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Fluorometric Determination of Ampicillin and Aminobenzylpenicilloic Acid in Presence of Pivampicillin in Body Fluids[1]

Katsumi Miyazaki, Osamu Ogino,[2a] Masahiro Nakano, and Takaichi Arita[2b]

Pharmacy, Hokkaido University Hospital[2a] and Faculty of Pharmaceutical Sciences, Hokkaido University[2b]

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In order to investigate the transfer of ampicillin and pivampicillin in biological systems, the sensitive separatory determination method of ampicillin and aminobenzylpenicilloic acid in presence of pivampicillin was developed. This method is based on the separation of these substances utilizing their different distribution behavior between the aqueous phase and the chloroform phase, followed by fluorometric determination. In the transfered studies in biological systems, marked differences were found in the rate of absorption and the amount of excretion between ampicillin and pivampicillin. Biotransformational studies were also discussed.

Pivampicillin is easily hydrolyzed to ampicillin in body fluids[3] and the procedures based on microbiological assay[3,4] have been used mainly for detection of formed ampicillin, at the low concentrations encountered in body fluids following the therapeutic dose of the antibiotics. These studies report apparent increase in blood levels of formed ampicillin after oral administration of pivampicillin. However these studies did not involve complete separation of unchanged pivampicillin from ampicillin in body fluids prior to microbiological assay.

Furthermore, the transformation products of ampicillin were reported in recent years.[5] Although identification of the metabolite has been discussed, the possibility that aminobenzylpenicilloic acid (AB–PA) is the major metabolite as well as other penicillin derivatives[6] cannot be excluded. The examination for the blood level and urinary excretion of AB–PA based on the chemical assay as well as ampicillin after administration of ampicillin and pivampicillin is of interest in view of the comparison of the transfer of these drugs in biological systems. This paper reports the direct separatory determination method of formed ampicillin and AB–PA in presence of pivampicillin in body fluids according to the fluorometric determination method of ampicillin reported previously[7] and the transfer of these drugs in biological systems were investigated.

Experimental

Materials and Reagents——Ampicillin and pivampicillin used were prepared as described previously.[7] All the reagents used in the experiment were of special grade, and were freshly prepared with redistilled

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2) Location: *a*) *Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo*; *b*) *Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo.*
3) *a*) W. von Daehne, W.O. Godtfredsen, K. Roholt, and L. Tybring, *Antimicrob. Ag. Chemother.*, **1970**, 431; *b*) E.L. Foltz, J.W. West, I.H. Breslow, and H. Wallick, *ibid.*, **1970**, 442; *c*) J.B. Wilcox, R.N. Brogden, and G.S. Avery, *Drugs*, **6**, 94 (1973).
4) H.J. Simon and E.J. Yin, *Appl. Microbiol.*, **19**, 573 (1970).
5) *a*) T. Murakawa, Y. Kono, and M. Nishida, *J. Antibiot.*, **25**, 421 (1972); *b*) W.J. Jusko and G.P. Lewis, *J. Pharm. Sci.*, **62**, 69 (1973).
6) J. Birner, *J. Pharm. Sci.*, **59**, 757 (1970).
7) K. Miyazaki, O. Ogino, and T. Arita, *Chem. Pharm. Bull.* (Tokyo), **22**, 1910 (1974).

water. Quinine sulfate solution which is a standard fluorescence solution used in this experiment was prepared as described previously.[7]

Apparatus——Fluorescence intensity was measured by a Hitachi spectrofluorometer, 203, equipped with Xenon lamp.

Urinary Excretion of Antibiotics in Man——Male normal volunteer received doses of oral ampicillin and pivampicillin after fasting overnight. The ampicillin and pivampicillin capsules contained 250 mg ampicillin equivalent, respectively. The dosage form of ampicillin was commercial capsules (anhydrous form) and pivampicillin was loosely packed with gelatin hard capsules (JP 8 No. 0), which were ingested with 100 ml of tap water and additional water (100 ml) was taken hourly for the next 6 hours.

Examination for the Blood Levels of Antibiotics in Rats——Male albino rats (Wistar strain) weighing 300—350 g, were starved overnight and dosed intraduodenaly with ampicillin and pivampicillin in dosages equivalent to 10 mg of ampicillin per kg body weight. Ampicillin and pivampicillin were dissolved in 0.9% NaCl solution. Blood samples were obtained from carotid artery.

