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International Journal of Pharmaceutics 121 (1995) 55–63

international journal of pharmaceutics

Biopharmaceutical studies on drug/conjugated-metabolite interactions. I. Fate of acetaminophen sulfate, a major conjugated metabolite of acetaminophen, in rats

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Received 17 October 1994; revised 9 December 1994; accepted 3 January 1995

Abstract

The plasma elimination kinetics and intestinal absorption kinetics of acetaminophen sulfate (APAPS), a major conjugated metabolite of acetaminophen (APAP), indispensable for the kinetic elucidation of drug/APAPS interactions, were examined in rats. Plasma elimination kinetics of APAPS after i.v. administration could be described by a two-compartment model with linear parameters to the dose. The deconjugation of intravenously administered APAPS, i.e., the formation of APAP, was recognized in neither plasma, urine nor bile. Approx. 80% of intravenously administered APAPS was excreted as the unchanged form in the urine in 4 h while biliary excretion was only a few percent of the dose. Plasma profiles of APAPS after oral administration showed two peaks, but the second one disappeared when the rat was pretreated with kanamycin sulfate. However, APAPS permeation through the small- and the large-intestinal walls determined in situ was not altered after kanamycin treatment. High APAPS-hydrolyzing activities present in the cecal and colonic contents and the feces, but not in the small-intestinal contents, completely disappeared after kanamycin treatment. Thus, part of the orally administered APAPS was absorbed as the unchanged form from both the small and large intestines, and considerable amounts of the remainder were absorbed from the large intestine as APAP after enzymatic hydrolysis by the intestinal microflora during transit through the lower bowel.

Keywords: Acetaminophen; Acetaminophen sulfate; Conjugated metabolite; Pharmacokinetics; Intestinal microflora; Kanamycin sulfate; Rat

1. Introduction

In multiple-drug therapy, considerable attention has been focussed on drug/drug interactions such as interactions in drug distribution, drug metabolism, drug absorption and receptor binding (Niwa and Nakayama, 1970; Thomas et al., 1974; Price and Gale, 1987; Tsuji et al., 1988). Besides drug/drug interactions, we reported the significance of drug/metabolite interaction(s) in the biopharmaceutical fate of the drug in the 1960's (Niwa and Nakayama, 1968a,b). In order to improve the effectiveness and/or the safety of

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SSDI 0378-5173(95)00003-8

multiple-drug therapy, much more attention on the drug-metabolite interaction(s) from the pharmacokinetic viewpoint is required.

Acetaminophen (APAP) is widely used as an oral nonprescription drug and is frequently administered with other analgesic and antipyretic drugs as a multiple-drug therapy. A large quantity of APAP is metabolized to two major conjugates, i.e., the sulfate (APAPS) and the glucuronide (APAPG), in the liver (Galinsky and Levy, 1981; Hjelle et al., 1985; Sweeny and Reinke, 1988; Tone et al., 1990). We have reported several biopharmaceutical aspects of the interactions between APAPS, one of the major metabolites of APAP, and analgesic/antipyretic drugs (Niwa et al., 1972a) or barbiturates (Niwa et al., 1972b). In our preliminary pharmacokinetic study on drug/conjugated-metabolite interaction, significant changes in the pharmacokinetic parameters of APAP and salicylamide in the presence of APAPS were reported. These changes were the decrease in total body clearance of APAP and, in contrast, the increase in total body clearance of salicylamide (Kimura et al., 1990). In this series, we intend to discuss the biopharmaceutical significance of drug/conjugated-metabolite interactions, especially changes in the pharmacokinetics of certain drugs in the presence of APAPS, one of the model conjugated metabolites in common multiple-drug therapy. Precise information regarding the pharmacokinetics of the conjugated metabolite itself are indispensable for a kinetic investigation of such a drug/conjugated-metabolite interaction study.

In the present study, the plasma elimination kinetics and intestinal absorption kinetics of APAPS, including the role of the intestinal microflora in the fate of orally administered APAPS, are clarified.

