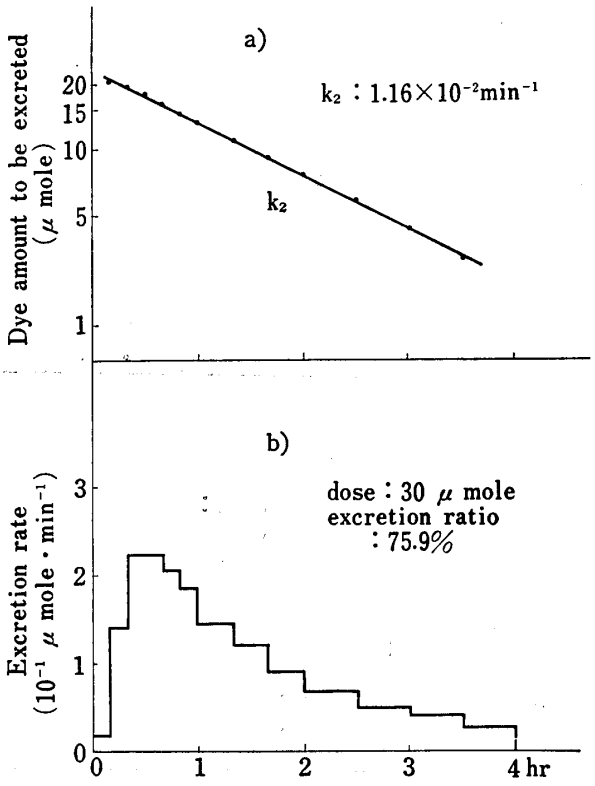


Fig. 12. a) Semilogarithmic Plots of Rose Bengal (RB) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Rose Bengal (RB)

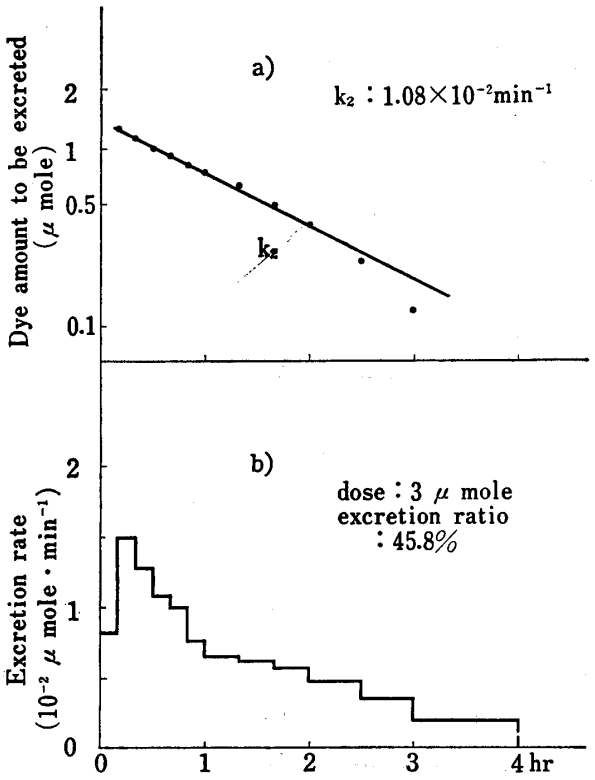


Fig. 13. a) Semilogarithmic Plots of Fluorescein Sodium (FS) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Fluorescein Sodium (FS)