Method of Separatory Determination of Ampicillin and AB-PA in Presence of Pivampicillin——Procedure 1; Method in Urine Sample: In the measurement of total pivampicillin (pivampicillin+ampicillin+AB-PA, C_1), the procedure for the determination of total ampicillin in urine sample described previously[7] was followed (Chart 1).

In the measurement of ampicillin and AB-PA, according to the procedure shown in Chart 1, a 1 ml aliquot of sample, if necessary, diluted adequately, was placed in a test tube containing 4 ml of PH 6.0 phosphate buffer solution. To this mixture, 5 ml of chloroform was added. The mixture was vigorously shaken for 5 minutes and centrifuged. Four milliliters of the aqueous layer was then placed in a brown test-tube. In the measurement of the sum of ampicillin and AB-PA (C_2), 0.5 ml of N NaOH was added to the 4 ml of centrifuged sample solution in the brown test-tube and the mixture was allowed to stand for 5 minutes, and 0.5 ml of N HCl was then added. To this mixture, 2 ml of 0.02% (w/v) $HgCl_2$ solution prepared with pH 2.5 buffer solution (citric acid–HCl–NaOH) was added. After 5 minutes, 1 ml of 1/4M Na_2HPO_4 solution was added to adjust pH of the mixture 6.1 ± 0.1. A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at 40° in water bath. After cooling, in order to separate the fluorescent product from the interfered materials in the body fluids, the fluorescent product was extracted with ethyl acetate at this pH medium and furthermore reextracted with pH 13.0 borate buffer solution as described previously.[7] And the fluorescence intensity was measured at an excitation wavelength of 340 nm and an emission one of 420 nm as described previously.[7]

In the measurement of AB-PA (C_3), 1 ml of distilled water instead of NaOH and HCl was added to the centrifuged aqueous layer in the another brown test-tube. And then the mixture was treated following the procedure mentioned in the sum of ampicillin and AB-PA (C_2) determination method. Consequently, the subtraction of C_2 from C_1 gives the concentration of pivampicillin and the subtraction of C_3 from C_2 gives the concentration of formed ampicillin.

Procedure 2; Method in Blood Sample: The procedure was shown in Chart 2. Four-tenth ml of whole blood sample was added to 4 ml of distilled water in a 10 ml glass-stoppered centrifuge tube. Three ml of 10% trichloroacetic acid was added to this hemolyzed blood sample solution and the mixture was centrifuged to obtain the clear supernatant. Three ml of clear supernatant was pipetted into a test tube and to this solution 3 ml of 1/4M Na_2HPO_4 was added to adjust pH of the mixture 5—6.5. To this mixture, 6 ml of chloroform was added and the mixture was vigorously shaken for 5 minutes and centrifuged. Two and three milliliters of the aqueous layer were then placed in a brown test-tube, respectively. In the measurement of the sum of ampicillin and AB-PA (C_1), 0.5 ml of N NaOH was added to the 2 ml of centrifuged sample solution in the brown test-tube and the mixture was allowed to stand for 5 minutes, and 0.5 ml of N HCl was then added. To this mixture, 2 ml of 0.005% (w/v) $HgCl_2$ solution prepared as mentioned above was added. After 5 minutes, 1 ml of 1/3M Na_2HPO_4 solution was added to adjust pH of the mixture 6.1 ± 0.1. A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at 40° in water bath. After cooling, the reaction mixture was treated following the procedure as described previously.[7]

