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โครงการ: เภสัชจลนศาสตร์ของสารต้านจุลชีพที่ใช้ในการเลี้ยงกุ้ง

คณะผู้วิจัย

ดำรงศักดิ์ ฟ้ารุ่งสาง

ภาควิชาเทคโนโลยีเภสัชกรรม คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

สมหมาย เชี่ยววารีสัจจะ

ภาควิชาวาริชศาสตร์ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

โครงการวิจัย DBG5180010

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บทคัดย่อ

โครงการวิจัยนี้ ได้ทำการศึกษาจลนศาสตร์การกระจายและขับออกของยาปฏิชีวนะออกซิเตตร้าไซคลิน (OTC) ในกุ้งขาวเลี้ยงจากฟาร์ม (*Litopenaeus vannamei*) ที่ให้ในขนาดต่างๆกันตั้งแต่ 10, 50, 500, 1,000, และ 2,500 mg/kg-น้ำหนักตัวกุ้ง ทั้งทางหลอดเลือด (เฉพาะ 10 mg/kg เท่านั้น) และทางปาก โดยได้พัฒนาแบบจำลองทางเภสัชศาสตร์ที่สามารถทำนายระดับยาที่เปลี่ยนแปลงตามเวลาหลังจากกุ้งได้รับยาที่วัดได้จาก เลือด, กล้ามเนื้อ, ต่อมย่อย (hepatopancreas), และเปลือกกุ้ง ได้เป็นผลสำเร็จ การวิจัยนี้ พบว่า OTC ไม่ได้มีการสะสมอย่างมีนัยสำคัญในเนื้อกุ้ง และ hepatopancreas เป็นอวัยวะที่มีบทบาทสำคัญในการดูดซึมและกำจัด OTC ยาปฏิชีวนะดังกล่าว กระจายแบบทางเดียวจากเนื้อเยื่อส่วนปลายไปยังเปลือก ด้วยระบบไหลเวียนเลือดของกุ้งเป็นแบบเปิดโดยเลือดอาบเนื้อเยื่อโดยตรง จึงมีหลักฐานชี้ว่า OTC ผ่านจากเลือดไปที่เปลือกได้ OTC อาจรบกวนกระบวนการ biomineralization โดยไปจับเป็นสารประกอบเชิงซ้อนกับ calcium และ magnesiumในเลือดทำให้ระดับเกลือแร่ดังกล่าวในเปลือกต่ำลง ค่าชีวะหิตประโยชน์หลังจากกุ้งได้รับ OTC ในขนาดยาสูง ลดลงอย่างมากเนื่องจาก ยาละลายไม่สมบูรณ์ และ/หรือ การดูดซึมยาผิดปกติ นอกจากนี้ ยังพบหลักฐานที่ชี้ว่า ระดับ OTC ที่สูง สามารถเหนี่ยวนำให้เกิดการทำลาย hepetopancreas จึงมีผลทำให้เกิดการเปลี่ยนแปลงการกระจายและขับออกของยาในหลายๆเนื้อเยื่อของกุ้งขาวได้

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Pharmacokinetics of antimicrobial agent used in shrimp aquaculture

Abstract

The time course of the distribution/elimination of oxytetracycline (OTC) antibiotic was done on farmed Pacific White shrimp (*Litopenaeus vannamei*) with various dose levels including 10, 50, 500, 1,000, and 2,500 mg/kg body weight via both intra-vascular (10 mg/kg only) and oral routes. The pharmacokinetic profiles of the drug in hemolymph, muscle, digestive gland (hepatopancreas), and cuticle have been successfully modeled. It was found that OTC did not accumulate in the edible muscle, and that hepatopancreas played an important role in drug absorption and elimination. The antibiotic was unidirectional distributed from the peripheral muscle to cuticle. There was evidence that some OTC present in the hemolymph may transfer to the shell by partitioning as the hemolymph directly bathes the tissues. OTC may disturb the biomineralization process via complex formation with calcium and magnesium lowering the mineral contents in shell. The bioavailability after oral administration with extreme doses markedly decreased due to incomplete dissolution and/or mild dysfunction in absorption. In addition, there has been evidence that OTC in extremely high levels can induce damage to the shrimp hepetopancreas that effect changes to the distribution/elimination of the drug in several tissues.

Keywords: Pharmacokinetics, Shrimp aquaculture, Pacific white shrimp, *Litopenaeus vannamei*, Antimicrobials, Antibiotics, Oxytetracycline

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Pharmacokinetics of antimicrobial agent used in shrimp aquaculture

Executive Summary

The time course of drug distribution/elimination is always modeled by multi-compartments, mostly 2. In the beginning of pharmacokinetic studies of shrimp, several investigators borrowed human pharmacokinetic models to describe their data sometimes with success. As the physiology and anatomy of Penaeidae are totally different from advanced vertebrates, for instances the differences in their circulatory system (open *vs*. closed) and tissues/organs responsible for the drug metabolism/elimination, the models used to explain the pharmacokinetics in Penaeid shrimps should be different from those of humans. Recently, an appropriate pharmacokinetic model for oxytetracycline antibiotics has been developed that explains more accurately the behavior of the antibiotic in adult *P. vannamei.* There is now evidence that the drug does not accumulate in the edible muscle. Also that there should be a peripheral compartment that eliminates the drug instead of a process linked directly to the central compartment. This elimination or disposal compartment was related to the physiological tissues/organ namely the hepatopancreas. Further studies need to be carried out to further investigate this role of the hepatopancreas in eliminating the antibiotic as well as their distribution and accumulation in the shrimp shell.

The pharmacokinetics of an orally administered oxytetracycline antibiotics in Penaeid shrimps can be assessed with respect to both the extent and rate of absorption. The bioavailability and absorption rate constants that correspond to the extent and rate of absorption, respectively, have been commonly reported. The method to determine the former is model-independent but not the latter. As a result the obtained absorption rate parameter

depends very much on the pharmacokinetic model. Most of the studies adopted the pharmacokinetic models used for humans which might not be appropriate in cases of the application to Penaeid shrimps and have problems of validity for the estimates of the absorption rate parameter. Faroongsarng et al (2007) developed the 3-compartment "first pass" model with absorption via the hepatopancreatic compartment and successfully fitted their data with an estimate of the valid absorption kinetic parameter. In addition, it has been demonstrated that the fraction unabsorbed *vs*. time plot and the method of deconvolution could alternatively result in determining an apparent absorption rate constant with regard to the pharmacokinetic model utilized.

Toxicokinetics, a kinetic study of the drug from an extreme dose level, did exhibit signs of abnormal processes in the distribution and elimination of the drug before causing damage to tissues/organs. As there have been very few antibiotics permitted for use in shrimp farms, there is very little information available in the literature on the toxicity of these drugs except for oxytetracycline. Recently we have carried out a kinetic study of OTC in extreme dose levels. It was found that the pharmacokinetics of OTC in shrimp was altered after only a single exposure. There is now evidence that these high levels can induce damage to the shrimp hepetopancreas that effect changes to the distribution/elimination of the drug in several tissues. In addition, the abnormally high oxytetracycline levels present in the muscle and shell has to be considered when deciding safe wash-out periods.

เนื้อหางานวิจัย

1. Pharmacokinetic Modeling in Penaeid shrimp

A model is any representation of a system that accounts for the properties of that system. It is used to characterize and summarize a set of data into a few simple cohesive parameters that may allow a prediction to be made. For example, the Penaeid shrimp pharmacokinetic model describes the relationship between the dose of a given antibiotic and its concentration in various biological matrices into a series of simple parameters. One important example is an ability to estimate the elimination half life of a drug from the shrimp body to foresee the wash-out period before harvesting, so the drug residues might not be detrimental to humans. There are two groups of models utilized in these studies: one a compartment based and the other a physiological based model. Comparisons of the two models indicate that the compartment model is probably the more useful as it could solve many problems dealing with pharmacokinetics (Wagner, 1993). Almost all pharmacokinetic studies of antibiotics in Penaeid shrimps previously published have utilized compartment models (e.g. Park et al, 1995; Reed et al, 2004, 2006; Uno, 2004; Uno et al, 2006b, 2006b; Chiayvareesaja et al, 2006; and Faroongsarng et al, 2007). However, to address specific problems with regard to the specific distribution and disposition in tissues/organs, a physiological based modeling can be more useful. This chapter will discuss in some details of pharmacokinetic modeling in penaeid shrimp as well as what we have found in white shrimp, *L. vannamei*.

One-compartment open model with the drug after direct introduction to the circulatory system

In each of the biological bodies, the transfer of a drug is at best described by a first order rate process. However, the events such as the rate of drug absorption, distribution,

and elimination are always many factored. For instance, the amount of drug present at any one time may be determined by a variety of processes including drug dissolution and degradation due to enzymes and other gastrointestinal (GI) conditions, gastric emptying, and first pass metabolism. These multi-process events could be simplified to a single process governed by one rate constant, e.g. for the above particular example, the absorption constant. Together with the rate constants, for the parts of the body to which the drug is homogeneously distributed is assumed to be a compartment. As a result, the body is thus made up of one or more compartments based on the distribution of the drug, i.e., each compartment represents a body volume or a group of similar tissues or fluids into which a drug is distributed (Welling, 1986). Schematically, a pharmacokinetic model can be represented by a diagram including the compartment(s) and the arrow(s) which illustrate the various transport steps for the drug. The simplest one is a one-compartment open model with the drug being directly introduced to the circulatory system either by an intravenous or intra-sinus administration. This was first published in 1967 (Smith et al, Figure 1.1). The equation that describes the pharmacokinetic profile of a systemic drug is:

$$C = C_0 \cdot e^{-kt} \qquad (1.1)$$

Where C_0: the initial concentration is D_0/V. This model assumes that the dose of the administered drug distributes instantaneously in the volume V. Thus, the maximum concentration is present at zero time. Figure 1.2 shows a Penaeid shrimp's hemolymph pharmacokinetic profile with this type of model. Notice that there are two graphic presentations in Figure 1.2: a linear and a semi-logarithmic plot that show an exponential decay curve and a linear line, respectively. As the model is a mono-exponential function

(Equation 1.1), a semi-logarithmic plot yields the linear line which illustrates the single phase of distribution.

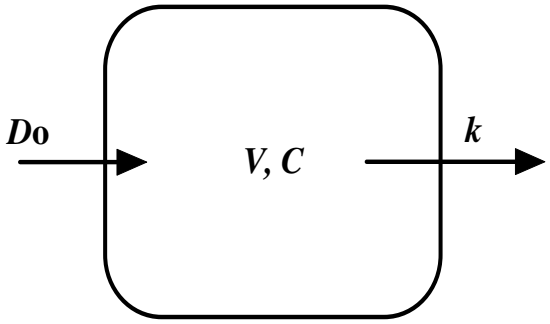

Figure 1.1 The diagram of a one-compartment model with intravenous administration where D_0, V, C, and k are the dose, volume of distribution, the drug concentration, and elimination constant, respectively.

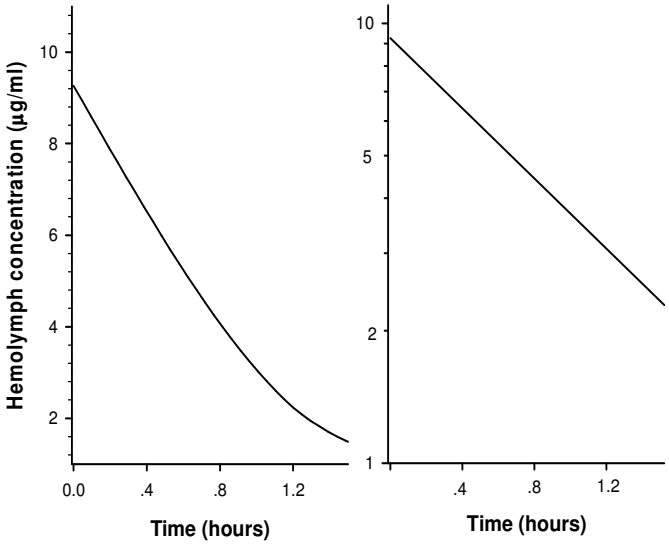

Figure 1.2 Linear vs. semi-logarithmic plot of a hemolymph pharmacokinetic profile representing the one-compartment model. The data were generated according to the very first pharmacokinetic profile of ometoprim in *P.vannamei* (Park et al, 1995).

Pharmacokinetic compartment models of the antimicrobials in Penaeid shrimps

To the best of my knowledge, there have been no pharmacokinetic studies of antibiotics in Penaeid shrimps reported in the literature that obey this one-compartment

model. Or in other words, the hemolymph pharmacokinetic profiles in Penaeid shrimps always exhibit a heterogeneity in which the data cannot be fitted into the one compartment model. Figure 1.3 shows some examples of these profiles for various antibiotics observed in Penaeid shrimp species. As can be seen each of the curves exhibits a refraction indicating that the drug is distributed in at least two phases, i.e., 2 compartments. Most of the studies have thus utilized a *classical* 2-compartment model to describe the data. The drug distribution in blood or hemolymph is usually referred as the *central* compartment whereas that in other tissues where the drug is distributed differently is the *peripheral* compartment. This model is schematically shown in Figure 1.4.

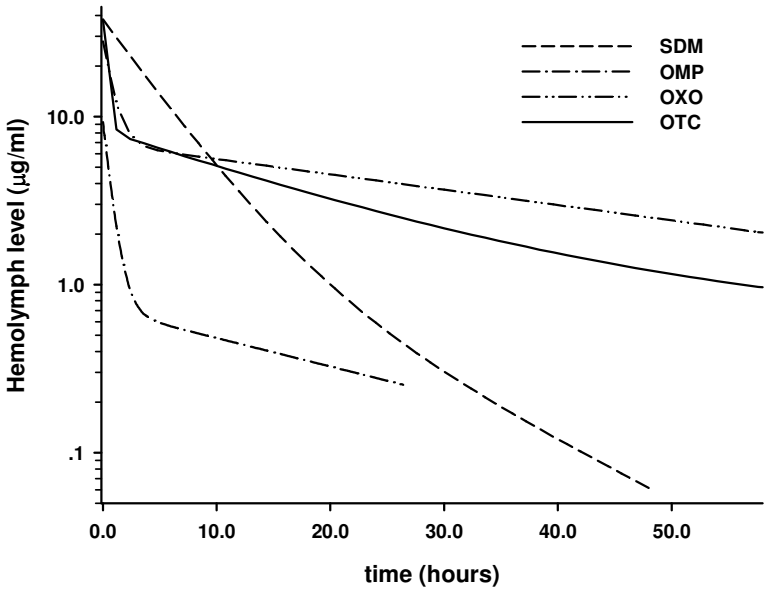

Figure 1.3 Hemolymph pharmacokinetic profiles of some antibiotics in Penaeid shrimp. Each curve was re-drawn using the authors' proposed models.

Key: SDM: sulphadimethoxine in *P. vannamei* (Park et al, 1995)
OMP: ormetoprim in *P. vannamei* (Park et al, 1995)
OXO: Oxolinic acid in *P. japonicas* (Uno, 2004)
OTC: Oxytetracycline in *L. vannamei* (Chiayvareesaja et al, 2006)

In this model (Figure 1.4) compartments 1 and 2 are always referred to as the central and peripheral compartments, respectively. The notations of each of first order fractional

rate constants (k's) include 2 numbers representing the compartments where the drug is from and to, for example, k_{12} refers to the rate constant of the drug being transported from compartment 1 to 2. Also k_{10} represents the elimination constant out of compartment 1. Once the dose of the drug: D_0 is introduced; it is instantaneously distributed into the volume V_C: the central compartment volume. The drug present in compartment 1 is then transported forwards and backwards between compartments 1 and 2 with the fractional rate constants of $k_{12}$and k_{21}, and was eliminated from compartment 1 by the fractional rate constant k_{10}. As a result the drug concentration in the hemolymph: C can be modeled with a bi-exponential function as:

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \tag{1.2}$$

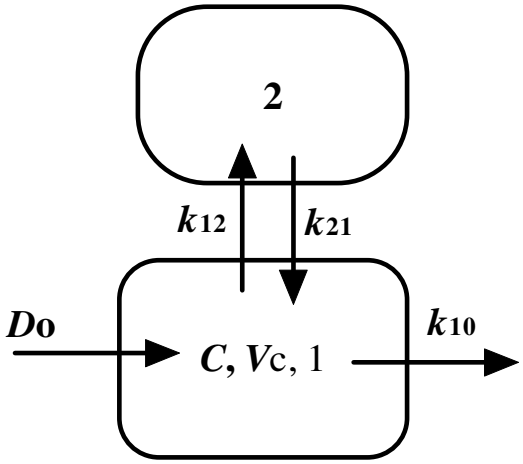

Figure 1.4 The schematic diagram of a classical 2-compartment open model after intra-sinus administration that is often utilized in antibiotic pharmacokinetic representations in Penaeid shrimp.

Where, A and B are coefficients that are defined as (Wagner, 1993):

$$A = \frac{D_0(k_{21} - \alpha)}{V_C(\beta - \alpha)}$$
$$B = \frac{D_0(k_{21} - \beta)}{V_C(\alpha - \beta)} \tag{1.3}$$

Many authors refer to the exponents: α and β as distribution and elimination rate constants which are not exactly correct as these exponents are compounded from several fractional rate constants (k's) as follows:

$$\alpha = \frac{1}{2}[k_{12} + k_{21} + k_{10} - \{(k_{12}+k_{21}+k_{10})^2 - 4k_{21}k_{10}\}^{1/2}] \quad (1.4)$$

$$\beta = \frac{1}{2}[k_{12} + k_{21} + k_{10} + \{(k_{12}+k_{21}+k_{10})^2 - 4k_{21}k_{10}\}^{1/2}] \quad (1.5)$$

The α and β phases should thus be referred to as the distribution and elimination *dominated* phases, respectively.

In order to obtain the coefficients and exponents of Equation 1.2, the hemolymph profile was fitted to the bi-exponential equation. Afterwards, the parameters of the model were estimated by Equations 1.6-1.9 (Wagner, 1993):

$$V_C = D_0/(A+B) \quad (1.6)$$

$$k_{21} = (A\beta + B\alpha)/(A+B) \quad (1.7)$$

$$k_{10} = \alpha\beta/k_{21} \quad (1.8)$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \quad (1.9)$$

Table 1 lists the model parameters of some antibiotics that were intra-sinusly administered to Penaeid shrimps. It is observed that the V_C for oxytetracycline (OTC) and oxolinic acid (OXO) in various Penaeid shrimps are comparable to the Penaeidaes' extracellular volume reported by Smith and Dall (1982) whereas the V_C values for sulphadimethoxine (SDM) and ormetoprim (OMP) in *P. vannamei* are far higher (Table 1.1). It is thus deduced that the extent of protein binding of OTC and OXO is not significant. However in the cases of SDM and OMP that exhibit high values for V_C, the

authors did not address the significance of protein binding but suggested a significant degree of extra-vascular distribution in the tissues that were part of the central compartment (Park et al, 1995).