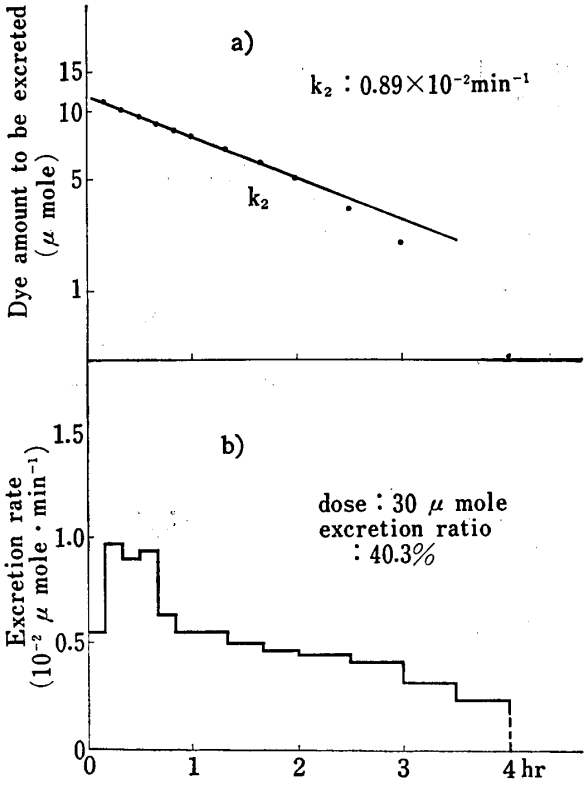


Fig. 14. a) Semilogarithmic Plots of Fluorescein Sodium (FS) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Fluorescein Sodium (FS)

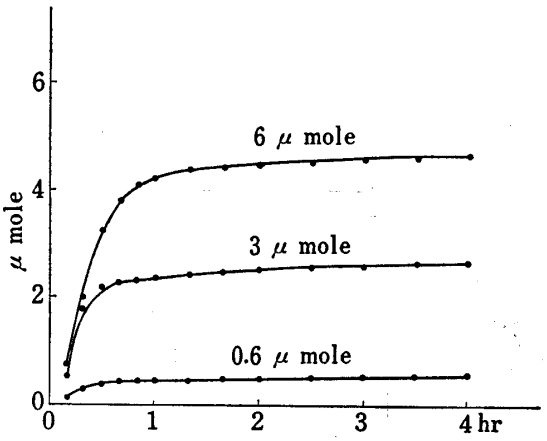


Fig. 15. Cumulative Bromsulphthalein (BSP) Excretion Curves in Bile

From these results, it was suggested that the effect of halogen group on the biliary excretion depended on the total electro negative strength of substituents, since -Cl had the most strong electro negativity among these three halogens and -Br was the second and -I had the weakest electro negativity. And furthermore this results coincided well with the report of Webb, *et al.*[8] And it was also suggested that the molecular weight was not always the main factor affecting the biliary excretion as shown in Table IV.

(2) **Excretion Pattern**——In the previous papers,[4,5] the authors reported that the type of the biliary excretion patterns could be divided into three types according to Nelson Plots[11] from the cumulative excretion data. Type A was composed of two straight lines and Type B of one straight line. And Type C gave one straight line with the early lag period. These types appeared in riboflavin, azo dyes and triphenylmethane dyes depending on dose. When a dose increased, the type changed from C to A. All of three types were not always observed for one compound in a studied dose range, but the above tendency of the type always appeared. In the present study of Xanthene dyes, the similar excretion patterns and dose dependency were observed. It was one of the common characteristic of Xanthene dyes except PB that Type B and Type C were observed in the same dose range as azo and triphenylmethane dyes. Typical examples for each dyes were shown in Fig. 4—15, and types were listed in Table V.

Table V. Biliary Excretion Rate Constant

Dye	Dose (μmole)	k_1 (10^{-2}min^{-1})	k_2 (10^{-2} min^{-1})	Type
Eosine Y (EY)	30		2.07	B
	15		2.00	B
	3		1.57	B
Phloxine B (PB)	30	4.58	1.61	A
	15	4.31	2.29	A
	3	3.65	1.77	A
Erythrosin B (EB)	30		1.64	B
	15		1.38	C
	3		1.97	C
Rose Bengal (RB)	30		1.16	B
	15		1.70	C
	3		1.62	C
Fluorescein (sodium) (FS)	30		0.89	B
	15		1.02	B
	3		1.08	B
Bromsulphthalein (BSP)	6	4.42	0.78	A
	3	6.79	0.93	A
	0.6	5.64	0.78	A

The standard compound of the present paper, FS which had no halogen group showed Type B excretion pattern in all doses (3—30 μ mole) and the delay in early period as shown in Type C was not seen, but the value of k_2 were smaller than other halogenated fluoresceins.