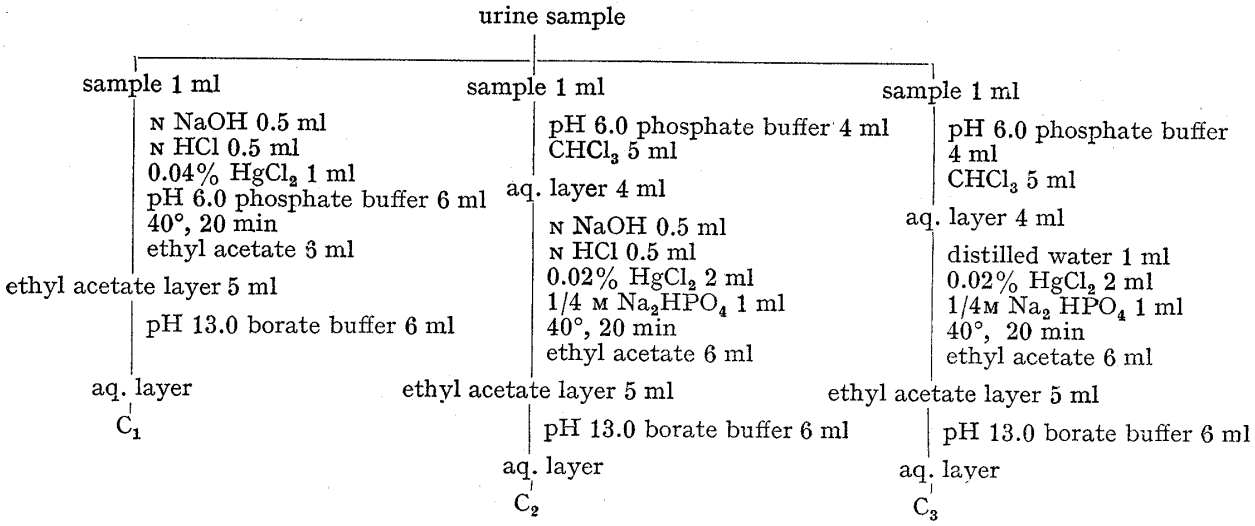
In the measurement of AB-PA (C_2), 1 ml of distilled water was added to the 3 ml of centrifuged sample solution in the brown test-tube. To this mixture, 3 ml of 0.002% (w/v) $HgCl_2$ solution was added. After 5 minutes, 1 ml of 5/12M Na_2HPO_4 solution was added to adjust pH of the mixture 6.1 ± 0.1. And then the mixture was treated as mentioned above. Consequently, the subtraction of C_2 from C_1 gives the concentration of formed ampicillin.

In this procedure, pivampicillin was not degraded in the acidic solution containing trichloroacetic acid and the fluorescence intensity of ampicillin in presence of pivampicillin was not altered in this determination method.

Result and Discussion

Extractibility of Pivampicillin

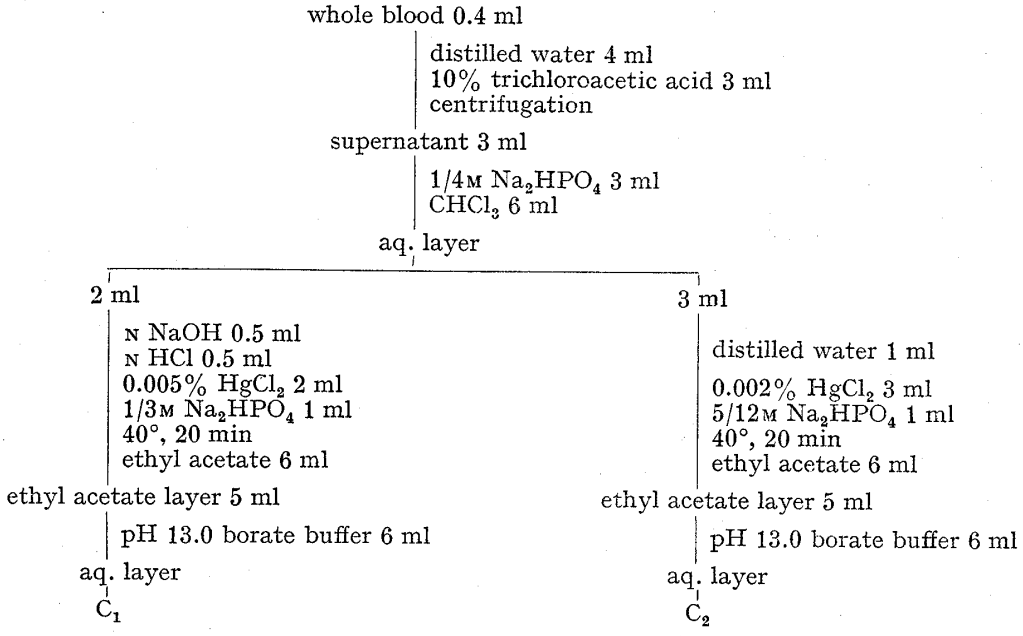
In order to separate pivampicillin from ampicillin and AB–PA, the extractibility of pivampicillin in various pH media with chloroform and ethyl acetate was studied (Fig. 1).