2. Materials and methods

2.1. Materials

APAP used was of JP grade. APAPS was synthesized as the potassium salt in our laboratory according to the method of Burkhardt and Wood (1972) with minor modifications. m.p., 214–220°C (sintering above 174°C). IR (KBr) (cm^{-1}): 3565 (N-H), 1668 (C=O). ^{1}H-NMR (D_2O) δ: 2.14 (3H, s, $COCH_3$), 7.36 (4H, m, aromatic H). Analysis: Calculated for $C_8H_8KNO_5S \cdot H_2O$: C, 33.44; H, 3.50; N, 4.87. Found: C, 33.19; H, 3.47; N, 4.52. The amounts of APAPS and APAPG in this paper were expressed as the amounts equivalent to APAP to facilitate balance study. Kanamycin sulfate and β-glucuronidase (type IX-A, from *E. coli*) were purchased from Wako Pure Chemicals Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. All other chemicals and reagents were of analytical grade or better.

2.2. Animals

Male 12-week-old Wistar rats (250–300 g) (Japan SLC. Inc., Hamamatsu, Japan) were used.

2.3. In vivo experiment

The rats were fasted overnight before use, but had free access to water. Polyethylene tubing was cannulated into both the left femoral vein and the bile duct under light ether anesthesia and the rat was settled in a Bollman cage (Bollman, 1948). After recovery from anesthesia, APAPS (10, 30, 60 or 120 mg APAP eg./kg) dissolved in saline was administered intravenously through the right femoral vein (bolus i.v. injection study) or intragastrically through a gastric sonde (oral administration study). The volume of the drug solution administered was 1.0 ml/kg. Blood and bile samples were collected through the cannula fastened to the femoral vein and the bile duct, respectively, at appropriate time intervals. Urine samples were also collected periodically. Normal saline solution (1.5 ml aliquot) was orally administered every 2 h in order to maintain the bile and the urine flows.

2.4. In situ absorption study

The rats were anesthetized with sodium pentobarbital. A small-intestinal loop from the duodenum to the ileocecal junction or a large-intestinal

loop from the beginning of the ascending colon to the anus was surgically prepared and the luminal contents of the loop was washed out with saline (37°C). 5 and 2 ml of the APAPS solution (2.5 mg APAP eq./ml) were administered into the small-intestinal loop and the large-intestinal loop, respectively. 2 h after administration, the remaining amounts of APAPS in the loop were determined.

2.5. APAPS-hydrolyzing activities in the intestinal contents

Intestinal contents in the small intestine, cecum and colon and the feces in the rectum were collected from the non-fasted rat. The contents were immediately transferred to a screw-capped bottle filled with nitrogen gas and APAPS solution (25 μg APAP eq./ml) was added to give a 10% wet weight slurry of the contents. The atmosphere of the mixture was made anaerobic by the nitrogen gas stream and the mixture was incubated for 6 h at 37°C under anaerobic conditions.

2.6. Pretreatment with kanamycin sulfate

In order to reduce the enzymatic hydrolyzing activities of the intestinal microflora of the rat, kanamycin sulfate (5 × 200 mg/animal) was administered orally twice daily for 2 days and 4 h before the experiment as described in our previous report (Yamaguchi et al., 1994).

2.7. Analytical method

APAP and APAPS in plasma were determined by a reversed-phase HPLC method (Adriaenssens and Prescott, 1978; Galinsky et al., 1979) with minor modifications. A high-pressure liquid chromatograph (LC-3A, Shimadzu, Kyoto) equipped with a UV detector (SPD-2A, Shimadzu) operated at 254 nm was used in a reversed-phase mode with an Inertsil™ ODS column (average particle size 5 μm, 4.6 mm i.d. × 250 mm, GL Sciences Inc., Tokyo). A mixture of 50 mM phosphate buffer solution (pH 7.4) and methanol (90 : 10 v/v) was employed as the mobile phase at a flow rate of 0.9 ml/min. The concentrations of APAP and APAPS were calculated based on the peak-area measurements. APAPG was determined as APAP after hydrolyzing the sample by incubation with β-glucuronidase solution (1000 units dissolved in 100 mM acetate buffer solution, pH 5.0) at 37°C for 20 h. To all plasma, urine and bile samples (100 μl each), methanol (150 μl) was added. After centrifugation (3000 rpm for 10 min), the supernatant of the resulting mixture was filtered through a 0.45 μm pore-size membrane filter (SJHVL04NS, Nihon Millipore Kogyo, Yonezawa, Japan) and the filtrate was used for HPLC measurements.