The most excretable PB showed Type A in all doses and the ratio of k_1 to k_2 were between 2 and 3. It was found that the halogenation with both –Cl and Br– gave great influence not only on the excretion ratio as described in the previous section, but also on the excretion pattern, and these effect may depend on the increasing of polarity ascribed to the introducing of halogen groups which had strong electro negativity. On the other hand, the following

11) E. Nelson, *J. Pharm. Sci.*, 132, 181 (1961).

retardation effect of –I on the excretion was observed. RB which had similarly two halogen groups, –Cl and –I, showed Type B even in 30 μ mole dose, and Type C, of which delay in early periods was very remarkable, in 15 and 3 μ mole doses. But EY which had only –Br showed Type B in all doses and EB which had –I, gave the same pattern as shown in RB.

From these results, it was suggested that the –I had some effect on the delay in early period. This delay was probably due to the uptake of RB and EB into liver cells because they disappeared from plasma very fast (about 5—6 min half life) and they had high tendency for the protein binding.[12] This results coincided well with the results of Suga, *et al.*[13] that in the liver homogenate test, large amount of ^{131}I-RB were found in the suppernatant fraction after intravenous injection of ^{131}I-RB in rats. And Mendeloff, *et al.*[14] also reported that Fluorescein of intravenous administered RB appeared only in hepatic polygonal cells.

Summarizing these results, as shown in Table IV, it was found that the halogenation had the great influence on both the biliary excretion and patterns, and this was ascribed to the increasing of polarity depending on the strength of electro negativity of halogen groups. And it was also found that the increasing of molecular weight had not so much effect than the increasing of polarity. And furthermore it was suggested that the –I gave the delay of excretion in the early period and this was probably due to the larger uptake into liver cells.

Bromsulphthalein

Bromsulphthalein (BSP) has been used clinically for hepatic function test as well as Indocianin Green and Azorubin S (AS) for a relatively long time. The molecular weight of it is 838.05 and it has the similar value to those of Phioxine B (PB) and Erythrosine B (EB) (Table I) and the structure is also similar to that of Xanthene dyes as shown in Chart 1, but this has both four halogen groups and two sulfonate groups.

Since BSP is the most useful drug for hepatic function test, there have been many reports about its biliary excretion, blood elimination, uptake into liver cells and metabolism up to this time. Brauer, *et al.*[15–18] reported the biliary excretion and uptake into liver in dogs. Cantarow, *et al.*[19] reported the biliary excretion of BSP and bilirubin. Cohen, *et al.*[20] studied the effect of other dyes on the biliary excretion of BSP. And Cohn, *et al.*[21,22] studied the excretion rate after intravenous injection of BSP in dogs. About the enterohepatic circulation of BSP, Lorber, *et al.*[23] reported. In the fields of kinetic analysis, Richards, *et al.*[24] reported the relationship of time course concentration of BSP among blood, liver and bile, and its analysis by mathematical model. In the methabolism of BSP, Krebs, *et al.*[25] reported that BSP was excreted in bile after metabolized and the species difference was observed. Combes[26] obtained glycine and glutamic acid from the main metabolite of BSP after hydrolysis. And recently, Priestly, *et al.*[27] reported that the metabolite of BSP was glutathion conjugation and in the same paper, the effect of Benziodorone on the glutation conjugation and

12) These results have been unpublished and will be reported in the latter paper.
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biliary excretion of BSP from the enzymatical point of view in rats. And the comparative study with Indocianin Green were reported by Baker,[28] Goresky,[29] and Hargreaves.[30]

In this study of BSP, the authors intended the comparative study with azo dyes and triphenylmethane dyes which had sulfonate groups and Xanthene dyes which had halogen groups. And the effect of sulfonate group and halogen group in the same structure, was discussed. Since BSP has the high sensitivity, the dose were used in the order lower range (0.6—6 μ mole) than other dyes. The biliary excretion of it was 85—90% as listed in Table III and these values belonged to the larger groups such as those of Brilliant Blue FCF (BB), Fast Green FCF (FG) and Phloxine B (PB). It was the most characteristic result of BSP that the concentrical excretion in early periods was seen and above 90% of the total excreted amount was found within 1 hr as shown easily in Fig. 15—17. And the same tendency was seen in riboflavin and Brilliant Blue FCF (BB). On the other hand, the excretion pattern showed also typical high dose type (Type A) as shown in Fig. 16—17, and the ratios of k_1 to k_2 were between 6 and 7 and these values were larger than those of riboflavin (4.7—5) and other tar dyes (1.3—4). It was suggested that BSP showed the clearer dose dependency than that of riboflavin, and the future study will be necessary in still lower doses.