C_1-C_2=unchanged pivampicillin, C_2-C_3=formed ampicillin
C_3=aminobenzylpenicilloic Acid

Chart 1. Method of Separatory Determination of Pivampicillin, Ampicillin and Aminobenzylpenicilloic acid in Urine

C_1: amt. of total pivampicillin equivalent to ampicillin in 1 ml of sample solution
C_2: amt. of the sum of ampicillin and aminobenzylpenicilloic acid equivalent to ampicillin in 1 ml of sample solution
C_3: amt. of aminobenzylpenicilloic acid equivalent to ampicillin in 1 ml of sample solution
Fluorescence intensity is measured at 340 nm excitation wavelength and 420 nm emission wavelength.



C_1-C_2=formed ampicillin, C_2=aminobenzylpenicilloic acid

Chart 2 Method of Separatory Determination of Ampicillin and Aminobenzylpenicilloic Acid in Presence of Pivampicillin in Blood

C_1: amt. of the sum of ampicillin and aminobenzylpenicilloic acid equivalent to ampicillin in 0.4 ml of blood
C_2: amt. of aminobenzylpenicilloic acid equivalent to ampicillin in 0.4 ml of blood
Fluorescence intensity is measured at 340 nm excitation wavelength and 420 nm emission wavelength.

Pivampicillin is readily and completely extracted into chloroform at the neutral pH range (5—6.5) and at this pH range 5% of pivampicillin was remained in aqueous layer in the case of ethyl acetate. In the alkaline solution, because of hydrolysis of pivampicillin, the extractibility is decreased. Furthermore, ampicillin and AB—PA could not be extracted with these

solvent. According to these results, chloroform was suitable solvent for the separation of pivampicillin from ampicillin and AB–PA.

Effects of the Concentration of $HgCl_2$ Reagent on the Fluorescence Intensity

The effect of concentrations of $HgCl_2$ reagent on the maximum intensity of fluorescence in urine sample was investigated according to procedure 1. As shown in Fig. 2, the maximum and the constant fluorescence intensity was obtained at the concentration range of 0.01—0.03 % (w/v) $HgCl_2$ reagent.

In the experiment in blood sample solution (procedure 2) the maximum fluorescence intensity was obtained at the concentration range of 0.001—0.01% (w/v) $HgCl_2$ reagent in the sum of ampicillin and AB–PA determination method and at the concentration range of 0.001—0.005% (w/v) $HgCl_2$ reagent in AB–PA determination method (Fig. 2).

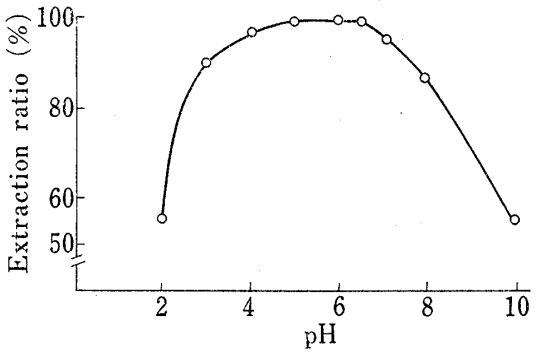


Fig. 1. pH Profile of the Extraction Ratio of Pivampicillin with Chloroform

buffer component, pH: 2.0—5.0 (citric acid-HCl-NaOH)
pH: 6.0—7.0 (Na_2HPO_4-KH_2PO_4)
pH: 10.0 (sodium borate-NaOH)

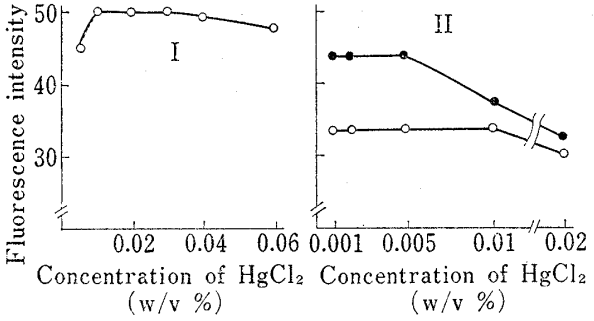


Fig. 2. Effect of Concentration of $HgCl_2$ Reagent on the Fluorescence Intensity