Agent	Specie	V_C (L/kg)	k_{21} (h^{-1})	k_{12} (h^{-1})	k_{10} (h^{-1})	Reference
Oxolinic acid	*P. japonicas*	.36	.31	.81	.08	Uno, 2004
Sulphadimethoxine	*P. vannamei*	1.1	.09	.02	.20	Park et al, 1995
Ormetoprim	*P. vannamei*	4.6	.15	.93	.38	Park et al, 1995
Oxytetracycline	*L. vannamei*	.27	.72	2.3	.19	Chiayvareesaja et al, 2006
	L. setiferus	.64	.17	.11	.11	Reed et al, 2004
	P. monodon	.22	.85	2.6	.18	Uno et al, 2006b
	P. monodon	.20	.34	.35	.06	Sangrungruang et al, 2004
	P. japonicas	.16	.30	1.1	.14	Uno, 2004

Table 1.1 The 2-compartment model parameters of some intra-sinusly administered antibiotics into various Penaeid shrimp. If not reported in the literature, the parameter values were re-calculated using Equations 1.6-1.9.

Among the fractional rate constants, k_{10} indicates the disposition of the drug out of the hemolymph. It was found that the elimination half-lives of antibiotics from the hemolymph (compartment 1) varied among Penaeid shrimps in the range of approximately 2-12 hours that corresponded to a k_{10} of 0.38 h^{-1} for OMP in *P.vannamei* and 0.06 h^{-1} for OTC in *P.monodon* (Table 1). Moreover, k_{10} of the same drug and specie reported by different groups of authors were surprisingly different, e.g., OTC in *P. monodon* reported by Uno et al (2006b) was 3-fold greater that that reported by Sangrungruang et al (2004) while the V_C obtained by these 2 groups of workers was essentially identical (Table 1). The pharmacokinetic profiles of these two groups based on a 2-compartment model as proposed by both groups of authors are illustrated in Figure 1.5. It is observed on the one hand that the OTC level in the α-phase of the profile of Uno et al (2006b) corresponding to

approximately one and a half hours was dissipated more rapidly than that of Sangrungruang et al (2004) as the rate of the drug transport out of compartment 1 was far greater (e.g. k_{12} of 2.6 h^{-1} for Uno et al (2006b) *vs*. 0.35 $h^{-1}$for Sangrungruang et al (2004): Table 1). It could be because there was a difference in the animal conditions and in the forms of the drug, as well as in the experimental settings that had an effect on the OTC distribution. On the other hand, the decreased OTC in the β-phase is essentially identical (Figure 1.5) as the curves were parallel to each other. Referring to the elimination dominated phase, it is unlikely that the identical pattern of the β phase has a different elimination parameter. Thus the fractional transfer constants, especially one dealing with the drug disposition out of the hemolymph, should be interpreted with caution.

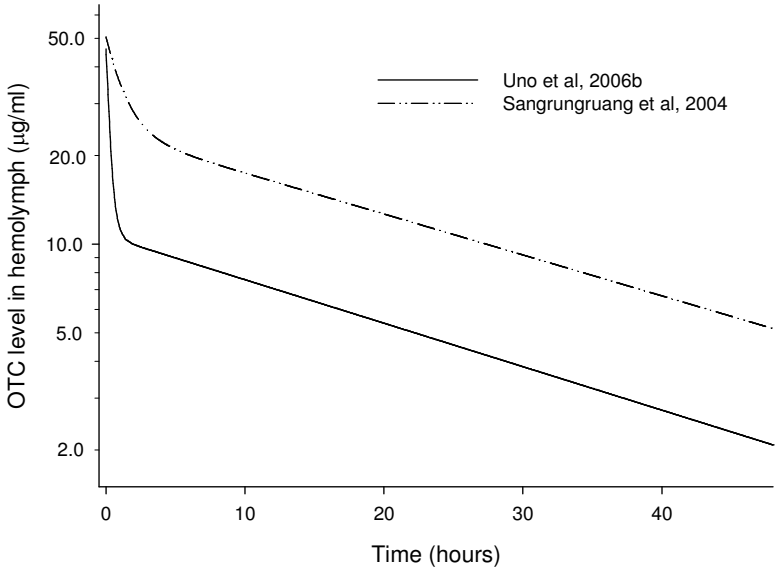

Figure 1.5 The 2-compartment pharmacokinetic modeled profiles of OTC intra-sinusly administered with 10 mg/kg body weight in P. *monodon* reported by 2 different groups of authors

Most of the compartmental pharmacokinetic studies in Penaeid shrimp used the model to describe their drug kinetics in central compartment but the drug residues in edible muscle which is always referred to peripheral one are of concern. Wang et al (2004) studied

on the residue elimination from muscular tissues of 3 antibiotics in shrimp *P. chinensis* including chloramphenicol (CAP), sulphamethoxazole (SMZ), and OTC. Instead of compartment model utilization, they simply fitted their data with a monoexponential equation discarding the very first beginning of the profiles where the drug level increased with time, and reported the elimination half-lives of OTC, CAP, and SMZ as 16.12, 10.04, and 5.68 h, respectively. These half-lives were only useful for the prediction of the "wash-out" time periods when the drugs reached the minimum residual levels allowing for safe consumption. But the data had no meaning in the pharmacokinetic modeling as the matter fact that shrimp's muscle, as the peripheral compartment, did not eliminate the drugs. A drug distributed into and out of muscular tissues, while it was centrally eliminated according to the compartment model. To demonstrate the examples of muscular pharmacokinetics, the model treatment of these antibiotics is revisited here. The concentration-time profile of each antibiotic in muscular tissues was taken from the literature (Wang et al, 2004) and, they were reanalyzed using this classical 2-compartment open model with the commercial software (Phoenix WinNonlin v. 6.1, Pharsight Co., USA).

Antibiotic	k_{01}**(SE)**	k_{10}**(SE)**	k_{12}**(SE)**	k_{21}**(SE)**	$\mathbf{R^2}$
CAP	0.071(.009)	2.273(1.268)	0.243(.068)	2.273(1.268)	0.9687
SMZ	0.122(.019)	1.586(1.067)	0.059(.019)	1.586(1.067)	0.9403
OTC	0.043(.005)	1.838(.054)	0.429(.142)	3.046(.897)	0.9954

Table 1.2 The estimates of fractional rate constants with their standard errors (SE) in parentheses obtained from data fitting according to the classical 2-compartment open model schematically described in Figure 1.4 with the pharmacokinetic profiles showed in Figure 1.6. k_{01} refers to the absorption constant as the antibiotics were orally taken into shrimp *P.chinensis* (Wang et al, 2004) whereas others were described previously.

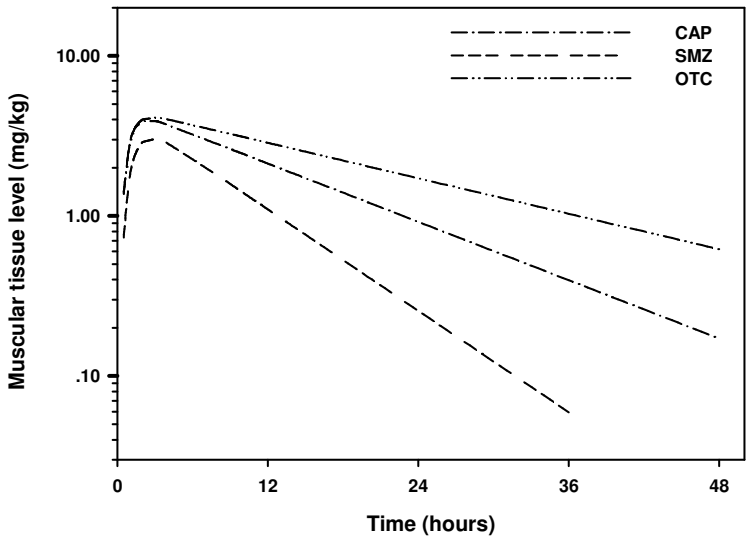

Figure 1.6 The muscular pharmacokinetic profiles of chloramphenicol (CAP), suphamethoxazole (SMZ), and oxytetracycline (OTC) in *P. chinensis* after orally fed with commercial medicated diet containing 2000 mg/kg of each antibiotic twice daily for 3 days. The data were taken from Wang et al (2004).

The muscular pharmacokinetic profiles according to the model fitting were illustrated in Figure 1.6 whereas their fitted parameters are tabulated in Table 1.2. It was found that the data were fitted into the model with very high correlations: r^2 of between 0.9403 and 0.9954. Among antibiotics, it was estimated that SMZ underwent fastest absorption (e.g. highest k_{01}) but slowest elimination (lowest k_{10}). CAP was the most quickly eliminated by shrimp *P. chinensis* while OTC was the most slowly absorbed. After multiple dosing when the drug reached steady state, the ratio: k_{12}/k_{21} is equal to the ratio of the extent to which the drug distribution into to that out of muscle. They were ranked from highest to lowest as OTC, CAP, and SMZ, respectively, which was in identical order of haft-live reported by Wang et al (2004) ranking.

The concept of the classical 2-compartment model describes the elimination of the drug from the body via k_{10} where the drug to be removed is present in compartment 1.This might not be the case in Penaeid shrimps as the evidence indicates that the digestive gland

(hepatopancreas) is responsible for elimination of the drug. Therefore the drug may be distributed differently than being in compartment 1 (Chiayvareesaja et al, 2006). This organ may include a *peripheral* compartment rather than a *central* one. Instead of a classical 2-compartment model, the Rowland 2-compartment model with peripheral elimination as described schematically by Figure 1.7 may be more appropriate. In this model, the drug is directly introduced to compartment 1. In compartment 1, the drug is instantaneously redistributed, and is transports forward and backward between compartments 1 and 2 with the fractional rate constants k_{12} and k_{21}. The drug is eliminated via compartment 2 with the fractional rate constant k_{20}. It is noted that the model of Figure 1.7 differs from that of Figure 1.4 only in the pathway of elimination located in a peripheral compartment (compartment 2).

From the coefficients and exponents of the bi-exponential function in Equation 1, the parameters of the model could be estimated by Equation 1.6 and 1.10 to 1.13 (Wagner, 1993).

$$F = (A\beta + B\alpha)/(A + B) = k_{21} + k_{20} \tag{1.10}$$

$$k_{12} = \alpha + \beta - F \tag{1.11}$$

$$k_{20} = \frac{\alpha \cdot \beta}{k_{12}} \tag{1.12}$$

$$k_{21} = F - k_{20} \tag{1.13}$$

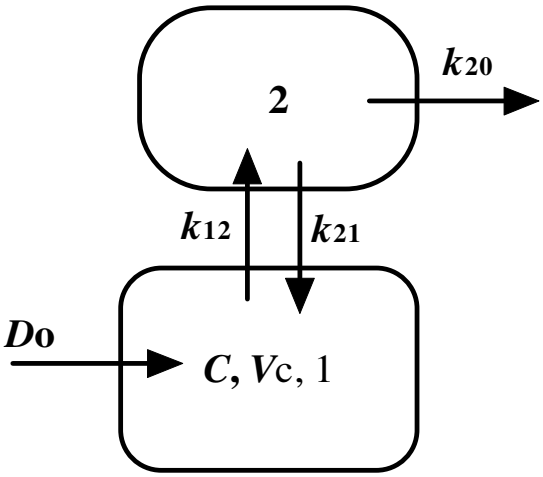

Figure 1.7 The schematic diagram of the Rowland 2-compartment open model with peripheral elimination and intra-sinus administration.

The Rowland 2-compartment model parameters were estimated especially for the OTC pharmacokinetics in *P. monodon* where two different profiles are seen in Figure 1.5 The elimination constant k_{20} for the profiles of Sangrungruang et al (2004) and Uno et al (2006b) were 0.059 h^{-1} and 0.056 h^{-1}, respectively, and show the same elimination characteristics. In addition, k_{20} for antibiotics in various Penaeid shrimps as listed in Table 1 were estimated to be in the range of between 0.028 h^{-1} and 0.084 $h^{-1.}$ This corresponds to an elimination half life of between 8 and 24 hours. This range of half-lives was consistent with the *practical* elimination half-lives for most antibiotics previously reported in a variety of Penaeid shrimps (e.g. Weifen et al, 2004; Faroongsarng et al, 2007; and Uno et al, 2006a).

More complicated compartment models

There has been a variety of the overall sampling time periods used in pharmacokinetic studies of antibiotics in Penaeid shrimps for monitoring the drug levels after administration. The shortest period was for 48 hours post dose (Sangrungruang et al,

2004), but most have used 120 hours (e.g. Park et al, 1995; and Uno et al 2006b). As the α-phase lasted only a couple of hours post dose, the pharmacokinetic profiles often exhibited 2 phases if the investigators sampling periods lasted for between 48-120 hours. However, Chiayvareesaja et al (2006) collected samples up to 504 hours post dose and found that the hemolymph profile of OTC in *L. vannamei* exhibited 3 phases rather than two. It had been demonstrated previously that a three-compartment model also best described the elimination of a lipophilic phenolic compound in for example the American lobster, *Homarus americanus* (Li & James, 1998). Thus the timing of the last sample might be important in order to obtain the entire profile. Although late samples can make only a small contribution to the overall pattern because the measured drug levels are very small, one could misinterpret the results if the data did not include the entire profile. Figure 1.8 shows the level-time profiles of OTC after intra-sinus administration into *L. vannamei* for up to 170 hours.This provided the profile of OTC levels with time not only in the hemolymph but also in the abdominal muscular tissues and in the digestive gland (hepatopancreas). The hemolymph profile exhibited three phases of OTC levels and the hemolymph concentration can be described by a tri-exponential function:

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + G \cdot e^{-\gamma \cdot t} \qquad (1.14)$$

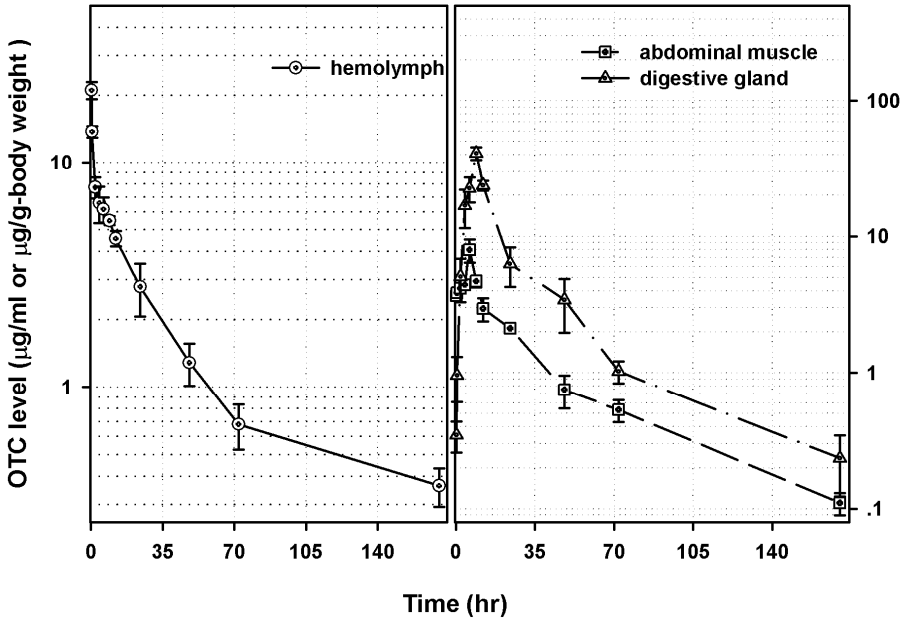

Figure 1.8 The OTC pharmacokinetic profiles up to 170 hours in the hemolymph, abdominal muscle and digestive gland of L. vannamei after intra-sinus administration at a dose level of 10 mg/kg-body weight.

It can be seen on the right-hand side of Figure 1.8, that the OTC levels in such *peripheral* compartments as the muscle and hepatopancreas are distributed differently. It is therefore deduced that the distribution of the drug in the hepatopancreas cannot be described by the *conventional peripheral* compartment (compartment 2 in two-compartment model). Chiayvareesaja et al (2006) modified the 2-compartment model by adding the elimination compartment (compartment 0) which is supposed to be the hepatopancreas in the model as illustrated in Figure 1.9. The authors have been successful in applying the model to the OTC kinetics in the hemolymph, muscle, and hepatopancreas. They also suggested that approximately 60% of the OTC intra-sinusly administered was disposed of via the hepatopancreas whereas the remaining portion was centrally eliminated via other route(s) displayed by the broken arrow in Figure 1.9.

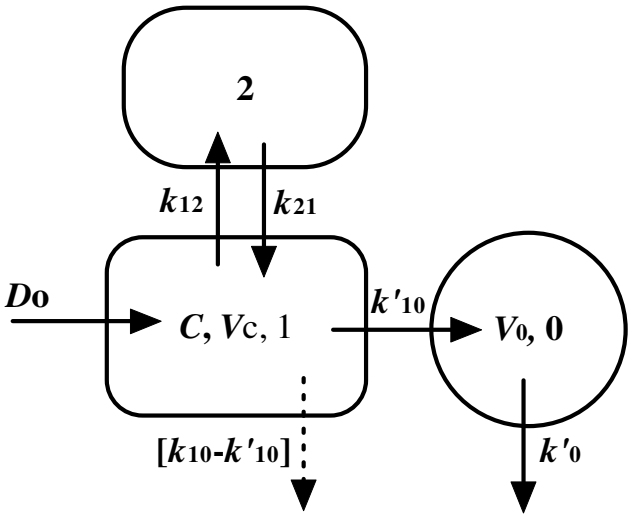

Figure 1.9 Schematic diagram of the 2-compartment model with an elimination compartment (compartment 0) for elimination of OTC in *L. vannamei* proposed by Chiayvareesaja et al (2006).

Although there are now three compartments, it should be noted that the diagram in Figure 1.9 is an incomplete 3-compartment model but is really only a *modified* 2-compartment one. In this model, the hepatopancreas was considered to be an elimination compartment analogous to the *open elimination* where the drug is transported between compartments 1 and 0 in a one-way fashion, i.e., the drug cannot travel back from compartment 0 to 1. The drug in compartment 0 is spontaneously and passively eliminated from the body. The elimination compartment is more or less isolated from the other two compartments. In a full three-compartment model the drug may be transported back and forth between compartment 0 and 1 as shown in Figure 1.10. The model in Figure 1.10 is said to be the first-pass 3-compartment open model with the drug being directly introduced to the circulatory system. As the drug is administered directly to compartment 1, it is instantaneously distributed in the compartment and is then transported back and forth between compartments 1 and 2 as well as between 1 and 0. The drug is then eliminated via compartment 0. The term: *first-pass* appears since when the drug was orally administered;

it is exposed to the elimination mechanisms prior to entering the circulatory system. The concentration C in the central compartment at time t is given by Equation 1.14. To estimate the parameters of the model, one needs to go through the algebraic calculations as follows, where K_0, K_1, and K_2 are turnover constants of compartments 0, 1, and 2, respectively. Other fractional rate constants refer to appear as in Figure 1.10.