2.8. Pharmacokinetic analysis

Plasma elimination profiles of intravenously administered APAPS were analyzed by a two-compartment model shown in Fig. 2 using the nonlinear least-squares regression program, 'MULTI' (Yamaoka et al., 1981). Total body clearance (CL_{total}) of APAPS was calculated as the dose divided by the area under the plasma concentration-time curve (AUC) value, which was estimated by the trapezoidal rule extrapolated to infinity.

3. Results and discussion

3.1. Plasma elimination of APAPS

Fig. 1 shows the plasma concentration-time profiles of APAPS after i.v. administration with varying doses (10, 30 and 120 mg APAP eq./kg). Plasma profiles of APAPS were apparently biexponential with time. The data were analyzed by a two-compartment model as shown in Fig. 2. The pharmacokinetic parameters in each rat were estimated by the nonlinear least-squares regression program, MULTI (Yamaoka et al., 1981), and are summarized in Table 1. Morris and Levy (1984) reported the existence of an active secretion process in renal APAPS excretion. Later studies by Savina and Brouwer (1992) implicated significant impairment of renal APAPS secretion by probenecid. However, the process of elimination of APAPS from the plasma could apparently be expressed as a first-order mechanism in the pre-

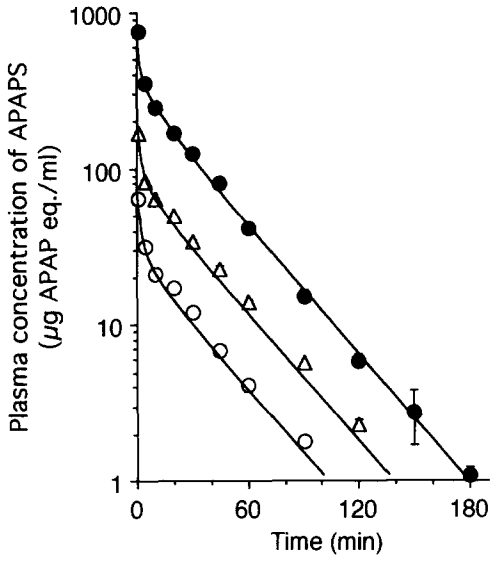

Fig. 1. Plasma concentration profiles of intravenously administered APAPS in rats. The doses of APAPS were 10 (○), 30 (△) and 120 (●) mg APAP eg./kg. Results are expressed as the mean ± S.E. The numbers of rats for each dose are listed in Table 1. Each line represents the simulation curve calculated according to the model shown in Fig. 2 by using the parameters listed in Table 2.

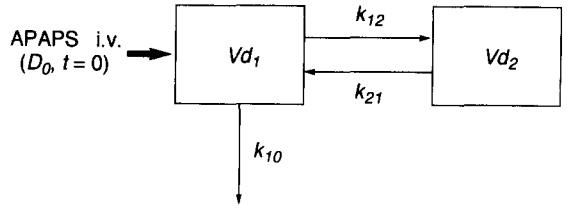

Fig. 2. Pharmacokinetic model for APAPS in rats. APAPS was administered intravenously at the dose of D_0. k_{12} and k_{21} are the first-order rate constants between two compartments. k_{10} denotes the first-order elimination rate constant. Vd_1 and Vd_2 are the volume of distribution of central and peripheral compartment, respectively.

sent dose range (10–120 mg APAP eq./kg), i.e., the parameters describing the elimination process, namely, CL_{total} and β, showed dose-independent phenomena. Further, no significant difference was observed in the other parameters at all doses (Table 1). In order to estimate the pharmacokinetic parameters as the dose-independent ones, simultaneous analysis of the three plasma APAPS elimination profiles shown in Fig. 1 was carried out and the estimated dose-independent pharmacokinetic parameters of APAPS in rats are listed in Table 2. As shown in Fig. 1, all plasma profiles were in close agreement with the simulation lines calculated using the model (Fig. 2) and the parameters (Table 2). APAP, the deconjugation product of APAPS, was not detected in the plasma at any doses. This suggests