I: following to the procedure 1
II: following to the procedure 2
—○—: in the sum of ampicillin and aminobenzylpenicilloic acid determination method
—●—: in aminobenzylpenicilloic acid determination method

Recovery Test of Ampicillin and AB-PA in Presence of Pivampicillin

A mixture containing a known amount of every standard substance in human urine and blood was separately determined following the procedure in Chart 1 and Chart 2, respectively. The results are given in Table I, in which agreement between added amount and found value is reasonable. Thus these separatory determination methods of ampicillin and AB–PA in

Table I. Recovery Test of Ampicillin and Aminobenzylpenicilloic Acid in Presence of Pivampicillin

Sample	Added (μg)			Found (μg)[a]		Recovery (%)[a]	
	PV-PC	AB-PC	AB-PA[b]	AB-PC	AB-PA	AB-PC	AB-PA
Urine	2.0	5.0	5.0	5.00 —5.01 (3)	4.95 — 4.99 (3)	100.0—100.3	98.9— 99.8
	2.0	5.0	10.0	4.90 —5.14 (3)	9.95 —10.00 (3)	98.2—102.8	99.5—100.0
	10.0	5.0	2.0	5.04 —5.08 (3)	1.96 — 1.98 (3)	101.2—101.6	98.0— 99.0
Blood[c]	0.4	0.4	0.4	0.397—0.397(3)	0.417— 0.419(3)	99.3— 99.3	104.3—104.8
	0.2	0.4	0.8	0.397—0.404(2)	0.804— 0.810(2)	99.4—101.1	100.5—101.3
	0.8	0.4	0.2	0.411—0.415(2)	0.202— 0.202(2)	102.9—103.7	101.1—101.1

a) Range of recovery is shown.
b) shown as ampicillin equivalent
c) Sample was added to 0.4 ml human whole blood.
Experiment no. is given in parentheses.
PV-PC: pivampicillin, AB-PC: ampicillin, AB-PA: aminobenzylpenicilloic acid

presence of pivampicillin were found to be applicable for the urine and the blood samples, at the low concentrations encountered following therapeutic dose.

Urinary Excretion of Ampicillin and AB-PA in Man

The urine specimens after oral administration of ampicillin were treated to determine separately ampicillin and AB–PA according to the procedure described previously.[7] And the urine specimens after oral administration of pivampicillin were treated to determine separately pivampicillin, ampicillin, and AB–PA according to the procedure in Chart 1. The result are shown in Fig. 3. In the urine during 6 hours after administration of ampicillin, amount of unchanged ampicillin recovered was about 33% of the dose. On the other hand, about 78% of the dose was recovered as formed ampicillin after administration of pivampicillin. And the small amount of 4% and 8% of the dose was excreted as AB–PA after administration of ampicillin and pivampicillin, respectively. Furthermore, unchanged pivampicillin was not excreted in urine.

It was demonstrated from these results that there were marked differences in the rate of absorption and in the amount of absorption between ampicillin and pivampicillin. Moreover, it was suggested that pivampicillin is completely hydrolyzed in the biological systems.[3a]

Identification of the Metabolite in Urine Sample

Identification of the small amount metabolite in urine sample that determined as AB–PA was investigated with thin-layer chromatography (TLC). It was observed that using Kieselgel G (Merck) and iso-PrOH–MeOH (3: 7) in TLC ampicillin (*Rf* value: 0.6—0.9) can be separated from AB–PA (*Rf* value: 0.2—0.4). Color reagents are the solution[8] which 10% aqueous ferric chloride and 5% aqueous potassium ferricyanide were mixed with 20% sulfuric acid and the vapour of iodine.