Let $X = A/C_0, Y = B/C_0, C_0 = A + B + G, K_1 = k_{10} + k_{12} = g_1 - K_0 - K_2,$ $K_0 = k_{01} + k_{00} = (R + Q)/2,$ and $K_2 = k_{21} = (R - Q)/2$, and

$$g_1 = K_1 + K_2 + K_3 = \alpha + \beta + \gamma \tag{1.15}$$

$$g_2 = k_{10}(k_{00} + k_{21}) + k_{00}(k_{12} + k_{21}) + k_{01}(k_{12} + k_{21}) = \alpha\beta + \beta\gamma + \alpha\gamma \tag{1.16}$$

$$g_3 = k_{10}k_{00}k_{21} = \alpha \cdot \beta \cdot \gamma \tag{1.17}$$

$$R = K_0 + K_2 = X \cdot (\gamma - \alpha) + Y \cdot (\gamma - \beta) + \alpha + \beta \tag{1.18}$$

$$Q = K_0 K_2 = [\{X(\gamma - \alpha) + Y(\gamma - \beta) + \alpha - \beta\}^2 + 4Y(\beta - \alpha)(\gamma - \beta)]^{0.5} \tag{1.19}$$

Then,

$$k_{12} = \{g_3 - K_2(g_2 - K_1K_2 - K_0K_2)\}/\{K_2(K_2 - K_0)\} \tag{1.20}$$

$$k_{10} = K_1 - k_{12} \tag{1.21}$$

$$k_{01} = \{K_0K_1 + K_0K_2 + K_1K_2 - g_2 - k_{12}K_2\}/k_{10} \tag{1.22}$$

$$k_{00} = K_0 - k_{01} \tag{1.23}$$

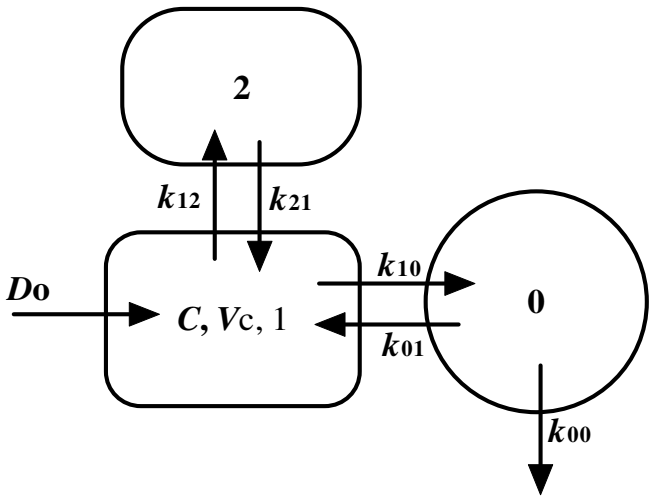

Figure 1.10 The schematic diagram of the first-pass three-compartment open model after intra-sinus administration.

Faroongsarng, et al (2007) utilized this model for their OTC pharmacokinetics in *P. vannamei* with a standardized molting stage of C-D_0 and estimated the elimination half-life via compartment 0: the *hepatopancreatic* compartment that corresponds to k_{00} in this model (Equation 23) to be 17.9$\pm$1.1 hours. The value appears to be an appropriate estimation as it is not only consistent with most other studies but it also is utilized in the updated corrected model.

Physiological based compartment model

Although the compartment models have been widely utilized for shrimp antibiotic kinetic studies, there do exist some limitations. Because many tissues having the same drug distribution are lumped together to form a compartment, the compartment model simply approximates the pharmacokinetic parameters and serves as a descriptive function with regard to the drug level-time profiles in such biological matrices. The parameters are only either overall or average values. They do not address the specific problems that relate to the actual time course of drug distribution, binding to cellular substances, and metabolism/elimination in a particular tissue and organ of interest (Welling, 1986). These limitations have led to the need for an alternative approach which the physiological based model could very well serve. The anatomical or physiological tissues/organs counted within the model are typically the tissues/organs that not only accumulate a considerable quantity of the drug but are also involved in the drug metabolism/elimination. In an advanced vertebrate, a *physiological* compartment is considered to be such an organ or a group of tissues of the same type including 3 well mixed phases referred as the vascular, interstitial, and intracellular sub-compartments (Gerlowski & Jain, 1983). However in Penaeid shrimp, the interstitial space is negligible due to their open circulation where their blood mostly

bathes the tissues directly. Thus, there may be only two, i.e., vascular (hemolymph) and cellular sub-compartments. Figure 1.11 is a schematic representation of a physiological compartment model as proposed for Penaeid shrimp. The drug in the hemolymph with concentration C_B enters the vascular sub-compartment by the flow of the hemolymph (Q) and has the vascular concentration C^{V} with volume V^{V}. The drug is then simultaneously transported from the vascular space to the intracellular space with a concentration C^{C} and volume V^{C} by the flux $n^{V\text{-}C}$. Notice that the drug may be transported back and forth between the sub-compartments and this may occur by either active transport or passive diffusion where C^{V} and C^{C} may be in equilibrium with each other. In addition, the drug may bind to endogenous substances, be degraded by metabolic reactions and/or be disposed. The specific binding may lead to either a therapeutic or toxic action of the drug. The mass balance equations on the drug in the vascular and intracellular sub-compartments are respectively written as:

$$V_i^{V}\frac{d\,C_i^{V}}{dt} = Q_i C_B - Q_i C_i^{V} - n_i^{V\text{-}C} \tag{1.24}$$

$$V_i^{V}\frac{d\,C_i^{C}}{dt} = n_i^{V\text{-}C} \tag{1.25}$$

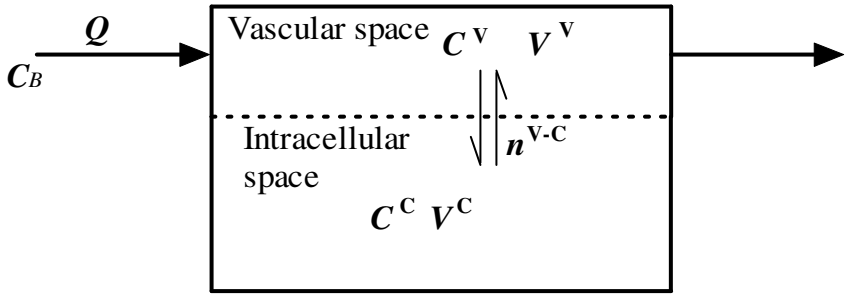

Figure 1.11 Schematic diagram of the vascular and intracellular sub-compartments included in a physiological compartment model as proposed for Penaeid shrimp. The substance transports back and forth across the dashed line representing the boundary of the sub-compartments by the flux, $n^{V\text{-}C}$.

It is noted that the diagram in Figure 1.11 illustrates only a single compartment. To produce a model with more than one compartment but interconnected through the body fluid systems. A mass balance of the drug is therefore made on the body fluid system which is basically plasma or blood to be close to the balance of the entire system (Gerlowski & Jain, 1983):

$$V_B \frac{d C_B}{dt} = -Q_B C_B + \sum_i Q_i C_i^{\mathrm{V}} + g(t) \qquad (1.26)$$

Where $g(t)$ is the drug input function. In practice, the concentration of a sub-compartment cannot be measured but only the total concentration C_I. A simplification needs to be made where one of the steps in the mass transfer is rate limiting. There are two cases: flow and membrane limitations where blood flow and transport by flux are rate limiting, respectively. It seems that the model for Penaeid shrimp might favor a flow limitation. This may be valid since the circulatory system of Decapods is classed as being open or more or less partially open (Dall et al., 1991). The term $n^{\mathrm{V\text{-}C}}$ in Equation 1.24 may be negligible and the equilibrium is instantly established yielding C^{V} equivalent to the total concentration C_I. Equation 1.24 then turns to Equation 1.27:

$$V_i \frac{d C_I}{dt} \approx Q_i C_B - Q_i C_I \qquad (1.27)$$

We developed a simple model that included two physiological based compartments: muscle and hepatopancreas to address the distribution/elimination of OTC in *P. vannamei* (see Output No.1). Because antibiotic residues in the shrimps' muscle has long been of primary concern because the residues cannot exceed the lowest values allowed for safe human consumption. It has been demonstrated that the hepatopancreas not only plays an

important role in antibiotic metabolism/elimination (Chiayvareesajja et al 2006) but also in its primary absorption (Verri et al, 2001, and Faroongsarng et al, 2007). These two organs/tissues of Penaeid shrimp are therefore the focus of the measurements. The model can be divided into 2 of the interested physiological compartments including muscle, and hepatopancreas denoted as *M*, and *L*, respectively. Together with the circulatory system that is represented by the hemolymph pool (*B*), a scheme of the OTC pharmacokinetic model based on physiology is shown in Figure 1.12. It is noted that the model also includes the shrimp's foregut. But, due to the fact that OTC is directly introduced into the hemolymph by intra-sinus administration, the foregut compartment is not involved

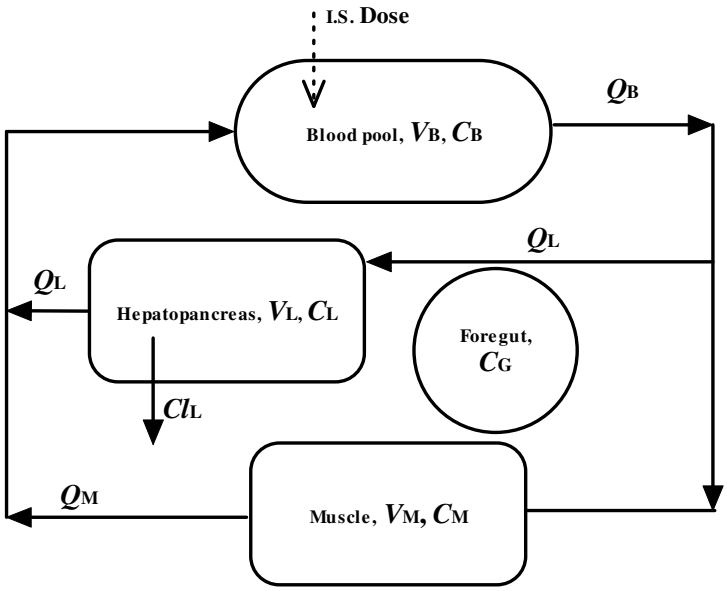

Figure 1.12 Schematic diagram of physiological based pharmacokinetic model after intra-sinus (I.S. Dose) administration as proposed by Faroongsarng et al (2009: Output No. 1).

The drug-time profiles in such a physiological based compartment model are governed on the basis of the blood flow rate denoted as Q_I through each physiological region (*I* is *B*, *M*, or *L*), and the relative affinity of the drug for the hemolymph and those other tissues R_I. By definition, the relative affinity R_I is the partition coefficient of the drug

while being distributing from the blood into tissues where the drug in the hemolymph leaving the tissues is rapidly in equilibrium with the drug within the tissues (Welling, 1986), i.e., $R_I = C_I/C_o$ where C_I and C_o are the total concentration in the tissue as previously denoted and the drug concentration in the blood leaving the tissues, respectively. As the hemolymph is conserved within the shrimp body, the blood flow rates are conditioned as: Q_B equals $Q_M + Q_L$. The law of mass balance describes the model by a set of 3 differential equations:

$$V_B \frac{d C_B}{dt} = -Q_B C_B + Q_M \frac{C_M}{R_M} + Q_L \frac{C_L}{R_L} \quad (1.28)$$

$$V_M \frac{d C_M}{dt} = Q_M C_B - Q_M \frac{C_M}{R_M} \quad (1.29)$$

$$V_L \frac{d C_L}{dt} = Q_L C_B - [\frac{Q_L + Cl_L}{R_L}] C_L \quad (1.30)$$

Where, C_I, and V_I are the total drug concentration and volume of distribution for each compartment, respectively. Cl_L is a drug clearance parameter that is assumed to occur in the hepatopancreas (hepatopancreatic clearance). Notice that Equation 1.28 is a special case of Equation 26 whereas Equations 29 is similarly a special case of Equation 1.27, and, Equation 1.30 is a modification of the Equation 26 type where the organ is involved in the drug clearance.

When it came to the estimation of the parameters of the model, it is not possible to do any differential equation regression without any data manipulation since $\frac{d C_I}{dt}$ could not be directly measured but C_I and the sampling intervals are too large to obtain the valid differential C_I with infinitesimal time. A numerical interpolation of the concentration-time profile during each time interval was necessary so that $\frac{\Delta C_I}{\Delta t}$ which is $\frac{(C_{I,n+1} - C_{I,n})}{(t_{n+1} - t_n)}$ could

approach $\frac{dC_I}{dt}$ when Δt approached zero, i.e., $\frac{(C_{I,n+1} - C_{I,n})}{(t_{n+1} - t_n)} \approx \lim_{t \to 0} \frac{\Delta C_I}{\Delta t} \approx \frac{dC_I}{dt}$. The numerical data were finally fitted to the differential equations of the physiological based model (Equations 1.28-1.30). With this technique, Faroongsarng et al (2009) were able to determine the model differential equations (multiple correlation: R^2) as:

$$\frac{d C_B}{dt} = -0.552 \cdot C_B + 0.396 \cdot C_M + 0.036 \cdot C_L \, (R^2 = 0.9373) \quad (1.28a)$$

$$\frac{d C_M}{dt} = 0.164 \cdot C_B - 0.198 \cdot C_M \, (R^2 = 0.8266) \quad (1.29a)$$

$$\frac{d C_L}{dt} = 0.412 \cdot C_B - 0.105 \cdot C_L \, (R^2 = 0.6571) \quad (1.30a)$$

By comparisons of the coefficients obtained between Equations 1.28 and 1.28a, 1.29 and 1.29a, and 1.30 and 1.30a, the authors could estimate the relative affinities of OTC: R_M and R_L. The model was able to successfully address how the drug was distributed in the muscular and hepatopancreatic tissues. The authors also suggested that OTC did not accumulate in the edible muscle whereas the drug present in the hepatopancreas was extensive. The value of R_M determined by Faroongsarng et al (2009) is comparable to those in advanced vertebrates such as rats (tetracycline antibiotics' R_M of 1.03-2.9: Poulin and Theil, 2000). It was also evident that OTC may be eliminated via the hepatopancreas. In addition, it was demonstrated that drug in the hepatopancreas may undergo first pass elimination with non-linear kinetics which seemed to be consistent with the elimination of drugs via the liver in advanced vertebrates.

Compartment versus physiological based model: An example of model fitting

To demonstrate that the pharmacokinetic model would fit the data, a set of OTC levels in the hemolymph taken at time intervals up to 170 hours using adult *P. vannamei* after intra-sinus dosing at 10 μg/g body weight was fitted into the models described in

Figures 1.9 and 1.11 using commercial software (Phoenix WinNonlin v. 6.1, Pharsight Co., USA). The obtained parameters are listed in Table 1.3. The data were successfully fitted into both models with acceptable standard errors of the estimates (S.E.). Unfortunately the software was provided with only a hemolymph level-time profile, so the volumes of the peripheral compartments 0 and 2 identified in Figure 1.10 could not be estimated but the volumes of blood V_B, hepatopancreras V_L and muscle V_M were determined using Figure 1.12. The extent to which the estimation of the volumes for V_B, V_L, and V_M is valid is questionable as they are based on volumes that were not actually measured. V_B in Table 1.3 is comparable to the estimates of the physiological blood volume of various crustaceans that have been previously reported (Smith & Dall, 1982; and Hurton et al, 2005). Thus it can be said that V_B might be very close to the actual hemolymph volume of the experimental animals. On the other hand, the estimated V_L was as high as 0.2415 mL/g BW and the hepatopancreas was less than 3% of the total wet weight; i.e., about 0.03 mL/kg BW (Barclay et al, 1983). There was also a large discrepancy in the value of V_M (3.4412 mL/g BW) as this far exceeds the volume of the shrimp's body. Note that these volumes are based on the distribution of OTC in particular tissues. Protein and other tissue binding sites and biotransformations of the drug may influence the values of the estimates.

In Figure 1.10: the first-pass 3-compartment open model with intra-sinus administration, each of the compartments interconnects to each other by an in and out transport process of the drug under study. In comparison with Figure 1.12: the physiological based model with systemic dosing and hepatopancreatic elimination, they are more or less similar in the sense of there being a number of tissue compartments and the drug being transport back and forth between them. The drug movement of the former is assumed to be first order kinetics whereas that of the latter is governed by the blood flow

rate. They both share a common principle in that the rate is dependent upon the level of the drug from where it is. Presumably, analysis of the models' rate constants reveals that the fractional rate constant (k_{ij}) of the compartment model might be equivalent to the corresponding blood flow rate (Q_I) of the physiological-based model divided by its volume. This is valid only if the distribution of the drug is negligibly affected by other pharmacokinetic factors such as protein/tissue binding and biotransformation/metabolism. It is observed from Table 1.3 that the blood flow rate of OTC into the hepatopancreas denoted as Q_L divided by V_B yields the value (S.E.) of 2.4611 (0.0113) h^{-1}, and that into the muscular tissues Q_M divided by V_B is 0.0899 (0.0065) h^{-1}. The numbers might be statistically identical to k_{10} and k_{12} of the compartment model (Table 1.3), respectively. Furthermore, the flow rate from the hepatopancreatic and muscular tissues Q_L/R_L and Q_M/R_M when divided by V_L and V_M equals 0.7238 (0.0058) h^{-1} and 0.0114 (1.2194) h^{-1}. Again, these numbers are not statistically significantly different from k_{01} and k_{21}. This evidence emphasize that OTC pharmacokinetics is not affected by protein binding and/or metabolism.

First-pass 3-compartment open model			**Physiological-based model**		
Parameter	Estimate	*S.E.	Parameter	Estimate	S.E.
V_C (ml/g BW)	.2678	.0053	V_B	.2678	.0031
			V_L (ml/g BW)	.2415	.0038
			V_M	3.4412	1.2192
k_{10} (h^{-1})	2.4615	.0823	Q_L (ml h^{-1}/g BW)	.6591	.0109
k_{01} (h^{-1})	.7244	.0182	Q_L/R_L(ml h^{-1}/g BW)	.1748	.0044
k_{12} (h^{-1})	.0900	.0213	Q_M(ml h^{-1}/g BW)	.0241	.0057
k_{21} (h^{-1})	.0115	.0061	Q_M/R_M(ml h^{-1}/g BW)	.0391	.0208
k_{00} (h^{-1})	.0387	.0072	Cl_L/R_L(ml h^{-1}/g BW)	.0093	.0006

*Standard error of the estimate

Table 1.3 Pharmacokinetic parameters of the first-pass 3-compartment open model illustrated in Figure 1.10 compared with the physiological based ones in Figure 1.12 of a hemolymph level-time profile after intra-sinus administered of 10 µg/g body weight OTC to adult *P. vannamei*.