Table 1
Pharmacokinetic parameters of APAPS in rats

Parameter		Dose (mg APAP eq./kg)		
		10 ($n = 4$)	30 ($n = 3$)	120 ($n = 3$)
A	(μg APAP eq./ml)	56.3 ± 3.5	142.5 ± 7.9	678.7 ± 34.6
B	(μg APAP eq./ml)	29.6 ± 0.3	78.7 ± 0.3	317.4 ± 15.8
α	(h^{-1})	27.5 ± 1.5	25.6 ± 1.0	25.0 ± 2.3
β	(h^{-1})	1.9 ± 0.1	1.7 ± 0.1	2.0 ± 0.1
k_{12}	(h^{-1})	13.8 ± 1.2	12.9 ± 0.8	12.4 ± 1.3
k_{21}	(h^{-1})	10.7 ± 0.2	10.2 ± 0.1	9.4 ± 1.0
k_{10}	(h^{-1})	4.9 ± 0.2	4.2 ± 0.2	5.3 ± 0.1
Vd_1	(ml/kg)	116.9 ± 4.8	135.9 ± 4.8	120.8 ± 4.2
Vd_2	(ml/kg)	149.0 ± 5.9	170.7 ± 4.5	159.7 ± 6.2
AUC	(μg APAP eq. h ml^{-1})	17.6 ± 0.3	52.2 ± 0.6	199.8 ± 4.1
CL_{total}	(ml h^{-1} kg^{-1})	569.0 ± 10.2	575.2 ± 6.1	601.2 ± 12.6

Results are expressed as the mean ± S.E. of the parameters estimated by individual pharmacokinetic analysis in three to four rats. The plasma profile of each rat was fitted to the following equation with the weight for the calculation of C_p^{-2}:

$$C_p = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$$

where C_p is the plasma concentration of APAPS at time t.

Table 2
Pharmacokinetic parameters of APAPS in rats estimated by the simultaneous analysis

A/D_0	(kg/l)	5.33
B/D_0	(kg/l)	2.85
α	(h^{-1})	28.2
β	(h^{-1})	1.91
k_{12}	(h^{-1})	14.2
k_{21}	(h^{-1})	11.1
k_{10}	(h^{-1})	4.87
Vd_1	(ml/kg)	122.3
Vd_2	(ml/kg)	156.3
AUC/D_0	(kg h l^{-1})	1.68
CL_{total}	(ml h^{-1} kg^{-1})	596

D_0, dose of APAPS (10, 30 or 120 mg APAP eq./kg).

that systemic deconjugation of APAPS was practically negligible.

3.2. Urinary and biliary excretion of APAPS

The urinary and biliary excretion characteristics of APAPS following i.v. administration were also investigated, the results being summarized in Table 3. APAPS was mainly excreted in the urine as the unchanged form and very small amounts of APAPS were excreted in the bile. These results are consistent with the report of Iida et al. (1989). Neither APAP nor APAPG was detected in the urine as well as in the bile. These results suggest that the deconjugation of APAPS, i.e., the formation of APAP, after the i.v. administration of APAPS can practically be negligible in this study.

3.3. Absorption kinetics of APAPS after the oral administration

The plasma concentration-time profiles of APAPS after the oral administration of APAPS (30, 60 and 120 mg APAP eq./kg) are shown in Fig. 3. The plasma APAPS profiles display two peaks and the T_{max} values were similar for the three doses. A small peak of APAP, the deconjugation product, could be detected in the plasma samples collected 4 h after administration. While the plasma disposition of intravenously administered APAPS was linear over the present dose range (Fig. 1), the rates of increase in AUC values of APAPS after oral administration were not proportional to the dose, especially for the high peroral dose (120 mg APAP eq./kg): the bioavailabilities of APAPS for oral doses of 30, 60 and 120 mg APAP eq./kg were 0.76, 0.70 and 0.51, respectively. The non-linear aspects of peroral APAPS will be discussed later.

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Table 3
Urinary and biliary excretion of APAPS, APAP and APAPG in 4 h after the i.v. administration of APAPS in rats

(a) Urinary excretion				
Dose (mg APAP eq./kg)	Excreted in urine in 4 h (% of dose)			
	APAPS	APAP	APAPG	Total
10	81.8 ± 3.6	ND [a]	ND	81.8 ± 3.6
30	77.3 ± 4.2	ND	ND	77.3 ± 4.2
60	75.6 ± 4.7	ND	ND	75.6 ± 4.7
120	80.9 ± 4.4	ND	ND	80.9 ± 4.4
(b) Biliary excretion				
Dose (mg APAP eq./kg)	Excreted in bile in 4 h (% of dose)			
	APAPS	APAP	APAPG	Total
10	1.92 ± 0.50	ND [a]	ND	1.92 ± 0.50
30	1.67 ± 0.67	ND	ND	1.67 ± 0.67
60	2.33 ± 1.38	ND	ND	2.33 ± 1.38
120	2.12 ± 0.62	ND	ND	2.12 ± 0.62

Results are expressed as the mean ± S.E. of four rats.
[a] Not detected.