In order to compare *Rf* values of the AB–PA and ampicillin fraction in the urine sample after administration of ampicillin with *Rf* values of the spots of the authentic samples, after

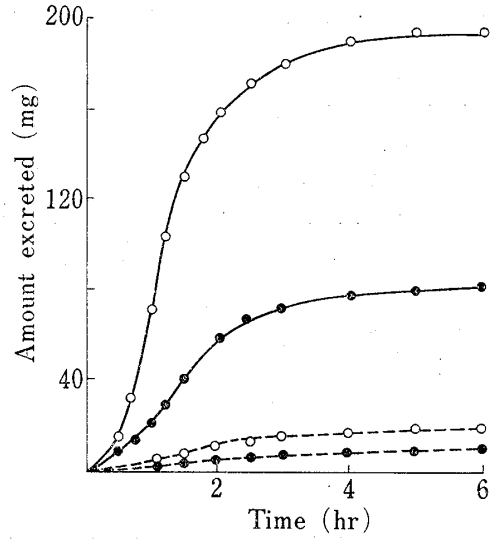


Fig. 3. Cumulative Urinary Excretion of Ampicillin and Aminobenzylpenicilloic Acid after Oral Administration of Ampicillin and Pivampicillin to Man

●: ampicillin capsule (250 mg)
○: pivampicillin capsule (ampicillin equivalent)
—: ampicillin in urine sample
---: aminobenzylpenicilloic acid in urine sample

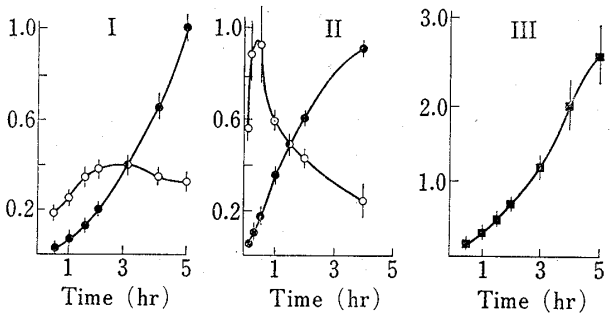


Fig. 4. Blood Levels of Ampicillin and Its Metabolite after Intraduodenal Administration of Ampicillin (I), Pivampicillin (II) and Aminobenzylpenicilloic Acid (III) to Rats

dose: 10 mg/kg equiv. of ampicillin
—○—: ampicillin, —●—: metabolite of ampicillin, —■—: aminobenzylpenicilloic acid
mean ±S.E. (3—4)

8) I.J. Mcgilveray and R.D. Strickland, *J. Pharm. Sci.*, **56**, 77 (1967).

developing the urine sample and drying the plate, zones (1 cm) were scraped off and the scrapings were shaken with 4 ml of aqueous water for 20 minutes. Each of the centrifuged supernatants (1 ml) was used for fluorometric measurements.[7] Fluorescence intensity were observed in the range of *Rf* values corresponding to the spots of authentic AB–PA and ampicillin, respectively. It is therefore suggested that the metabolite in the urine sample is aminobenzylpenicilloic acid (AB–PA).

Examination for the Blood Levels

Blood levels of ampicillin and its metabolite after intraduodenal administration of ampicillin, pivampicillin and AB–PA to rats were measured according to the procedure described previously[7] and in Chart 2. The results are shown in Fig. 4. Pivampicillin gave rise to substantialy higher ampicillin concentration in the blood than did ampicillin. Mean peak concentration of 0.9 μg/ml was obtained as formed ampicillin in pivampicillin dosage. The corresponding peak concentration of ampicillin was 0.4 μg/ml. Furthermore, the peak concentration of ampicillin occurred about 0.5 hour after administration of pivampicillin, whereas the peak concentration of ampicillin was reached 2—3 hours after administration of ampicillin.

In contrast to the blood levels of ampicillin, the time course of blood metabolite levels (Fig. 4, I and II) indicates marked different features. The similar result is obtained in the case of AB–PA dosage (Fig. 4, III). Since the blood levels of the metabolite is estimated based on the direct determination method of AB–PA and then the time course of blood levels of AB–PA (Fig. 4, III) is similar to the results of blood metabolite levels (Fig. 4, I and II), the possibility that the metabolite in the blood is AB–PA itself cannot be excluded. Further studies of the identification, the toxity and the transfer in the biological systems of the metabolite would be of interest since its higher blood levels may lead to accumulation during multiple dosing.[5b]