The model for antibiotic distribution in the shrimp exoskeleton

There is evidence that an amount of OTC antibiotic has been detected in shrimp shell (Uno et al, 2006) even after receiving a dose of as low as 10 mg/kg (Uno et al, 2010). An abnormal soft exoskeleton was observed in *L. vannamei* fed with the maximum recommended therapeutic dose of OTC as well as those in excess of this dose level (Bray et al, 2006). It may be a specific characteristic of this antibiotic that the drug formed a complex with calcium and magnesium ions which build up the strength of the shell. As a result these minerals might be deficient and limit the formation of cuticle during molting. Uno et al (2010) reported the distribution profile of OTC in the shrimp shell but without a full kinetic description. In the profiles, the drug level immediately increased and, with no evidence of an elimination phase, persisted at concentrations of approximately 0.7 and 2.0 μg/g for more than 5 days following oral administration at doses of 10 and 50 mg/kg, respectively.

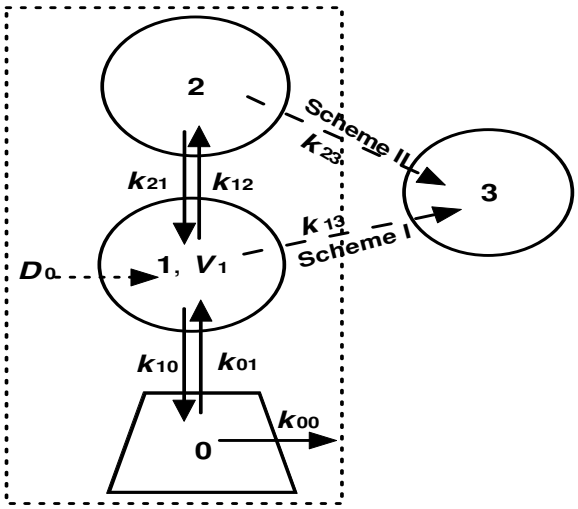

Figure 1.13 The proposed compartment model for OTC distribution in shrimp shell. Scheme 1 represents the distribution of OTC from the central compartment (compartment 1). Scheme II represents that from the peripheral *muscular tissues* compartment (compartment 2). Where compartment 0 and 3 represent the first-pass *hepatopancreatic* elimination compartment and *shell* compartment, respectively. k_{ij}'s are a fractional constant for inter-compartmental transfers.

Exuviations represent a net loss especially from the shell materials such as chitin and minerals. The shrimp exoskeleton is shed from time to time. Together with old cuticle, OTC in the shell might also be disposed of. Faroongsanrg et al (see Output No. 3) hypothesized the unidirectional distributions of OTC into the shrimp shell with two possible sources: from the hemolymph and peripheral tissues mainly muscle. Hemolymph serves as the blood circulation (compartment 1 in Figure 1.13) which supplies essential substances such as oxygen, nutrients, hormones, and cytokines as well as compounds dissolved in it including the drug. While the epidermis, a peripheral tissue connecting the muscle to the exoskeleton, is a part of the sampled muscular tissues (compartment 2), and might play a storage role to provide supplies for the synthesis of cuticle (Dall et al, 1990). OTC might be transported via these storage cells that differentiate from the epidermis during the D stage of the molting cycle into a new cuticle that possibly makes compartment 2 to be a source of OTC. Figure 1.13 provides a diagrammatic representation of the compartmental model for OTC distribution into the shell. The model is developed based on the first-pass 3-compartment open model previously proposed by Faroongsarng, et al (2007: a dash-line box in Figure 1.12). There are 2 schemes: Scheme I represents the distribution of OTC from the central *hemolymph* compartment (compartment 1) whereas Scheme II represents that from the peripheral *muscular tissue* compartment (compartment 2). It was demonstrated by groups of adult *L. vannamei* standardized at the C-D_0 stage of molting dosed with 500 and 1,000 mg/kg BW OTC that scheme II was a major pathway with a first order distribution process (Faroongsarng et al, Output No 3). In addition, there was evidence that some OTC present in compartment 1 may transfer to the shell. As the hemolymph directly bathes the tissues, the drug becomes distributed between the two. Or in

other words, OTC in compartment 1 transported to shell in scheme I via partitioning rather than the first order distribution process. Apparently, OTC in compartment 1 contributed to the shell level when the systemic concentration was high enough to allow for a sufficient amount of the drug to partition when it occurred very soon after the OTC was administered while the OTC contributed from the peripheral compartment was not yet fully in operation.

2. Oral absorption analyses in Penaeid shrimp

In shrimp aquaculture, disease preventing agents are usually fed to the animals orally with their food. Only a portion of the dose would enter the shrimp's body due to the fact that not only does some food remain uneaten but the orally administered drug needs to be absorbed via the gastro-intestinal (GI) tract whereas in the mean time it may be in forms that either are not ready to be absorbed, or they are metabolized and degraded. In a natural feeding process, a portion of the agent entering the shrimp's systemic circulation may be as low as 10% (Limpoka et al, 1993). After the drug is taken orally, there are a number of pharmacokinetic processes that occur including absorption, distribution, biotransformation, and elimination. This chapter will focus on the absorption process. It is noted that not only is the amount of the drug critical but the rate to which the drug is available systemically is of importance. The extent of absorption is normally evaluated based on the area under the curve of a pharmaco-kinetic profile (*AUC*). While there have been a number of methods to evaluate the rate of absorption including curve-fitting, an amount absorbed-time plot, the method of data deconvolution, and from a mathematical intercept method (Gibaldi and Perrier, 1982).In this work an assessment of the extent as well as the rate of dispersal of disease preventing agents has focused on oxytetracycline (OTC) antibiotics entering the shrimp body. Only previously reported methods used for Penaeid shrimps will be discussed.

Bioavailability

In order to achieve the disease preventing goal, where the systemic drug is available at a therapeutic level, it is important to assess the amount of drug entering the shrimp body.

The amount of the drug available systemically via absorption after oral administration can be evaluated by comparing the area under the curve (*AUC*) of a drug concentration-time pharmacokinetic profile with that of the drug that has been directly introduced into the systemic circulation. As 100% of the dose of the drug is present systemically when introduced by intra-sinus administration, the so-called "*Bioavailability*" of the drug administered via a route that requires the process of absorption such as orally can be determined by comparing its *AUC* with that after intra-sinus administration of the same drug in a similar amount as a reference. Equation 1 mathematically illustrates the determination of bioavailability denoted as *F* for the drug orally administered.

$$F = \frac{AUC_{oral}^{0\rightarrow\infty}}{AUC_{IS}^{0\rightarrow\infty}} \cdot \frac{D_{IS}}{D_{oral}} \qquad (2.1)$$

Where $AUC_{oral}^{0\rightarrow\infty}$ and $AUC_{IS}^{0\rightarrow\infty}$ are areas under the curve to infinite time of the hemolymph level-time profile after oral administration and that after one administered intra-sinusly. D_{IS} and D_{oral} are the amount of the drug dose for intra-sinus and oral administration, respectively. The *AUC*s need to be normalized for doses as sometimes the amounts of the drug administration are not equal. *F* sometimes refers to the fraction of the drug absorbed. Since the orally administered drug may enter the hepatopancreas which is considered to be an important organ for eliminating drugs from Penaeid shrimp, some might be eliminated prior to its presence systemically. This is usually referred to as *first-pass* elimination. The fraction of the drug absorbed is then governed by the extraction ratio (*ER*) of the drug after first passing through the hepatopancreas:

$$F = f_{GI}(1 - ER) \qquad (2.2)$$

The notation f_{GI} refers to the fraction of the drug ready for absorption. Notice that all of the drug could not be readily absorbed due to incomplete dissolution, and the instability of the drug when exposing to the GI conditions. *ER* includes the fractions of both the metabolized and eliminated (without metabolism) drug.

Table 2.1 summarizes some oral bioavailability data previously obtained for shrimp species. It can be seen that oxytetracycline (OTC) has been a focus of these studies due to its popularity as an antibiotic approved by the FDA for disease control in aquaculture. The OTC bioavailability values calculated based on Equation 1 have varied among species in the range of between 35 and 90%. It is noted that all the studies used a single dose administration and as a bioavailability study requires an accurate dose, all of the agents in Table 1 were introduced to shrimps by force feeding with no portion of the drug being uneaten.

Specie	**Agent (*dose in mg/kg body weight)**	**Bioavailability (%)**	**Reference**
Macrobracium rosenbergii	OTC (22)	64.7	Poapolathep et al (2008)
Penaeus vannamei	OTC (50)	80.6	Faroongsarng et al (2007)
Litopenaeus setiferus	OTC (100)	49.5-92.2	Reed et al (2006)
P. monodon	OA (50)	7.9	Uno et al (2006a)
P. monodon	OTC (50)	35.6	Uno et al (2006b)
P. monodon	OTC (10)	59.9	Sangrungruang et al (2004)
P. japonicas	OTC (50)	43.2	Uno (2004)
	OA (50)	32.9	
P. vannamei	SDM (210)	30	Park et al (1995)
	OMP (42)	32	

OTC: Oxytetracycline, OA: Oxolinic acid, SDM: Sulphadimethoxine, OMP: Ormetoprim
*Shrimp were dosed orally by force feeding.

Table 2.1 The summary of some oral bioavailability data carried out on cultured shrimp species.

Based on the *AUC*s data, Faroongsarng et al (2007) and Chiayvareesaja et al (2006) showed that the fractions of OTC that were distributed to the *P. vannamei*'s hepatopancreas after oral and intra-sinus administrations were approximately 0.8 and 0.6, respectively. Assuming the drug clearance solely happens in this organ, one could determine that the *first pass* extraction ratio (*ER* in Equation 2.2) is approximately 0.8 minus 0.6, i.e., 0.2. The authors also reported that the bioavailability of OTC after oral administration in *P. vannamei* calculated by Equation 2.1 was 80.6% (Faroongsarng et al, 2007). The numbers from Equation 2.1 and 2.2 are consistent where f_{GI} in Equation 2.2 approaches unity, i.e., the unabsorbed drug due to incomplete dissolution and instability during passing the digestive tract of the animal is negligible. This demonstrates the success in formulation of the medical feed in their experiments. In addition, it has been reported that the oral bioavailability of the tetracycline antibiotics in humans was approximately 75% (Barr et al, 1972). This is comparable to what several authors have found in Penaeid shrimp (Table 2.1).

Kinetics of absorption in compartment models

In an analogous way to the situation to absorption of drugs in humans, several authors have treated the oral absorption kinetics of the disease preventing agents in Penaeid shrimps as an apparent first order process (Park et al, 1995; Sangrungruang et al, 2004; Reed et al, 2006; and Faroongsarng et al, 2007). Together with the absorption kinetics, the compartment models that best described their pharmacokinetic profiles after oral administration were applied. Based on data fitting, a variety of compartment models were reported including one-compartment (Park et al, 1995; and Sangrungruang et al, 2004), two-compartment (Reed et al, 2006), and three-compartment with first pass elimination

models (Faroongsarng et al, 2007). On the other hand, some authors were reluctant to use the compartment models to describe their oral pharmacokinetic profiles even though they successfully modeled with these profiles after intra-sinus administrations of the same drugs in the same Penaeid species (Uno, 2004; Uno et al, 2006) because the errors of fitting were unacceptably high.

As previously discussed, a compartment model treats the part of the body where the drug is homogeneously distributed into a compartment. The body may be made up of one or more compartments based on the drug distribution. The input and elimination processes may or may not be to and from the same compartment but, they were located in a central compartment for most of the models. The schemes of the compartment models utilized in the oral pharmacokinetic studies of antibiotics in Penaeid shrimps share common features with those used for intra-sinus administrations as discussed in a previous chapter and which differ only in the input process. Figure 2.1 illustrates the scheme of the simplest one-compartment open elimination model with an absorption rate constant k_a and an elimination rate constant k_{el}.

The integrated equation describing the drug concentration C for the model is as follows:

$$C = \frac{F \cdot D \cdot k_a}{V(k_a - k_{el})}[\exp(-k_{el} \cdot t) - \exp(-k_a \cdot t)] \qquad (2.3)$$

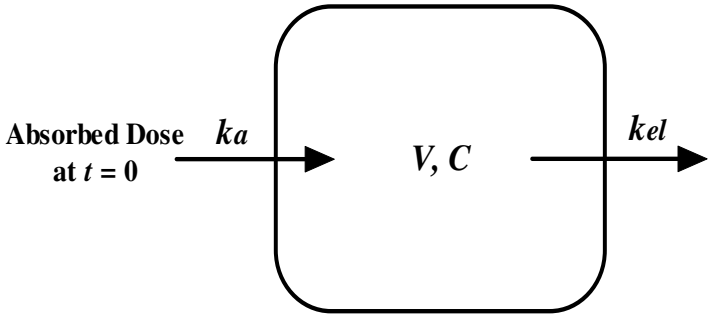

Figure 2.1 A schematic diagram for a one-compartment open model with absorption. Where, *F* is the fraction of the dose *D* that reaches the circulation, i.e., oral bioavailability and, *V* is the volume of distribution. This model was used in Penaeid shrimp pharmacokinetic studies after oral administration of oxytetracycline (OTC in *P. monodon*: Sangrungruang et al, 2004), and Sulphadimethoxine and Ormetoprim (SDM and OMP in *P. vannamei*: Park et al, 1995). Figure 2.2 shows the pharmacokinetic profiles of these antibiotics after oral administration to Penaeid shrimp. Notice that the profiles are re-drawn from the equations proposed in the literature. According to the model, the distribution of the drug is so homogenous that the drug levels in the hemolymph could represent those of the whole body. Two parameters involved with the drug uptake include the fraction of absorption *F* discussed earlier and the absorption constant k_a. A variety of k_a values of this model were reported ranging between 0.138 h^{-1} (OTC in *P. monodon*: Sangrungruang et al, 2004) and 2.00 h^{-1} (OMP in *P. vannamei*: Park et al, 1995) and corresponded to the time required for half of the given drug to be absorbed into the systemic circulation of 0.5 - 5 hours. Notice also that these authors utilized a one-compartment model for oral administrations whereas they modeled those of the intra-sinus admimistration as a two-compartment model.

It is difficult to see how a one compartment model for the disease preventing agents given orally can apply to Penaeid shrimp. Most investigators dealing with intra-sinus administration successfully used a two-compartment model for their pharmacokinetic schemes whereas none of them used a 1-compartment model. It does not seem logical to treat the data differently since the same drug is introduced to identical species so should exhibit similar pharmacokinetics even though the drug is given by different routes. Thus, a 2-comparartment treatment for the orally administered drugs might be more appropriate. Reed et al (2006) successfully fitted their pharmacokinetic data of various forms of OTC orally administered to *L. setiferus*. Figure 2.3 and 2.4 show a schematic diagram of their model and their pharmacokinetic profiles, respectively. The integrated equation describing the drug concentration *C* in the central compartment for the model is as follows:

$$C = \frac{F \cdot D \cdot k_a}{V_c}\left[\frac{(k_{21}-\alpha)}{(\beta-\alpha)(k_a-\alpha)}e^{-\alpha \cdot t} + \frac{(k_{21}-\beta)}{(\alpha-\beta)(k_a-\beta)}e^{-\beta \cdot t} + \frac{(k_{21}-k_a)}{(\alpha-k_a)(\beta-k_a)}e^{-k_a \cdot t}\right] \quad (2.4)$$

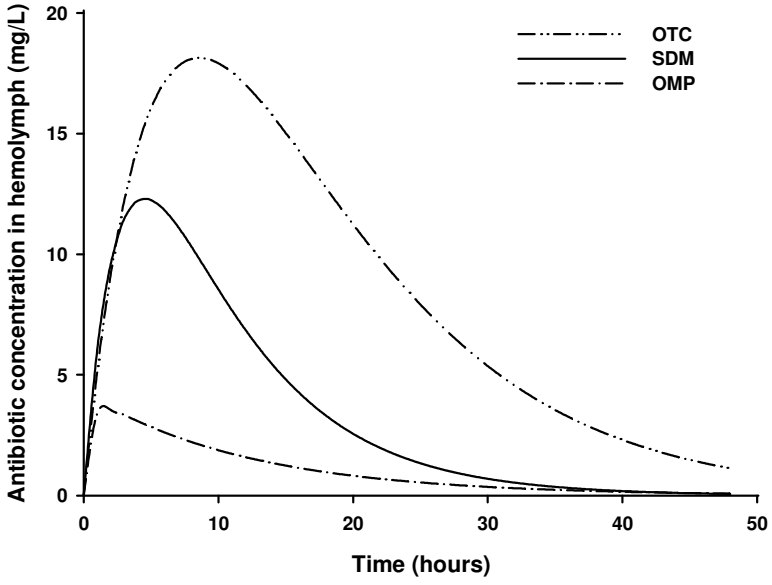

Figure 2.2 The one-compartment modeled pharmacokinetic profiles of oxytetracycline (OTC), Sulphadimethoxine (SDM) and Ormetoprim (OMP). The profiles are simulated according to the fitted equations as proposed by Sangrungruang et al (2004) and Park et al (1995).

Where V_c is the volume of the central compartment. Other parameters are defined in the previous chapter of Pharmacokinetic Models. It is noted that those parameters are also illustrated in Figure 2.3. Equation 2.4 and may be written for the sake of data fitting as:

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + I \cdot e^{-k_a \cdot t} \quad (2.5)$$

Where;

$$A = \frac{k_a FD(k_{21} - \alpha)}{V_c(\beta - \alpha)(k_a - \alpha)}; B = \frac{k_a FD(k_{21} - \beta)}{V_c(\alpha - \beta)(k_a - \beta)}; I = \frac{k_a FD(k_{21} - k_a)}{V_c(\alpha - k_a)(\beta - k_a)} \quad (2.6)$$

Using the coefficients (A, B, and I) as well as exponents (α, β, and k_a) from a non-linear fitting of data to Equation 2.5, the model parameters may be obtained from Equations 2.7-2.10 (Wagner, 1993).The reader should compare these Equations with those of 1.6-1.9 of the same model but after intra-sinus administration as described in a previous chapter.

$$\frac{V_c}{F} = \frac{k_a D}{A(k_a - \alpha) + B(k_a - \beta)} \quad (2.7)$$

$$k_{21} = \frac{B\alpha k_a + A\beta k_a + I\alpha\beta}{B(k_a - \beta) + A(k_a - \alpha)} \quad (2.8)$$

$$k_{10} = \alpha\beta / k_{21} \quad (2.9)$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \quad (2.10)$$

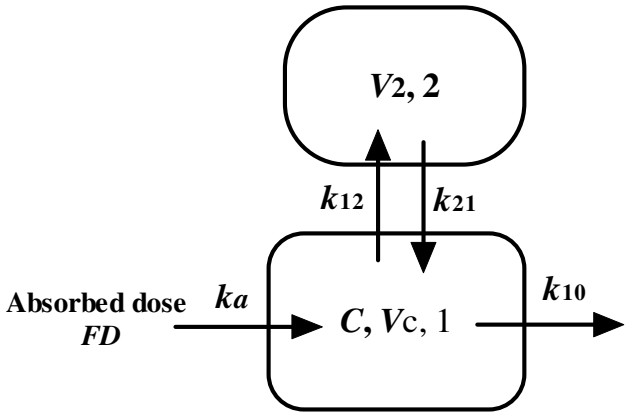

Figure 2.3 Scheme of a two-compartment open elimination absorption model with first order kinetics utilized by Reed et al (2006).