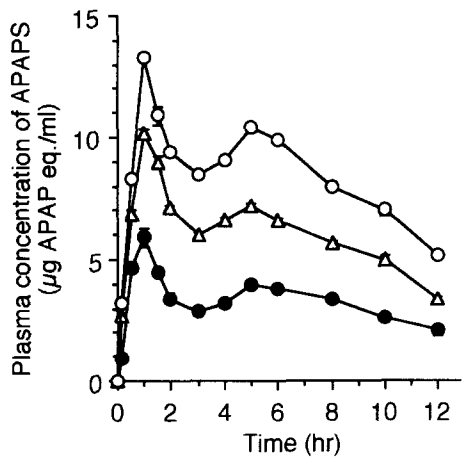

Fig. 3. Plasma concentration of APAPS after the oral administration of APAPS at a dose of 30 (●), 60 (△) and 120 (○) mg APAP eq./kg. Results are expressed as the mean ± S.E. of four rats.

conjugated metabolite and the intestinal resorption followed by the deconjugation of the metabolite in the lumen of the intestine, is well known in certain drugs. The enterohepatic circulation system sometimes provides a considerable reservoir for a drug. Recently, the possibility of the generation of a drug from its conjugated metabolite by the action of the intestinal microflora has been pointed out. The usefulness of the application of amino acids (Nakamura et al., 1992a) and dipeptides (Nakamura et al., 1992b) as conjugants of salicylic acid for colon-specific prodrugs of salicylic acid has been reported. Siegers et al. (1983) reported the existence of marked enterohepatic circulation of APAP, where APAP was excreted into bile as APAPS and APAPG, in rats. However, when the sulfated metabolite, APAPS, was administered to rats intravenously, the deconjugated product, APAP, was not detected in plasma at all. In addition, only a few percent of APAPS were excreted into the bile after i.v. administration (Table 3). Consequently, the enterohepatic circulation of APAPS scarcely contributes to the absorption kinetics of orally administered APAPS. Thus, the second plasma peak of APAPS (Fig. 3) and the synchronous detection of APAP in the plasma after the oral administration were assumed to result from the absorption of APAP which originated from APAPS during transit through the gastrointestinal tract.

3.4. Effect of intestinal microflora on APAPS absorption

In order to clarify the effect of intestinal microflora on the absorption of conjugated metabolite, an oral administration study of the kanamycin-treated rats was also carried out. Kanamycin treatment of rats was performed according to the method of Yamaguchi et al. (1994). The role of the intestinal microflora in the metabolism of peroral drugs and the effect of treatment with antibiotics to suppress metabolic activities of the microflora have been reported by Gingell et al. (1971). Walsh and Levine (1974) reported that treatment with antibiotics, such as neomycin sulfate, penicillin V potassium, tetracyclines, etc., affects the intestinal microflora but does not change drug absorption.

Firstly, the effect of kanamycin treatment on the permeability of the gut wall was investigated by an in situ intestinal loop method in order to avoid the effect of intestinal contents. Table 4 summarizes the results. There was no significant change in the disappearance of APAPS after the 2 h experiment in either small or large intestine, suggesting that the pretreatment with kanamycin sulfate has no effect on intestinal permeability to APAPS, in agreement with the results reported by Walsh and Levine (1974).

Changes in the plasma profile of APAPS after oral administration of APAPS (30 mg APAP eq./kg) in kanamycin-treated rats are summarized in Fig. 4. The second plasma peak of APAPS

Table 4
Effect of kanamycin treatment on in situ intestinal absorption of APAPS in rats

Site	Absorption in 2 h (% of dose)	
	Normal	Kanamycin-treated
Small intestine	16.7 ± 3.6	14.7 ± 2.4
Large intestine	5.3 ± 0.6	7.4 ± 1.6

Initial concentration of APAPS in each loop was 2.5 mg APAP eq./ml. Results are expressed as the mean ± S.E. of four rats.

observed 5 h after administration in normal rats (Fig. 3) completely disappeared in the kanamycin-treated rats. Further, almost no urinary excretion of APAP and APAPG, another major conjugated metabolite of APAP, was observed in the kanamycin-treated rats (data not shown). This suggests that APAP formation after the oral administration of APAPS in the kanamycin-treated rats during the experiment was negligible.