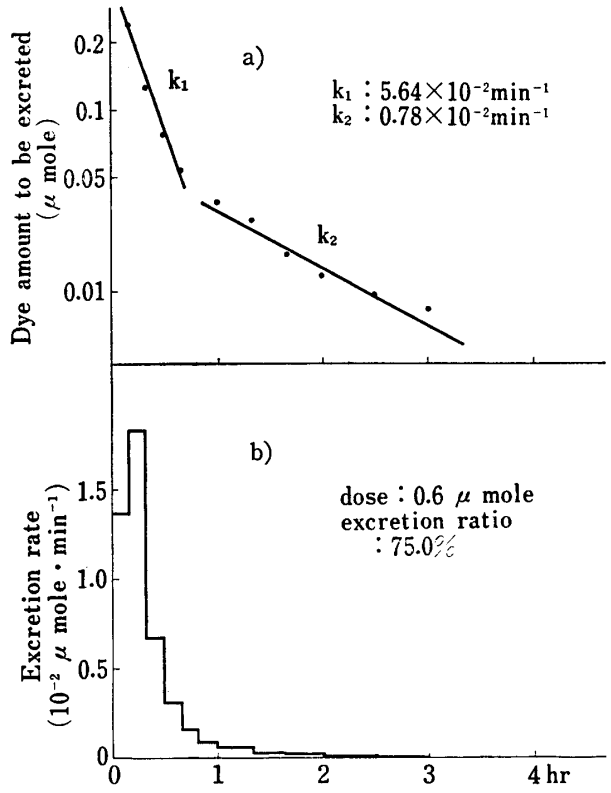

Fig. 16. a) Semilogarithmic Plots of Bromsulphthalein (BSP) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Bromsulphthalein (BSP)

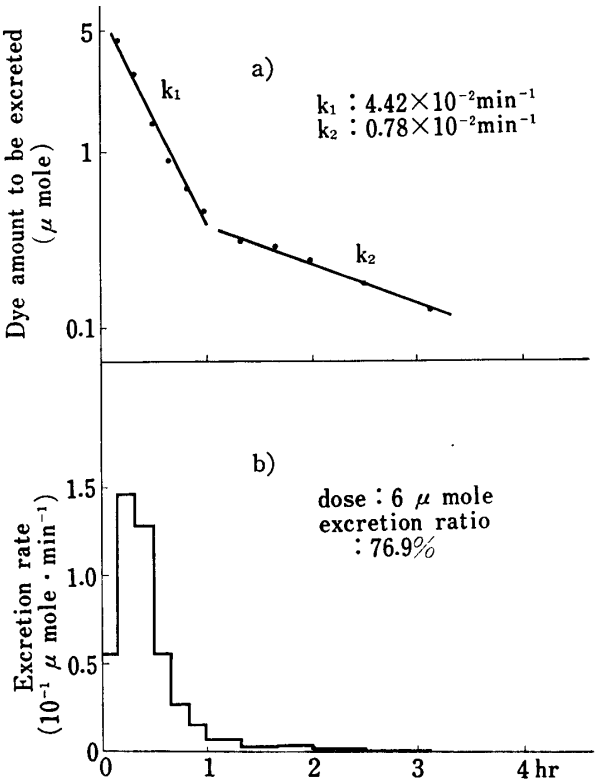

Fig. 17. a) Semilogarithmic Plots of Bromsulphthalein (BSP) in the Body to be excreted in Bile; b) Averaged Excretion Rate of Bromsulphthalein (BSP)

From these results, it was found that BSP showed markedly concentrical excretion pattern and large excretion ratio in a short period. And furthermore, it was found that as the drug for hepatic function test, BSP was superior than Azorubin S (AS) which showed not so

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so concentrical excretion pattern and had lower excretion ratio and sensitivity as shown in the previous paper.[4] It was also suggested that these characteristic behavior of BSP was mainly ascribed to both halogen groups and sulfonate groups which had great influence on the biliary excretion.

As for BSP, a future detail pharmacokinetic study including the metabolism in liver on the enzyme level, will be necessary.