The k_a's are much slower when compared to the 1-compartment treatment with the time required for half of a given dose to be absorbed being between 5-14 hours (Reed et al, 2006). In contrast while the authors found that the apparent bioavailability was as high as 92%, the estimation of the absorption rate using this model seems to be too slow to be realistic. In addition, the estimated time needed for the drug to be systemically available corresponded to the absorption rate constant and was far greater than the gastric emptying time after food ingestion in a variety of Penaeid species (Dall, 1967; Marte, 1980; and Soares et al, 2005). This may imply insufficient absorption presumably exhibiting a low bioavailability rather than what was reported by these authors. Thus, the pharmacokinetics of an antibiotic agent in Penaeid shrimp following a 2-compartment model with central absorption and elimination seems less likely.

Most models include the input and elimination processes to and from a central compartment, i.e., systemic circulation. This is similar to the human pharmacokinetics as these compartment models used for Penaeid shrimps were those adopted from human studies. Faroongsarng et al (2007) addressed some of the limitations of extrapolating the human model leading to misinterpretation of the obtained pharmacokinetic parameters. They then proposed a 3-compartment model where the drug absorption and elimination did not occur to and from the central compartment (compartment 1) but from the hepatopancreatic compartment. The scheme of this model is illustrated in Figure 2.5.

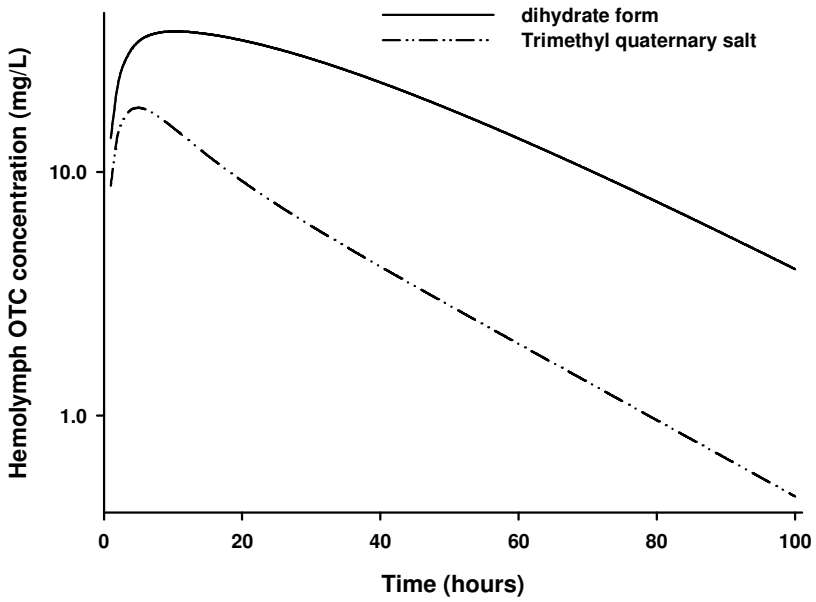

Figure 2.4 Pharmacokinetic profiles of 2 forms of oxytetracycline (dihydrate and trimethyl quaternary salt) orally administered to *L. setiferus*. The profiles are redrawn from the literature (Reed et al, 2006) according to the model shown in Figure 2.3.

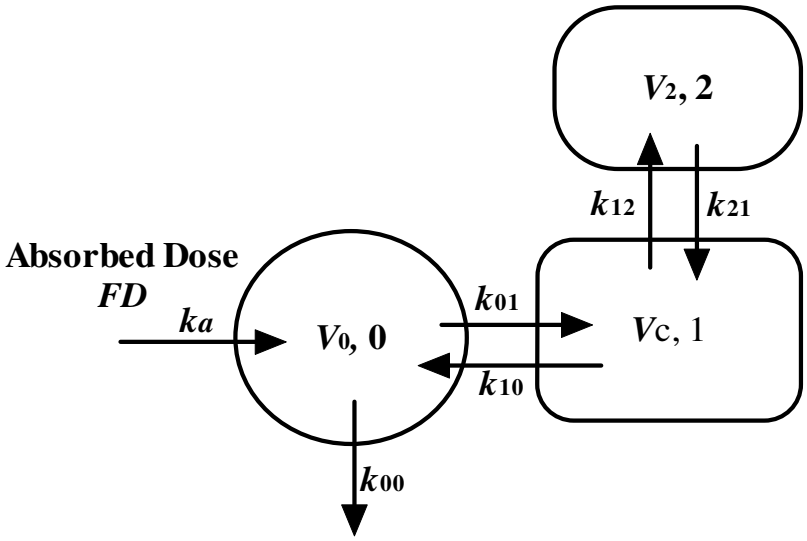

Figure 2.5 Scheme of a 3-compartment "first pass" elimination model with absorption via the hepatopancreatic compartment (compartment 0) proposed by Faroongsarng et al (2007).

The concentration C, in the central compartment at time t is given by Equation 2.11 where the coefficient Γ is defined as: $\Gamma = \frac{F \cdot D \cdot k_{01}}{V_c}$ (Faroongsarng et al, 2007).

$$C = \Gamma \cdot k_a \cdot [\frac{(k_{21} - \alpha) \cdot e^{-\alpha \cdot t}}{(\beta - \alpha)(\gamma - \alpha)(k_a - \alpha)} + \frac{(k_{21} - \beta) \cdot e^{-\beta \cdot t}}{(\alpha - \beta)(\gamma - \beta)(k_a - \beta)} + \frac{(k_{21} - \gamma) \cdot e^{-\gamma \cdot t}}{(\alpha - \gamma)(\beta - \gamma)(k_a - \gamma)} + \frac{(k_{21} - k_a) \cdot e^{-k_a \cdot t}}{(\alpha - k_a)(\beta - k_a)(\gamma - k_a)}] \quad (2.11)$$

The notations in Equation 11 have been described in a previous chapter of Pharmacokinetic Models and can be simplified as:

$$C = A\,e^{-\alpha \cdot t} + B\,e^{-\beta \cdot t} + G\,e^{-\gamma \cdot t} - I\,e^{-k_a \cdot t} \quad (2.12)$$

The relationships among fractional rate constants appear in Figure 2.5 and the coefficients: *A*, *B*, and *G*, as well as the exponents: α, β, and γ are expressed by Equations 1.15-1.23 of the previous chapter. Or in other words, these parameters could be obtained from the same model after intra-sinus administration. Whereas k_a is obtained by a non-linear fitting of Equation 2.12 and *F* is from the bioavailability data. We successfully modeled their pharmacokinetic data of OTC given orally to *P. vannamei* at a dose level of 50 mg/kg-body weight. We demonstrated how the model successfully fitted into not only the OTC profiles in the hemolymph but also in the hepatopancreas. Figure 2.6 illustrates the characteristic OTC profiles according to their model equations both in the hemolymph and the hepatopancreas. The k_a value of 0.2986 h^{-1} was reported. This value seems to be realistic as it was comparable to the gastric emptying time in Penaeid shrimp previously reported by several authors (Dall, 1967; Marte, 1980; and Soares et al, 2005).

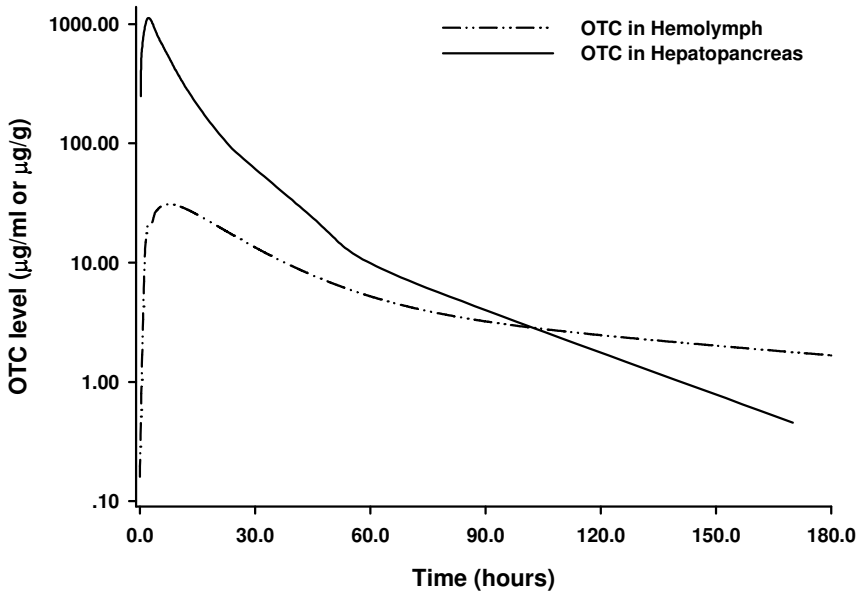

Figure 2.6 OTC Pharmacokinetic profiles after oral administration to *P. vannamei* based on a 3-compartment "first pass" model as proposed by Faroongsarng et al (2007).

Apparent absorption rate constant from a hepatopancreatic profile data fitting

Faroongsarng, et al (2007) have provided an equation that explains hepatopancreatic OTC levels by curve fitting into the model of Figure 2.5 that is similar to Equation 2.12 with the plug-in specific values of α, β, γ, and k_a, i.e.,

$$C_{0,t} = A \cdot e^{-3.2647 \cdot t} + B \cdot e^{-0.0555 \cdot t} + G \cdot e^{-0.0061 \cdot t} - I \cdot e^{-0.2986 \cdot t} \qquad (2.12')$$

Unfortunately, they reported that some of the estimates of the coefficients (A, B, G, and I in Equation 2.12′) exhibited such considerably high standard errors that the values of the estimates were meaningless. To determine k_a with the reduction of the variability in curve fitting, only the compartments directly involved with the hepatopancreatic OTC can be considered. As a result the model in Figure 2.5 is "*reduced*" to be, apparently a 2-compartment model as shown in Figure 2.7. The data of OTC orally administered to the standardized C-D_0 molting stage adult male *P. vannamei* at a single dose level of 50 mg/kg-

body weight similar to those reported in Faroongsarng et al (2007) were utilized. With the aid of a commercial software (Phoenix WinNonlin, Pharsight Corporation, USA), the determined k_a (standard error) is found to be 0.2954 (0.0970) h^{-1}.

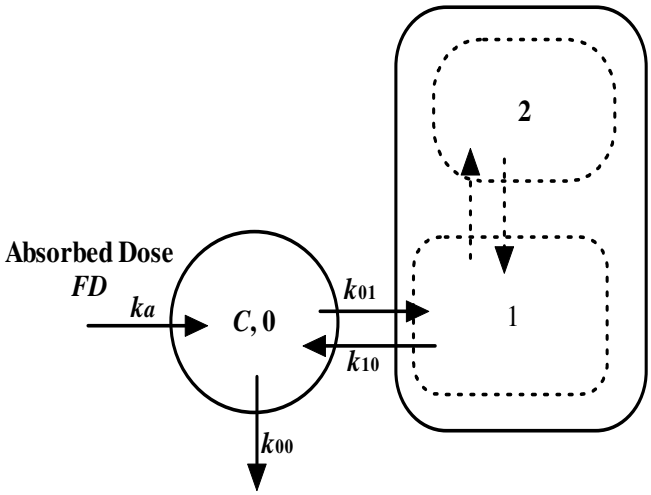

Figure 2.7 The reduced model applied to determine the apparent hepatopactreatic absorption constant k_a. Compartment 1 and 2 are confined into one compartment as the fractional rate constants are no more of concern.

Apparent absorption rate constant based on a fraction of the absorption analysis

One of the difficulties in curve fitting for the estimation of the absorption kinetics based on compartment models has been the deviations of the data from the models and errors of estimation that sometimes are too large to allow for acceptance of these correlations and making the authors reluctant to report their results especially those that include multi-exponential equations (for examples: Uno et al, 2004 and 2006). Many investigators then seek to use the alternative methods and lower numbers of parameters with more easy data fitting. One of them is the percentage absorbed-time plot that does not require any assumption of the order of absorption (Gibaldi and Perrier, 1982). In 1964, Wagner and Nelson published the application of this method using both blood levels and urinary excretion data after a single dose of drug administered to humans. Based on mass

conservation, they proposed that the amount of the drug that has been absorbed into the systemic circulation at any time after administration should equal the sum of the amount of the drug in the body and the cumulative amount of the drug eliminated (Wagner and Nelson, 1964). Assuming one-compartment characteristics, the fraction absorbed *F* is expressed as:

$$F = \frac{A_t}{A_\infty} = \frac{C_t + k_{el}\int_0^t C dt}{k_{el}\int_0^\infty C dt} \qquad (2.13)$$

Where, A_t, and A_∞ are the amount of the drug absorbed at time *t*, and that of the ultimately absorbed. C_t is the hemolymph concentration at time *t* and k_{el} is the elimination rate constant. The assessment of absorption is carried out by plotting the fraction unabsorbed, i.e., 1-*F*, versus time. If the plot on the semi-logarithmic coordinates, for example, approximates a straight line, apparent first order absorption is indicated. k_a is then estimated from the slope of the plot. Sangrungruang et al (2004) utilized Equation 13 to treat their OTC pharmacokinetics after oral administration into *P. monodon*. They described the percentage absorbed-time curve and found that the absorption followed first order kinetics with the estimated k_a of 0.138 h^{-1}. Unfortunately, the Wagner-Nelson method is rigorously applicable to only the one compartment model, and there has been only one pharmacokinetic study done on a Penaeid shrimp that utilized this method since then (Sangrungruang et al, 2004). This is mainly because the pharmacokinetics of the disease preventing agents in Penaeid shrimps always exhibit multi-compartment characteristics in which the Wagner-Nelson method excludes the important peripheral tissue compartment. It has been demonstrated that using the Wagner-Nelson plot to assess the drug absorption in a

multi-compartment model results in underestimation of the time when the absorption ceases and provides an overestimation of the absorption rate (Loo and Riegelman, 1968). While the latter could lead to misinterpretation of the kinetics of absorption, the former is also important since the absorption process continues far beyond the time when the concentration reaches its maximum level. In addition, it has been reported that the estimation of the k_{el} error results in an upward bias in the Wagner-Nelson estimates whereas the truncation error in AUC results in a downward bias (Wang and Nedelman, 2002). This joint effect of the estimation of the error could result in a non-monotonic fraction-of-drug-absorbed-*vs.*-time plot. However with some conditions, k_a from the Wagner-Nelson plot may serve as a reasonable approximation of the absorption kinetics after oral administration that cannot be fitted into, for example, a 2-compartment but only a one-compartment model (Wagner, 1970).

To assess more appropriate values of k_a, Loo and Riegelman (1968) introduced a method for determining the fraction of absorption of the drug following two-compartment characteristics (Figure 2.3). Again, based on mass conservation, they included the amount of the drug in a peripheral compartment together with that in the central one and the amount eliminated. The amount of the drug available via absorption at time t (A_t) divided by the volume of distribution of central compartment V_c is as follows:

$$\frac{A_t}{V_c} = C_t + k_{el}\int_0^t Cdt + \frac{A_2}{V_c} \qquad (2.14)$$

Where, A_2 is the amount of the drug in the peripheral compartment (compartment 2) at time t. Alternatively, Equation 2.14 can be rewritten as (Wagner, 1983):

$$\frac{A_t}{V_c} = C_t + k_{el}\int_0^t C dt + k_{12}e^{-k_{21}\cdot t}\int_0^t C\, e^{k_{21}\cdot t} dt \qquad (2.15)$$

and, with the graphical technique as well as integrated Laplace transformation and the Taylor simplification (Loo and Riegelman, 1968), the term representing the amount of the drug in compartment 2 could be approximated as:

$$\begin{aligned}\frac{A_2}{V_c} &= k_{12}\cdot e^{-k_{21}\cdot t}\int_0^t C\cdot e^{k_{21}\cdot t}\cdot dt \\ &= \left(\frac{A_t}{V_c}\right)_{t,n-1} e^{-k_{21}\Delta t} + \frac{k_{12}}{k_{21}} C_{t,n-1}[1-e^{-k_{21}\Delta t}] + \frac{k_{12}}{k_{21}}\Delta C - \frac{k_{12}}{k_{21}}\frac{\Delta C}{\Delta t}[1-e^{-k_{21}\Delta t}]\end{aligned} \qquad (2.16)$$

Where $\left(\frac{A_t}{V_c}\right)_{t,n-1}$ is the amount of the drug in the body normalized by volume of that distribution to the central compartment up to the previous time interval, i.e., at time *t, n*-1. k_{12} and k_{21} are fractional rate constants between compartments 1 and 2 as they appear in Figure 2.3. $C_{t,n-1}, \Delta C$, and Δt are the hemolymph concentrations at the previous time interval, the difference in the consecutive central compartment concentration: $C_{t,n} - C_{t,n-1}$, and that in consecutive time: $t_n - t_{n-1}$, respectively. It is seen that the estimation of the drug fraction absorbed, based on the Loo-Riegelman method, required concentration levels in the central compartment and an evaluation of the distribution and elimination, i.e., the determinations of k_{12} and k_{21}, from a previously administered intra-sinus dose.

In the Loo- Riegelman treatment, there are three important differences compared to the previous Wegner-Nelson one (Loo and Riegelman, 1968): First, the very important peripheral compartment(s) may be included. Second, the model is forced to distinguish

between the rate constant for the drug disappearance from the body and the specific elimination constant. The drug disappearance rate constant is a complex constant including the fractional distribution constants e.g. k_{12} and k_{21} as well as one for elimination (k_{el}). The reader should refer to the previous chapter of Pharmacokinetic Models for the description of this rate constant, and should learn that k_{el} in Equations 2.14-2.17 is k_{10} in Figure 2.3.Third, the volume constant used to convert the amount to concentration is the volume of the central compartment rather that the apparent volume of distribution. As long as the absorption and elimination are taking place in the central compartment, Equation 2.16 could be applicable to the calculation of other peripheral compartment(s) in which the fractional rate constant between the corresponding peripheral and central compartments can be assessable beforehand. For instance, Wagner (1983) extended the Loo-Riegelman equation to a 3-compartment open model with first order absorption and elimination via the central compartment. The equations describing the amount absorbed per unit volume are as follows (Wagner, 1983):

$$\frac{A_t}{V_c} = C_t + k_{el}\int_0^t C dt + k_{12}e^{-k_{21}\cdot t}\int_0^t C\, e^{k_{21}\cdot t} dt + k_{13}e^{-k_{31}\cdot t}\int_0^t C\, e^{k_{31}\cdot t} dt \qquad (2.17)$$

However, it appears that the Loo-Riegelman method of determination of the fraction absorbed might not be applicable to the 3-compartment pharmacokinetic model of such a disease preventing agent as OTC in Penaeid shrimps as proposed by Faroongsarng et al (2007). This is because the agent was absorbed and eliminated via the hepatopancreas which has been considered to be a peripheral compartment not a central one. The amount eliminated is no longer determined from the *AUC* of the central compartment whereas the

estimation of the amount of the drug presenting in the peripheral compartments as shown in Equation 2.16 are no longer valid.