3.5. *APAPS-hydrolyzing activity in intestinal contents*

A preliminary study on gastric absorption of APAPS in surgically pylorus-ligated rat clarified that the absorption and deconjugation of APAPS in the stomach were negligible in both the normal and kanamycin-treated rats because APAPS was not detected in plasma and almost 100% of the dose was recovered as intact APAPS from the stomach 6 h after administration. Localization of APAPS-hydrolyzing activity in the intestinal contents was investigated by incubating APAPS with the intestinal contents collected from the non-fasted rat with or without kanamycin treatment.

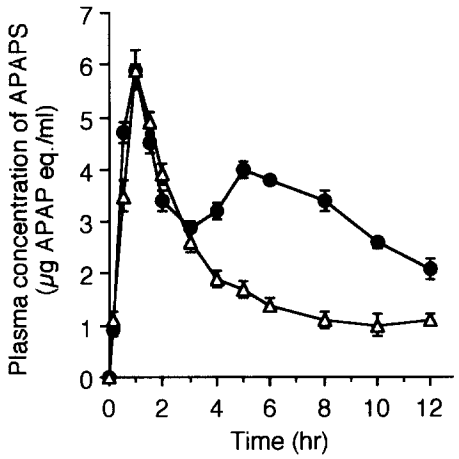

Fig. 4. Effect of kanamycin pretreatment on plasma profile of APAPS after oral administration. APAPS (30 mg APAP eq./kg) was administered to normal (●) and kanamycin-treated (○) rats. Results are expressed as the mean ± S.E. of four and five rats for normal and kanamycin-treated group, respectively.

Table 5
APAPS-hydrolyzing activity in the intestinal contents and the feces of normal and kanamycin-treated rats

Rat	APAP formation in 6 h (%)			
	Intestinal contents			Feces
	Small intestine	Cecum	Colon	
Normal	0	100	62.3 ± 4.6	55.2 ± 2.1
Kanamycin-treated	0	0	0	0

APAP formation (%) was calculated from the following equation:

$$\text{APAP formation (\%)} = [\text{APAP}]/([\text{APAP}]+[\text{APAPS}]) \times 100$$

Results are expressed as the mean ± S.E. ($n = 4$).

As summarized in Table 5, considerable APAPS-hydrolyzing activity was found in cecal and colonic contents, whereas the small-intestinal contents had no hydrolyzing activity. In addition, the activities localized in the cecal and colonic contents and in the feces completely disappeared following kanamycin treatment. Nakamura et al. (1988) have reported on the hydrolysis of salicyluric acid, a glycine conjugate of salicylic acid, by the intestinal microflora in rats. The above findings (Fig. 4 and Table 5) suggest that the intestinal absorption of APAP formed via hydrolysis by intestinal microflora is responsible for the second plasma peak of APAPS observed after oral APAPS administration (Fig. 3). Moreover, the major deconjugation site for APAPS is thought to be the hind gut.

Although the elimination kinetics of intravenously administered APAPS was linear (Fig. 1) over the present dose range, the plasma AUC values of APAPS after oral administration showed non-linear aspects (Fig. 3). The existence of the following two non-linear processes seems to be acceptable for the explanation. The first is the hydrolyzing process of orally administered APAPS, i.e., the process of APAP formation in the hind gut, which might be governed by the hydrolyzing activity of the intestinal microflora as well as by the residence time of APAPS in the hind gut. The second is the process of APAPS

formation, i.e., the hepatic first-pass sulfation process of absorbed APAP.

The findings clarified in this study are indispensable for pharmacokinetic investigations of drug/APAPS interactions. Firstly, we will make a pharmacokinetic approach to elucidate the biopharmaceutical significance of drug/conjugated-metabolite interactions between APAP and APAPS which we have recognized in rats (Kimura et al., 1990) in a subsequent paper.

4.Conclusions

The plasma elimination kinetics of APAPS could be described by a linear two-compartment model. The plasma profiles of peroral APAPS showed two peaks, the second one being derived from the absorption of APAP, which originated from APAPS after enzymatic hydrolysis of APAPS by the intestinal microflora, in the lower bowel.

Acknowledgment

The authors wish to thank Dr. Kenji Sasaki of Okayama University for invaluable assistance in synthesis and analysis of APAPS.

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