Unlike the studies in humans which have to be carried out under good clinical practices as well as with strictly ethical concerns, the data obtained from shrimp aquaculture are more freely assessable. While only blood and/or urine could be collected from human subjects, hemolymph, muscle, digestive gland as well as other tissues can be utilized with shrimp samples. This provides an advantage as the amount of both the central and important peripheral compartment(s) A_i can be directly determined. Based on the mass conservation in the model shown in Figure 2.5, it follows that:

$$A_t = C_{0,t} \cdot V_0 + C_{1,t} \cdot V_c + C_{2,t} \cdot V_2 + Cl_0 \int_0^t C_{0,t} \cdot dt \tag{2.18}$$

Where, subscripted notations 0, 1, and 2 are the hepatopancreas (compartment 0 in Figure 2.5), hemolymph (compartment 1), and muscle (compartment 2) compartments, respectively. $C_{i,t}$ and V_i are the drug concentrations in compartment i at time t and the volume of that corresponding compartment. Cl_0 is the intrinsic clearance via the hepatopancreas after oral administration. Notice that the amount eliminated is determined from the AUC of compartment 0. The amount ultimately absorbed via compartment 0 A_∞ is then equal to $Cl_0 \int_0^\infty C_{0,t} \cdot dt$, and the fraction absorbed F will be:

$$F = \frac{A_t}{A_\infty} = \frac{\sum_{i=0}^{2} C_{i,t} \cdot V_i + Cl_0 \int_0^t C_{0,t} \cdot dt}{Cl_0 \int_0^\infty C_{0,t} \cdot dt} \tag{2.19}$$

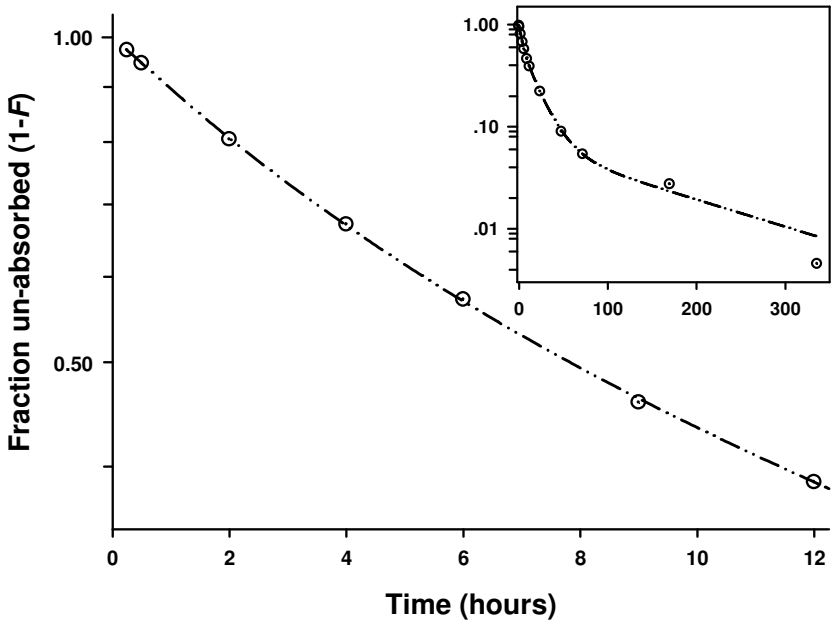

Figure 2.7 Fraction of unabsorbed-time plot of OTC up to 12 hours after oral administration into *P.vannamai*. A_t was calculated by Equation 18 where V_0, V_c, V_2, and Cl_0 were taken from Faroongsarng et al (2007). And, F was calculated by Equation 19. The inset illustrates the entire 1-*F* -time plot.

Figure 2.7 shows the plot in semi-logarithmic coordinates of the unabsorbed fraction 1-*F* of the OTC antibiotics *versus* time up to 12 hours after oral administration to the standardized C-D_0 molting stage of adult male *P. vannamei* at a single dose level of 50 mg/kg-body weight. *F* has been calculated according to Equation 2.19 where the OTC levels in biological matrices including hemolymph, muscle, and hepatopancreas were obtained from Faroongsarng, et al (2009). The volume of each compartment V_i as well as the hepatopancreatic clearance Cl_0 was also taken from the literature (Faroongsarng, et al, 2007 and 2009: Output No. 1). As seen in Figure 2.7, the majority portion of the drug was absorbed in 8-12 hours after dosing. A curvilinear characteristic is observed indicating that the absorption process may follow first order kinetics. Moreover, the entire 1-*F vs.* time

plot in the inset of Figure 2.7 illustrates a decreasing curve that could be best described by a tri-exponential function:

$$1 - F = I_1 \cdot e^{-\lambda_1 \cdot t} + I_2 \cdot e^{-\lambda_2 \cdot t} + I_3 \cdot e^{-\lambda_3 \cdot t} \qquad (2.20)$$

The data in Figure 2.7 successfully fit into Equation 20 having the correlation coefficient r^2 of 0.9999. The estimates of the coefficients and exponents in Equation 2.20 as well as their standard errors (SE) are tabulated in Table 2.2. In an analogous way to that of Wagner (1983), it is proposed that the exponents: λ_i may be estimates of the apparent absorption rate constants. From a mathematical point of view, the term possessing the largest λ_i, i.e., $I_1 \cdot e^{-\lambda_1 \cdot t}$ contributes most of the drug disappearance. The λ_1 exponent is then of particular interest as it corresponds to a very high proportion of the rate of the drug disappearance especially in approximately 12 hours after administration which is presumably thought to be mainly due to the absorption process. Notice that the calculations of Equations 2.18-2.19 are based on the amount of the drug entering the body, while the other processes result in drug loss, for examples, incomplete drug dissolution and drug instability during the GI transit are discarded. As previously mentioned (Chiayvareesajja et al, 2006), the shrimps' digestive gland (hepatopancreas) may play a major role in the drug absorption. It is thus hypothesized that the term $I_1 \cdot e^{-\lambda_1 \cdot t}$ in Equation 2.20 may correspond to the absorption process and it is deduced that λ_1 may be a valid alternative estimate of k_a for absorption via the shrimp's hepatopancreas.

Parameter	Estimate	SE
Coefficient		
I_1	0.3404	0.0391
I_2	0.5952	0.0315
I_3	0.0659	0.0148
Exponent		
λ_1	0.2469	0.0242
λ_2	0.0549	0.0047
λ_3	0.0061	0.0015

Table 2.2 Coefficients and exponents of Equation 20 obtained from non-linear fitting of the fraction unabsorbed 1-*F* –time coordinates in Figure 2.7. The non-linear regression shows the correlation coefficient r2 of 0.9999 with a standard error of estimate of 0.0035.

In addition to the term of $I_1 \cdot e^{-\lambda_1 \cdot t}$, the other terms in the right-hand side of Equation 2.20 may be due to either the drug input via other GI components or an artifact that is influenced by distribution from the peripheral compartmental since the calculation was based on the amounts in these compartments and, the absorption may cease long before the terms $I_2 \cdot e^{-\lambda_2 \cdot t}$ and $I_3 \cdot e^{-\lambda_3 \cdot t}$ predominate. In addition, it is noted that λ_2 and λ_3 are coincidently essentially identical to β and γ of Equation 20 (λ_1 *vs.* β of 0.0549 *vs.* 0.0555 h^{-1}, and λ_2 *vs.* γ of 0.0061 *vs.* 0.0061 h^{-1}). The evidence indicates that the artifact may be influenced by the amounts distributed to the peripheral tissues.

The absorption kinetic assessment based on a deconvolution method

As compartment pharmacokinetic modeling refers to one or multi-compartments having first-order transfer kinetics and accordingly is described by linear differential equations, a drug level-time profile may be considered as the response function of a convolution-type. The operational principle of the convolution is based on molecular stochastic independence that exhibits the linearity between the kinetic response variables

with linear superposition. It is a mathematical operation on two functions, an input function *I* and a unit impulse response function *UIR*, producing a response function *R* (Veng-Pedersen, 2001):

$$R = UIR * I \tag{2.22}$$

Where, the symbol $*$ represents the convolution operant. Alternatively, *R* is typically viewed as a modified version of one of the original functions for which the fundamental equation is written as:

$$R \equiv c(t) = \int_0^t UIR(t-u) \cdot I(u) \cdot du \tag{2.23}$$

In Equation 2.23, it is seen that the pharmacokinetic response function is simply $c(t)$ which is the systemic drug concentration at time *t* after the drug administration with the input function *I*. *UIR* is an original kinetic function given by a linear drug disposition *L* for instantaneous input, i.e., in this particular case, intra-sinus administration I_{IS}, normalized by a given dose D_{IS}.

$$UIR \equiv \frac{L(I_{IS})}{D_{IS}} \tag{2.24}$$

The disposition function *L* of Equation 2.24 can be approximated by a multi-exponential function:

$$L(I_{IS}) \equiv c(t) = \sum_i A_i \exp(-\alpha_i \cdot t) \tag{2.25}$$

In practice, 2- or 3-exponential functions may be fitted into the drug level data of intra-sinus administration. The reader should go to the chapter of pharmacokinetic modeling to obtain the specific exponential functions describing the pharmacokinetics of some antibiotics in Penaeid shrimp. The operational procedure to determine *I* in Equations 2.22-

2.23 is called the method of deconvolution. In addition to $c(t)$, *UIR* needs to be determined prior to the determination of *I*.

There have been a number of sophisticated numerical methods proposed by numerous investigators in order to de-convolve *I*. In principle, the deconvolution through a convolution iterative procedure consists of three steps. First, the input function is adjusted by changing its parameter values. Second, the new input function is convolved to produce a calculated drug level response. Third, the agreement between the observed data and the calculated drug level data is quantitatively assessed according to some objective function. The three steps are repeated until the objective function is optimized (Phoenix WinNonlin, 2009). A number of input functions have been applied, for examples, gamma distribution, analytical, spline function, and uniform inputs, to evaluate deconvolution measurements of a systemic absorption (Levitt, 2003). The mathematics of deconvolution however is beyond the scope of this discussion. Fortunately, a number of software packages are available such as a freely distributed package PKQuest (http://www.pkquest.com) and the commercial package Phoenix WinNonlin (Pharsight Corporation, USA). An example of deconvolution for the input function, i.e., absorption of antibiotics introducing into a Penaeid shrimp is demonstrated here using the data set of OTC orally administered to the standardized C-D_0 molting stage adult male *P. vannamei* at a single dose level of 50 mg/kg-body weight similar to those in Figure 2.7. The data were analyzed using a commercial software package (Phoenix WinNonlin v. 6.1, Pharsight Corporation, USA). *UIR* was pre-determined by using the tri-exponential equation proposed by Chiayvareesajja et al (2006) as $L(I_{IS})$ with a D_{IS} of 10 µg/g-body weight. Figure 2.8 illustrates a plot of the de-convolved *I* as the cumulative fraction of absorption *F vs.* time *t*. As the absorption process is presumably first

order with an apparent absorption rate constant k_a, the solid trend line shown in Figure 2.8 is then obtained by data fitting according to Equation 2.26:

$$F = F_\infty[1 - \exp(-k_a \cdot t)] \quad (2.26)$$

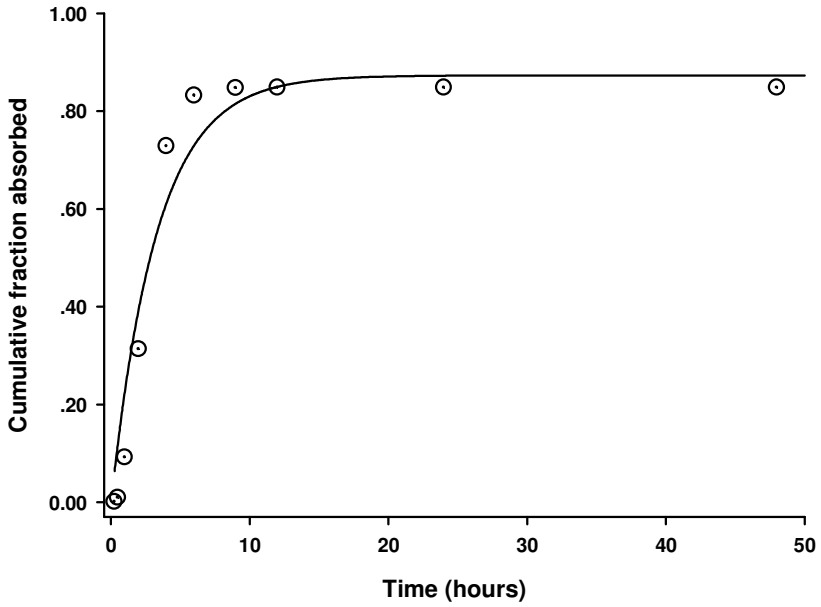

Figure 2.8 The plot of the cumulative fraction of OTC absorbed after being orally administered into adult *P. vannamei* obtained by the method of deconvolution against time.

The data did successfully fit into Equation 26 with correlation coefficient r^2 of 0.9487 having k_a and F_∞ with the standard error in parentheses of 0.3022 (0.0523) h^{-1} and 0.8733 (0.0371), respectively.

Comparison among apparent absorption rate constants obtained by various methods

Method	**Estimate of k_a (h^{-1})**	**SE**
Curve fitting	0.2954	0.0970
Fraction of absorption	0.2469	0.0242
Deconvolution	0.3022	0.0523

Table 2.3 Estimates of an apparent absorption constant obtained from different methods.

In this chapter, three methods for the assessment of absorption kinetics have been explored. They are summarized in Table 2.3. Although the data for these 3 examples came from the same group of experimental animals, they were rather different set ups. The curve fitting method utilized the time course drug level in the shrimp's hepatopancreas. The fraction of absorption analysis used the hemolymph, hepatopancreas, and muscle data, whereas the method of deconvolution used only the hemolymph data. To compare the estimates of the apparent absorption rate constant, one-way analysis of variance with F-test was carried out as shown in Table 2.4. The apparent absorption constants are not significantly different among the methods of assessment at the 95% confidence (p-value is far greater than 0.025). Thus, statistically speaking, these parameters are essentially identical. Furthermore, these numbers are comparable to those previously reported in an identical cultured specie (k_a of 0.2986 h^{-1}: Faroongsarng et al, 2007) have demonstrated that each of the methods of assessment is valid for these experimental settings.

Source	d.f.	Sum Square	Mean Square	F	p-value
Method	2	.0126	.0063	1.846	0.175
Error	30	.1023	.0034		

Table 2.4 One-way analysis of variance (ANOVA) of the estimates listed in Table 3. (d.f. stands for degrees of freedom)

3. Pharmacokinetics of antibiotics in Penaeid shrimps
Toxicokinetics

There are a number of requirements for a substance to be approved as an antibiotic for use with food-producing animals. These include proof of efficacy, animal, human and environmental safety, and manufacturing stability (Bray et al, 2006). Animal safety deals with the issues of toxicity, i.e. to establish animal safety the animals are exposed to the substance in extreme doses over a sufficiently long period of time to allow for any histological changes and toxic responses to be observed. Due to survival problems animal's lives might be lost. As a result the lethal dose parameters need to be established first. On the contrary, Human's food safety is based on an assessment of the amount of the drug, free metabolites, and metabolites that are covalently bound to endogenous molecules in any edible animal product, i.e., the total residues. In this case, not animal safety but the intermittent and chronic exposure of humans to relatively low concentrations of residues is of concern (US FDA, 2006). Establishing the exact level of antibiotics used and potential dangers is difficult mainly due to lack of information (Tu et al, 2010) whereas antibiotic residues may be destined for human consumption and result in the development of antibiotic resistance in potentially pathogenic bacteria (Park et al, 1994). To date, several regulatory authorities have established the maximum residue limits (MRLs) based on the concept of the acceptable daily intake with toxicological safety, pharmacological and microbiological considerations (Woodward, 2004). In addition, pharmacokinetic studies have been providing recommendations for proper handling protocols to ensure the safety of food-producing animals for example the establishment of a wash-out period before

harvesting so as to allow the residual level lower than the MRL. In shrimp aquaculture, another concern is the use of antibiotics at levels that exceed the recommended toxic level. While pharmacokinetic information for antibiotics used in adult shrimps has been published, the toxicity studies tend to be done on the animals of larval life stages (For example: Soto-Rodriguez et al, 2006). And, it is not realistic to assume that the drug at these extreme levels could be biologically handled in the same manner as for normal therapeutic use. *Toxicokinetics*: the kinetics of systemic drug levels following an extreme dose might be useful in this situation. This chapter will cover the toxicokinetic issue of an example of a common antibiotic used in Penaeid shrimp culture.

The nature of toxicokinetics

Like pharmacokinetics, toxicokinetics uses the model to quantitatively describe the time course of the drug or chemical in the body. The model may be either compartment or physiological based mathematically described by a set of differential equations as discussed in Chapter 5. Despite their similarity, toxicokinetics differs from pharmacokinetics in some ways: Firstly, a pharmacokinetic study is done with low pharmacological dose where the kinetic processes are always linear. While a toxicokinetic one generally deals with non-linear processes since the maximum capacity of each of the processes is approached due to the considerably large amount of the drug or chemical involved. Secondly, a normally low pharmacological dose in a pharmacokinetic study is not susceptible to solubility-related dosing problems leading to the drug precipitation in gastrointestinal tract as is the toxicokinetic counterpart. As a result the pattern of absorption may be different. And, it might not be done with intra-vascular route that requires completely soluble drug prior to administration as the amount of the drug/chemical may exceed its solubility. Thus, the

information regarding bioavailability, elimination half life, volume of distribution, as well as clearance obtained from a pharmacokinetic study cannot be straightforwardly extrapolated to a high dose as used in toxicity study. It is noted that the drug research and development is generally conducted on the purpose of human use. Thus, the toxicokinetic studies are normally done for preclinical safety evaluation as the animal toxicity data cannot be inferred to human without an understanding of the kinetics of absorption, distribution, biotransformation/metabolism, and elimination in preclinical species (Dixit et al, 2003). Toxicokinetics serves as a bridge for extrapolating the drug concentrations across different species which is very important for the drug product development. There is no toxicokinetic information available to date for antibiotics. It is because shrimp is not a model animal for human toxicity assessment. As previously mentioned, the shrimp toxicokinetics for an antibiotic may be of importance when it comes to the use at the dosage levels exceeding the recommended toxic dose.

Antibiotic toxicity in Penaeid shrimp: An oxytetracycline case

The risks associated with harmful effects on human health have led to bans on the use of certain antibiotics in animal food production (Tu et al, 2010). For examples: Nitrofurans and their metabolites, that have exhibited carcinogenic and mutagenic characteristics, have been banned in the EU since 1993. No residues of chloramphenicol, an antibiotic potentially causing fatal aplastic anemia; are permissible in food producing animals in many countries around the world. In Japan, a zero residue level is applied for sulfonamides, antibiotics that might cause serious allergic reactions. Quinolone/fluoroquinolone antibiotics are not approved for use in shrimp farms especially in the US. Even through the drugs disappear from shrimp body very quickly as they are

biotransformed to the active metabolites. In contrast, oxytetracycline (OTC) has been commonly used worldwide to fight against bacterial infections especially vibriosis and necrotizing hepatopancreatitis in shrimp aquaculture. It has long been recognized as an investigational New Animal Drug (Reed, et al., 2006) that may be permitted in specific conditions in the US. Recently, the Center for Veterinary Medicine, US FDA approved a few antibiotics including OTC for use in medicated feeds to control bacterial disease in fish aquaculture (FDA/CVM, 2007).

A toxicity study of OTC carried out on white shrimp has been reported (Bray et al, 2006). Toxic effects due to doses between 4.5 g/kg (the recommended maximum therapeutic dose) and 13.5 g/kg OTC in feed was checked in various tissues including gills and gill appendages, foregut, nerve cord/ganglia, antennal gland, hematopoeitic organ, maxillipeds, heart, lymphoid organ, hepatopancreas, midgut, anterior and posterior caeca, hindgut, and gonads of sub-adult *L. vannamei* were evaluated. No histological changes in those tissues were found but a decline in lipid reserves and evidence of tissue abnormalities, i.e., tubule atrophy and necrosis were detected in the hepatopancreas. A dose-dependent pattern of decline in lipid storage was noted. The development of gonad maturity was slow but this was not related to the effect on somatic growth that was also observed. In addition, although it was found that shrimp fed with OTC at the recommended maximum therapeutic dose level did not show either any significant toxic responses or any negative impact on growth rate, slight histological changes in the hepatopancreas, i.e., tubule atrophy were still observed. It might be due to the fact that multi daily dosing caused the drug to accumulate up to a level that adversely affected the hepatopancreas. Faroongsarng et al (2007) demonstrated that OTC was present in the hepatopancreas at a

highly significant level after oral administration. It is thus possible that not only was the shrimp sensitive to the drug but the hepatopancreatic tissues contained a sufficiently high concentration of OTC after the shrimp was exposed to OTC to be considered toxic.

Excessive exoskeleton softness has also been observed in shrimp treated with extreme dose levels (Bray et al, 2006). The prevalence of soft-shell shrimp may not be due to an effect of the different molting stages as the majority of samples in all groups were in the inter-molt stages of C and C-D transition where shrimp usually exhibit a hard shell. As OTC exhibits strong chelating properties with divalent metal ions, it may form complexes with calcium and magnesium, the major shell hardening components, and disturb shell growth by making those minerals unavailable. However, whether or not OTC interferes with shell formation at the cuticle epithelium level is yet to be studied.

OTC concentration-time profiles following high dose administrations: Exploratory toxicokinetics

An OTC kinetic study of high single-dose administrations into Penaeid shrimp has recently been completed (see output No. 2). C-D_0 molting stage male sub-adult *P. vannamei* were randomly assigned to being force fed by oral administrations of OTC present in medicated feeds at accurate dose levels of between 500 and 2,500 mg/kg body weight (BW). The medicated shrimp were sampled at different time intervals up to 7 days after dosing. Hemolymph, hepatopancreas, muscle, and shell samples were collected, and subjected to OTC determinations using the validated HPLC method. It has been demonstrated that systemic OTC levels following a 1,000 mg/kg BW single-dose was comparable to that of a steady state after the administration of the recommended maximum therapeutic multi daily dose. It is therefore assumed that a single dose of 1,000 mg/kg BW

may be the maximum dose that allows shrimp to essentially retain their normal functions. Thus, the profiles after 1,000 mg/kg BW administration serve as the representatives of the drug levels in presumably a normal pharmacokinetic experiment for comparison with those where a toxic response may be present.

The concentration-time profiles of OTC in various tissues are illustrated in Figure 3.1. It was observed that the concentration levels of OTC in the hepatopancreas (Figure 3.1 (b)) were an order of magnitude greater than those in the hemolymph (Figure 3.1 (a)). The results were consistent with the low-dose studies of oral administration (50 mg/kg BW dose: Faroongsarng et al, 2007) as well as after an intra-sinus administration (10 mg/kg BW dose: Chiayvareesajja et al, 2006). It is not surprising that a large amount of the drug was found in the hepatopancreas as it seems to be an organ for both absorption (Verri et al, 2001) and elimination (Chiayvareesajja et al, 2006). However, the underlying toxic responses after the white shrimp was exposed to OTC in extreme levels were tubule atrophy in the hepatopancreas and the sloughing of the hepatopancreatocyte epithelial even in the case of using the maximum recommended therapeutic dose with a long exposure (Bray et al, 2006). As seen in Figure 3.1 (a) and (b), the drug is distributed to both the hemolymph and hepatopancreas somewhat differently between the 2 dose levels as the decreasing pattern of the curves of the distribution/elimination phase were not parallel to each other. This evidence may indicate that alterations of the normal pharmacokinetic function may occur due to some hepatopancreatic tissue damage following exposure to extremely high levels of OTC.

In a previous experiment the peak level of OTC found in the peripheral muscular compartment, after a 50 mg/kg BW OTC-dose study, were similar to those found in the

systemic compartment (Faroongsarng et al, 2009: Output No. 1), however muscular levels following administration of an extreme dose (Figure 3.1 (c)) were significantly greater. Faroongsarng et al (Output No. 2) have found that following an extreme dose of OTC, levels in the peripheral tissues were more than 3 times higher than in the hemolymph. Furthermore, not only did OTC levels increase rapidly but they tended to persist in the muscular tissues and did not instantaneously decrease after reaching the maximum level (Figure 3.1 (c)). Thus we suggest that the wash-out time for safe harvesting practice of 7-14 days after treatment ceased, as previously recommended by several pharmacokinetic studies (Chiayvareesajja et al, 2006; Gomez-Jimenez et al, 2008; and Faroongsarng et al, 2009) cannot be applied in this case. It was hypothesized that the malfunction of the hepatopancreatic due to the drug toxicity might negatively impact on the prolonged OTC levels in muscle due to difficulties in redistributed the drug from the tissues to an organ for further disposal . This finding recommends caution and awareness not to use antibiotics that exceed recommended levels, as the drug residue may be persistent when it comes to harvesting the product.

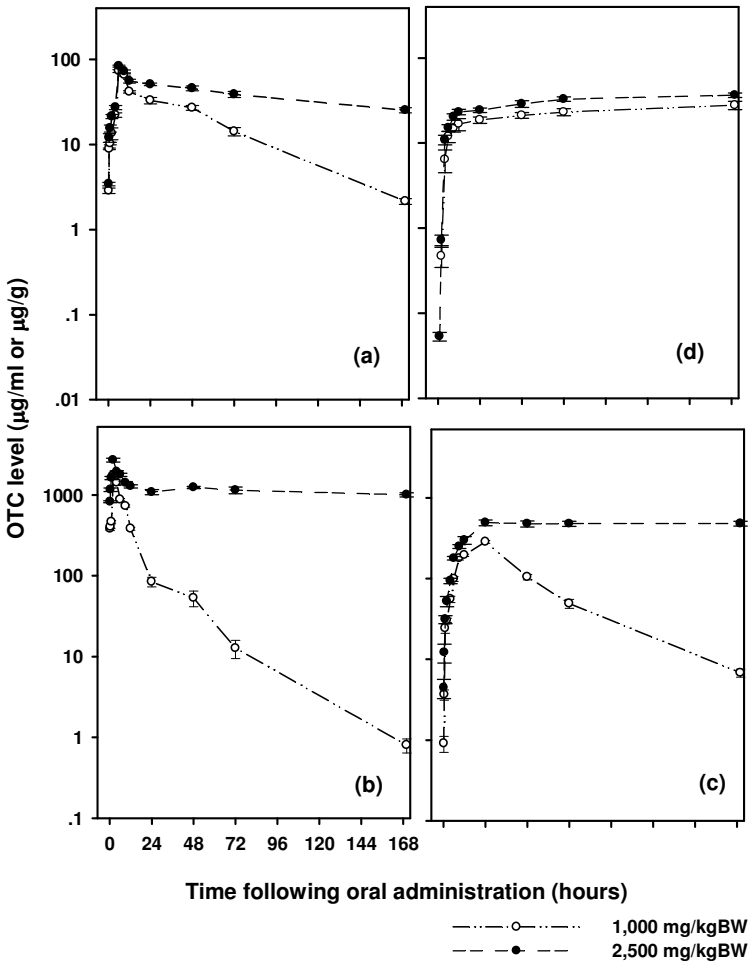

Figure 3.1 The OTC concentration-time profiles following force fed oral administrations of 1,000 (open circles) and 2,500 mg/kg BW (closed circles) in various tissues including: (a) hemolymph, (b) hepatopancreas, (c) muscular tissues, and (d) shrimp shells. Notice that the OTC concentration in the hemolymph is expressed as µg/mL while those of other tissues are µg/g.

A considerable amount of OTC was present in the shrimp shell after extreme doses were given to white shrimp (Figure 3.1 (d)). The drug level increased and persisted at concentrations of approximately 28 and 37 µg/g for more than 7 days after administration of oral doses of 1,000 and 2,500 mg/kg BW, respectively. These findings were consistent with those reported in a 10 and 50 mg/kg BW-dose study of the same drug in white shrimp (Uno et al, 2010). In that case the amount of OTC in the shell was independent of the dose.

Unlike in other tissues, the drug distribution pattern in the shrimp shell was not altered by the changes in the functions of the hepatopancreas. The distribution of OTC in the exoskeleton was modeled in a previous chapter where it was suggested that there may be a unidirectional transport of the drug from peripheral muscular compartment as well as from the blood circulation. In addition, it seems that the transport process is spontaneous and uncontrolled as it could not be disrupted by pharmacokinetic alteration due to the toxic effects on the hepatopancreas.

Physiological-based toxicokinetic model

As discussed in 1, physiological-based model may better address the drug distribution and metabolism/disposition in particular tissue and organ of interest. An attempt to fit the OTC level-time profiles in hepatopancreatic and muscular tissues was done with the physiological based model (Faroongsarng et al, Output No. 2). It was a limited hemolymph flow model described in 1 where OTC was absorbed via foregut to hepatopancreas with a first order rate. It was estimated that the drug affinity in muscular tissues was approximately three-fold higher after an extreme single oral dosage administration compared to that after the normal daily multiple dosing at maximum therapeutic dose level. At this toxic level, the authors proposed that OTC might not only exceed the maximum capacity of the drug clearance but damage the hepatopancreatic tissues resulting in the persistent OTC level in such an organ as muscle. And, the drug may be no longer eliminated via shrimp hepatopancreas.

As seen in Figure 3.1 (a) at 2,500 mg/kg BW dosage level, unlike OTC in other tissues, the systemic drug seemed to gradually decline as time went by despite the major organ of elimination, hepatopancreas was in dysfunction. It is thus suspicious that there

might be systemic elimination via other process. To determine whether or not the "extra" elimination was evident, the modified physiological based model was employed to fit the OTC profiles after white shrimp was orally dosed at 2,500 mg/kg BW in single dose fashion. Figure 3.2 illustrates the physiological based model for OTC distribution in the tissues/organs of interest modified form that utilized by Faroongsarng et al (2011: Output No. 2). Notice that this model was somewhat different from that of Faroongsarng et al (2009: Output No. 1; and 2011: Output No. 2) as it additionally included the "extra" systemic clearance denoted as Cl_B in Figure 3.2. In addition to in shrimp body, OTC may distribute to exoskeleton as the drug in a significant concentration level was found in it (Figure 3.1 (d); and Uno et al, 2010). The unidirectional first order distribution into shell with rate of k_{23} (arrow with dotted line in Figure 3.2) was also proposed according to the model for antibiotic distribution in shrimp exoskeleton described in 1.

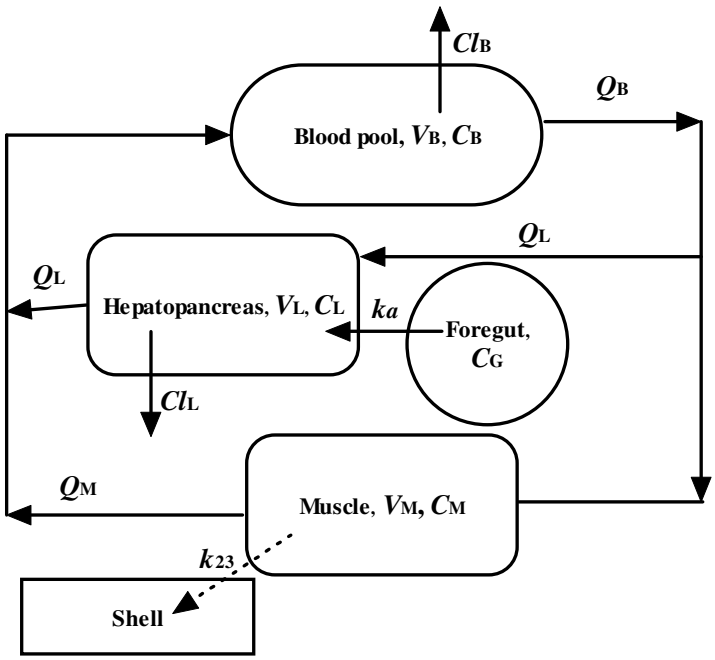

Figure 3.2 Physiological-based toxicokinetic model for OTC distribution/disposition after orally administered into white shrimp at extreme doses of 1,000 and 2,500 mg/kg BW.

With the aid of volume and flow parameters previously reported (Faroongsarng et al, 2011) the OTC level-time data were simultaneously modeled using commercial software (Phoenix WinNonlin, Pharsight Corporation, USA). The data fitting yielded fair correlations (r^2 of 0.699: for shell, 0.718: for blood pool, and 0.732: for hepatopancreas) as well as a good one (0.984: for muscle). The estimates of Cl_B and Cl_L with their standard errors in parentheses were 0.375 (0.162) and 0.022 (0.004) ml h^{-1}/g BW, respectively. While the maximum capacity of hepatopancreatic clearance, Cl_L according to the physiological based model employed by Faroongsarng et al (2011) was estimated to be 0.341 ml h^{-1}/g BW. It is thus evident that hepatopancreatic elimination function might be deteriorating altered due to toxicity, e.g., Cl_L decreased from 0.341 to 0.022 ml h^{-1}/g BW. But, the drug may still be systemically eliminated with Cl_B of 0.375 ml h^{-1}/g BW. However, the process responsible for this "*extra*" elimination is yet to be investigated.

4. Experimental

Chemicals

Oxytetracycline (OTC) dihydrate (Sigma, St. Louis, MO, USA) was utilized as the antibiotic under study. Chlortetracycline hydrochloride (Sigma, St. Louis, MO, USA) was used as an internal standard for the determination of OTC by HPLC in the shrimp biological matrices, including the shrimp shell. Unless otherwise indicated, other chemicals used were of analytical or HPLC grade.

Experimental Animals and Aquaria Conditions

Sub-adult male Pacific white shrimp, *L. vannamei*, weighing 14-22 g with a carapace length of 2.30-3.00 cm were collected from a local farm (Damrong Farm, Pattani, Thailand) and preconditioned by acclimating in a 3-ton concrete tank filled with continuously oxygenated natural seawater and conditions as per: 28±2 ^{o}C, 20±2 ppt salinity, pH 7.6±0.5, 200 ppm total hardness, and 12 h of light per day. Shrimp were fed with commercial pellets once a day at a rate of approximately 2-3% of body weight. The healthy shrimp were randomly divided into 4 groups with dosage levels of 0 (control), 50, 500, and 1,000 mg OTC /kg-BW respectively.

Dosage Administrations and Sample Preparations

OTC aqueous solution of pH 8.0 for intra-sinus administration was prepared as previously described (Chiayvareesajja et al., 2006). A clear solution of OTC was prepared by dissolving OTC dihydrate in 0.01 M phosphate buffer saline (pH 7.4) and adjusted to pH 12 by 0.1 M NaOH. After OTC was completely dissolved, it was adjusted to pH 8.0 with 1 M HCl without precipitation. The final concentration was 10 mg/ml. In the intra-sinus administration group, shrimps were dosed through the

arthrodial membrane at the junction of the carapace and abdomen on the ventral side into the ventral intra-sinus cavity at a dose level of 10.0 μg/g-body weight.

The medicated feeds were prepared by mixing commercial feed pellets previously ground to powders with OTC, to provide concentrations of 20, 200, and 400 mg/g. The mixture was then suspended in purified water at 1:1 w/v to form a paste. Each medicated feed paste was packed into a 1-mL syringe fitted with a blunt-end 18G needle, and the tip of the needle was fitted with a silicone tube to protect the shrimp's mouth. The volume of the paste was calculated individually for each of the sampled animals in order to meet an accurate dose on the basis of the shrimp's body weight. After molting when the animals were in the C-D_0 stage, each of the shrimp samples was kept individually in a 2.5-L glass aquarium to prevent contamination of the antibiotic between different aquaria. Each individual animal was then force- fed orally with the medicated feed to fully fill the foregut (approximately 0.5% of body weight). After administration, normal feeding was restarted 24 h post-dosing and continued once a day during the study. No mortality was observed during the study. Three individual shrimp were sampled for each time interval of 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, and 170 h after the oral administration of the antibiotic.

After the shrimp were sampled, 1.5-mL of hemolymph was removed through the ventral-sinus cavity by using a 5-mL syringe, fitted with a 19-G needle and containing 98% N-ethylmaleimide crystals (~0.01g) as an anticoagulant. For shrimp administered with 50 mg antibiotic /kg-BW, the muscle and hepatopancreas were collected later. For animals dosed with 500 and 1,000 mg/kg-BW, shell samples which were a cuticle mixture of abdominal shell, swimmerets, and telson were collected. All samples were

frozen and maintained at -70 C prior to OTC extraction. Individual samples were assayed for OTC levels in all the shrimp biological matrices.

Analytical Procedures

The HPLC system (Agilent 1,200 series binary LC system, Palo Alto, CA, USA) with a photodiode array detector and an analytical column of 5 μm, 4.6X250 mm (Purospher star RP-18 end-capped, Merck, Darmstadt, Germany) was used to determine OTC in the shrimp biological matrices. The mobile phase with a flow rate of 1 mL/min as well as the method of HPLC separation was modified from Li et al (2008).

Prior to the drug determination, the frozen hemolymph, muscle, and hepatopancreatic samples were prepared to make them suitable for the analytical procedure in the manner previously described (Faroongsarng et al, 2011: Output No. 2). OTC was extracted from shell samples as follows: The samples were frozen-dried and gently pulverized to powders. The powders were then accurately weighed and chlortetracycline hydrochloride used as an internal standard was accurately added to a concentration level of 20 μg/g. An equal volume of 0.01 M ethylenediamine tetraacetic acid- McIlvaline buffer was added to the powdered sample and after mixing was centrifuged for 15 min at 4,000 x g at 4 C. The supernatant was collected while the precipitate was re-extracted before being discarded. The collected supernatants were cleaned by C18 sorbent solid phase extraction (SPE: 3 mL Sep-Pak Vac C18, Waters, Milford, USA), pre-conditioned with methanol (3 mL) followed consecutively by Milli-Q water (5 mL), and 0.01 M Ethylenediamine tetraacetate-McIlvaline buffer (2 mL). The samples were then eluted by methanol. The methanol solutions were dried by

purging with nitrogen at 40 C and dissolved in the mobile phase of the analytical column.

While the analytical procedure used for OTC in hemolymph, muscle, and hepatopancreas had been previously validated (Faroongsarng et al, 2011), validation for the determination of OTC in shrimp shell was as follows: the mean recovery was 93.58% with the repeatability expressed as a relative standard deviation of 2.88%, the limit of quantitation was 0.05 μg/g whereas the linearity of the method with an r^2 of 0.9998 was observed up to a concentration of 100 μg/g, after a 3-month storage period at -70 C, 99.13% of the drug remained in the samples with a mean recovery of 92.76%, and the reproducibility expressed as a relative standard deviation was 4.55%.

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Output ที่ได้จากโครงการ

- **Output No 1**: Faroongsarng, D., Chiayvareesajja, S., Chandumpai, A., and Theapparat, Y. 2009. Hepatopancreatic and muscular distribution of oxytetracycline antibiotics in farmed pacific white shrimp (Penaeus vannamei): a physiological-based pharmacokinetic model approach. *Aquacult. Res*., 41: 143-152.

- **Output No 2**: Faroongsarng, D., Chiayvareesajja, S., and Theapparat, Y. 2011. A toxicokinetic study of Oxytetracycline antibiotics in farmed Pacific white shrimp *Penaeus vannamei*. Accepted and In press.

- **Output No 3**: Faroongsarng, D., Chiayvareesajja, S., and Theapparat, Y. Complete analysis of the distribution kinetics of the oxytetracycline antibiotic in the exoskeleton of farmed pacific white shrimp, *Litopenaeus vannamei. Aquacult. Res*., In review.

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Hepatopancreatic and muscular distribution of oxytetracycline antibiotics in farmed pacific white shrimp (*Penaeus vannamei*): a physiological-based pharmacokinetic model approach

Damrongsak Faroongsarng[1], Sommai Chiayvareesajja[2], Ausa Chandumpai[3] & Yongyuth Theapparat[4]

[1]Drug Delivery System Excellent Center, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand

[2]Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand

[3]Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand

[4]Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand

Correspondence: D Faroongsarng, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand. E-mail: damrongsak.f@psu.ac.th

Abstract

Oxytetracycline (OTC) pharmacokinetic models previously used to investigate *Penaeus vannamei* have not addressed the specific problems related to drug distribution/disposition in particular tissues. This study aimed to provide an insight into OTC kinetics in the hepatopancreas and muscle based on a physiological model approach. Adult male *P. vannamei* at the C-D_0 inter-moulting stage were randomly assigned to intra-sinus and oral administrations. In the intra-sinus group, shrimps were dosed via the ventral sinus at an OTC level of 10.0 $\mu g\, g^{-1}$ body weight, while in the oral one, they were force fed at a dose level of 50.2 $\mu g\, g^{-1}$. The medicated animals were sampled at various time intervals until 170 h after dosing. Haemolymph, muscle and hepatopancreas samples were taken and OTC levels were determined using the validated HPLC method. A model focused on the hepatopancreas and muscle was developed. Oxytetracycline pharmacokinetic profiles in particular tissues were fitted into the model with an R^2 of between 0.6568 and 0.9904. Oxytetracycline muscular distributions were essentially identical for both groups and the drug did not accumulate in muscle. The distributions in the hepatopancreas for both groups were extensive, whereas that for oral administration was approximately 2.3 times greater than that for the intra-sinus one. It was demonstrated that hepatopancreatic OTC may undergo significant first-pass elimination with non-linear kinetics.

Keywords: oxytetracycline (OTC), physiological-based pharmacokinetic model, *Penaeus vannamei*, hepatopancreas, relative affinity

Introduction

All studies to date describing the pharmacokinetics of oxytetracycline (OTC) in shrimp, e.g. those by Reed, Siewicki and Shah (2004, 2006), Uno (2004), Sangrungruang, Chotchuang and Ueno (2004), Uno, Aoki, Kleechaya, Tanasomwang and Ruangpan (2006), Chiayvareesajja, Chandumpai, Theapparat and Faroogsarng (2006) and Faroongsarng, Chandumpai, Chiayvareesajja and Theapparat (2007), have utilized the compartmental models. Although the models yielded the detailed rate kinetics of OTC among compartments, this type of model uses simple approximations and result in a descriptive function regarding drug profiles in biological matrices. The parameters obtained are either only overall or average values depending on the model utilized. They do not address specific problems related to the actual time course of drug metabolism/disposition in particular tissues or organs (Welling 1986). Such drug

kinetics in particular tissues/organs can be approached using a physiological-based model. Typically, the anatomical or physiological tissues/organs of interest used in such a model are (1) that contain or accumulate a considerable quantity of the drug and (2) that are involved in drug metabolism/disposition. The drug residue in shrimp muscle has long been a primary concern, and also several lines of evidence have indicated that the digestive gland (hepatopancreas) also plays an important role in both OTC disposition (Chiayvareesajja *et al.* 2006) and primary absorption (Verri, Mandal, Zilli, Bossa, Mandal, Ingrosso, Zonno, Vilella, Ahearn & Storelli 2001; Faroongsarng *et al.* 2007). Thus, it is of interest that OTC kinetics in shrimp hepatopancreas and muscle could be provided by the pharmacokinetic analysis using a physiological-based approach. The current study aimed to examine the distribution of OTC after both intra-sinus and oral administrations into pacific white shrimp (*Penaeus vannamei*) with a pharmacokinetic model developed by approaching the of interest physiological compartments including hepatopancreas and muscle.

Materials and methods

Oxytetracycline dihydrate and chlortetracycline were used, respectively, as the antibiotic under study and the internal standard for the determination of OTC in the biological matrices (Sigma, St Louis, MO, USA). The other chemicals used in the HPLC system were analytical grade.

Experimental animals

Approximately 3-month-old male *P. vannamei* weighing 16.0 ± 4.1 g with carapace length 2.30–3.00 cm were used. Shrimps were collected from a local farm (Pattani, Thailand) and held in a 3-tonne concrete tank filled with continuous oxygenated natural seawater. Seventy-two individuals were sampled and acclimated inside laboratory cages immersed in seawater conditioned as: 28 ± 2 °C, 20 ± 2 ppt salinity, pH 7.6 ± 0.5, 200 ppm total hardness, 12 h of light per day and constant aeration. The samples were fed with commercial pellets once a day at an approximately 2–3% body weight. After moulting, the shrimp samples were kept individually in 2.5 L glass aquaria to protect against cross-contamination during the drug dosing. The pre-conditioned shrimp samples were randomized into two parallel groups: either intra-sinus or oral dosing according to the routes of administration. Before OTC administration, they were fasted for approximately 2 days for their moult cycle entered the C-D_0 stage (Dall, Hill, Rothlisberg & Staples 1991). The feeding was restarted at 24 h after medication and continued throughout the study. There was no mortality of the experimental animals throughout the study.

Medications and sample preparations

An OTC aqueous solution of pH 8.0 was prepared for intra-sinus administration as described previously (Chiayvareesajja *et al.* 2006). A clear solution of OTC was prepared by dissolving OTC dihydrate in 0.01 M phosphate buffer saline (pH 7.4) and adjusted to pH 12 by 0.1 M NaOH. After OTC was completely dissolved, it was adjusted to pH 8.0 with 1 M HCl without precipitation. The final concentration was 10 mg mL^{-1}. In the intra-sinus administration group, shrimps were dosed through the arthrodial membrane at the junction of the carapace and abdomen on the ventral side into the ventral intra-sinus cavity at a dose level of 10.0 μg g^{-1} body weight. Three medicated shrimps were sampled at time intervals of 0.25, 0.5, 2, 4, 6, 9, 12, 24, 48, 72 and 170 h after administration. For each of the shrimp samples, approximately 1 mL haemolymph was collected through the ventral sinus cavity. After haemolymph removal, the specimen was sectioned and so each physiological sample included, muscle and digestive gland (hepatopancreas) areas. All sub-sectioned samples were frozen at − 70 °C until extraction.

The medicated feed was prepared by mixing OTC with commercial feed previously pulverized to an OTC concentration of 20 mg g^{-1}. Water was added at a ratio of 1:1 w/v to form an injectable paste. The concentration of OTC in the feed paste was 1.027 ± 0.022 mg mL^{-1}. The oral administration group, pre-conditioned as above, was medicated via oral force feeding using a 1-mL syringe fitted with a blunt-end 18 G needle where its tip was covered by a silicone tube to prevent injury of shrimp's mouth to fill the foregut (approximately 0.5% of body weight) with a dose level of 50.2 μg g^{-1} body weight. The sampling intervals and the method of sub-sectioned sample preparation were the same as in the intra-sinus administration group.

The methods of sample preparation were modified from the literature (Chiayvareesajja *et al.* 2006). In brief, frozen haemolymph was thawed and vigorously mixed to assure homogeneity before extrac-

tion. The individual haemolymph samples (100 μL) were placed in 1.5 mL Eppendorf tubes. Protein was denatured by the addition of 20 μL of trifluoroacetic acid. The samples were vigorously mixed for 10 s and centrifuged for 15 min at 13 900 × *g* at 4 °C. An aliquot with a volume of 20 μL of the resulting clear supernatant was subjected to the OTC determination.

After being thawed, a frozen specimen sample of either muscle or hepatopancreas was homogenized. The homogenate was frozen-dried and pulverized to powders. The extraction buffer (0.01 M ethylenediamine tetraacetic acid-Mc II valine buffer) in equal volume was added and the sample was centrifuged for 15 min at 4000 × *g* at 4 °C. The supernatant was collected whereas the precipitate was re-extracted before being discarded. The collected supernatant was further purified using a 3-mL Sep-Pac C18 column (Waters, Milford, MA, USA) pre-conditioned with methanol (3 mL), followed by Milli-Q water (5 mL), and 0.01 M ethylenediamine tetraacetate-Mc II valine buffer (2 mL) respectively. The samples were eluted using methanol. The eluent was dried by purged nitrogen at 40 °C and dissolved in the mobile phase before the OTC determination.

Oxytetracycline determination in white shrimp biological matrices

The OTC levels in the biological matrices (haemolymph, muscle and hepatopancreas) were determined using a validated HPLC method, modified from the literature (Faroongsarng *et al.* 2007). The reversed-phase HPLC system (Waters) consisting of a pump (Waters 510) connected to an injector (Rhedyne 7125 with injector loop 20 μL), an integrator (Waters 745 data module) with an analytical column of 4 μm, 3.9 × 150 mm, Nova Pak C_{18} protected by a column guard and a UV-visible detector (Waters 484; wavelength of 360 nm) was used. With a flow rate of 1.2 mL min^{-1}, the mobile phase program included: (1) 1 min at 100% 0.01 M oxalic acid, (2) a 17-min linear gradient to 0.01 M oxalic acid:methanol:acetonitrile (70:8:22 v/v/v), (3) 5 min isocratic under the final condition, (4) a 5-min linear gradient to 100% 0.01 M oxalic acid and (5) 3 min under an equilibration condition. Figure 1 illustrates the chromatograms of OTC as well as the internal standard (IS:Chlortetracycline) of the samples extracted from the haemolymph, digestive gland and muscle. As can be seen in Fig. 1, the OTC and IS chromatograms resolved very well in each of the biological matrix samples, indicating the specificity/selectivity of the

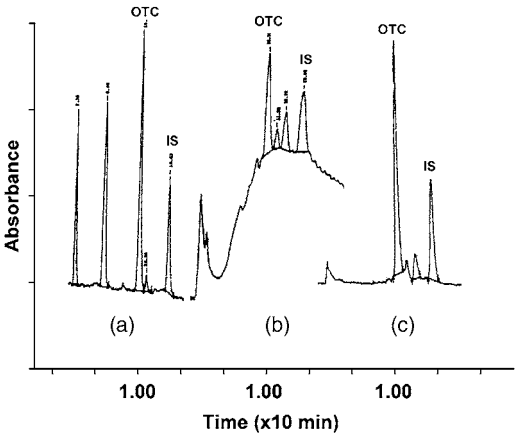

Figure 1 Oxytetracycline (OTC) and internal standard (IS: chlortetracycline) chromatograms of the samples extracted from the haemolymph (a), muscle (b) and digestive gland (c).

Table 1 Summary of the validation parameters for the method of OTC determination in biological matrices of Pacific white shrimps under study

Parameter	OTC in the haemolymph	OTC in muscle	OTC in the digestive gland
Specificity/ Selectivity*	+	+	+
Linearity (r^2)†	0.9994	0.9980	0.9990
LLOQ (μg mL^{-1}, μg g^{-1})‡	0.12	0.18	0.14
Recovery (%)	102.70–107.90	91.07–105.59	94.60–103.33
Precision (% RSD)§	1.72–4.82	3.45–3.68	4.21–10.30
Stability¶	90.01–99.78	90.01–99.78	90.01–99.78

*All chromatograms showed a good resolution for OTC and IS peaks.
†The linearity expressed as the square of correlation coefficient.
‡Lower limit of detection.
§The precision expressed as relative standard deviation on a percentage basis.
¶Stability means the percentage of OTC remaining after a 3-month storage at − 80 °C.
OTC, oxytetracycline; LLOQ, lower limit of quantitations.

method. Table 1 summarizes the validation parameters for the method under study. The recoveries (%*R*) of OTC extracted from the haemolymph, digestive gland and muscle were assessed, indicating a good accuracy (Table 1). The calibration curves for haemolymph, muscle and hepatopancreas samples ranging from 0.38 to 45.00 μg mL^{-1}, 0.50 to 25.00 μg g^{-1} and 1.00 to 1200.0 μg g^{-1}, respectively, showed very good linearity, with an acceptable lower

limit of quantitations (Table 1). Five replications were performed for each of five concentration levels having low relative standard deviations with a good precision. And to ensure that OTC in biological matrices is stable during the storage, the spiked biological matrices were stored at $-80\,^{\circ}C$ for 3 months. The samples were thawed and determined for OTC. The obtained $\%R$ was in the range between 90.01% and 99.78%, indicating that OTC in biological matrices was stable up to 3 months of storage. In sum, it was found that the determination of OTC in the haemolymph, muscle and hepatopancreas of *P. vannamei* using this method was valid.

Pharmacokinetic analysis

Intra-sinus administration

The intra-sinus administration of OTC uses the circulatory system as a medium to transport the drug to sites of action. In the model, the shrimp body was divided into three compartments based on the physiological tissues of interest: the haemolymph, muscle (peripheral tissue) and hepatopancreas, denoted as B, M and L respectively. A scheme of the OTC pharmacokinetic model is illustrated in Fig. 2. The model also includes a foregut, but as the OTC is directly introduced into the haemolymph, the foregut compartment was not involved in the parameters or variables of the study.

The drug-time profiles in the different compartments were assessed on the basis of the drug flow rates, where the shrimp haemolymph was the medium (denoted as Q_I) through each physiological region (the subscript I denoted as B, M or L), and the relative affinity of the drug for the haemolymph and those tissues (R_I). It was assumed that flow was rate limiting and the drug concentration in the haemolymph rapidly reached equilibrium with the drug concentration in the tissue space of each physiological compartment, i.e., the diffusion time for the drug to cross the tissue cell membrane and enter the tissue space is relatively fast compared with the transport of the drug via haemolymph flow. As the haemolymph is conserved within the shrimp body, the flow rates were conditioned as: Q_B equals Q_M+Q_L. Using the law of mass balance, the model is then described by a set of three differential equations:

$$V_B\frac{dC_B}{dt} = -Q_BC_B + Q_M\frac{C_M}{R_M} + Q_L\frac{C_L}{R_L} \quad (1)$$

$$V_M\frac{dC_M}{dt} = Q_MC_B - Q_M\frac{C_M}{R_M} \quad (2)$$

$$V_L\frac{dC_L}{dt} = Q_LC_B - \left[\frac{Q_L + Cl_L}{R_L}\right]C_L \quad (3)$$

where C_I is the drug concentration ($\mu g\,mL^{-1}$ in the haemolymph, or $\mu g\,g^{-1}$ in other tissues) and V_I the volume of distribution for each compartment (mL). Cl_L is the OTC hepatopancreatic clearance ($mL\,h^{-1}$).

Oral administration

As the drug was administered via the gastrointestinal tract, the foregut compartment where OTC is introduced is involved in the model (Fig. 2). It is further assumed that OTC is systemically absorbed via the hepatopancreas, and the drug transport from the haemolymph to the foregut is negligible. The differential equations describing the rate of OTC levels in haemolymph (Eq. (1)) and muscle (Eq. (2)) between intra-sinus and oral administrations are identical, while the OTC level in the hepatopancreas is shown in the following equation:

$$V_L\frac{dC_L}{dt} = Q_LC_B - \left[\frac{Q_L + Cl_L}{R_L}\right]C_L + K_GC_G \quad (4)$$

in which the subscript G represents the foregut compartment while the other variables remain as described for the intra-sinus administration. The foregut connects to the hepatopancreas via the hepatopancreatic ducts, i.e., the drug flows directly from the foregut to the hepatopancreas with an input rate of K_G.

It was impossible to perform the differential equations' regression without data manipulation as the

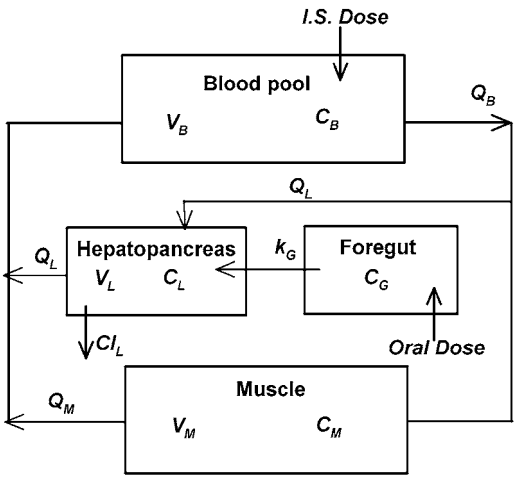

Figure 2 Schematic diagram representing the oxytetracycline (OTC) physiological model. There are two routes of administraion: intra-sinus (is dose) and oral (oral dose).

sampling time intervals selected were too large. It was necessary to perform numerical interpolation of the data among the time intervals so that $\frac{\Delta C_I}{\Delta t}$, which is $\frac{(C_{I,n+1}-C_{I,n})}{(t_{n+1}-t_n)}$ could approach $\frac{dC_I}{dt}$, where Δt approaches zero. The individual pharmacokinetic profile in each of the biological matrices was divided into 2–4 portions to explore the possible best-fit functions for each of the portions. Each was non-linearly fitted with an empirical function having R^2 of not <0.995 (SIGMAPLOT® for WINDOWS v. 10.0, Systat Software, Chicago, IL, USA). The best-fit functions then generated OTC level-time coordinates at pre-assigned 40-s intervals. These generated data were gathered to yield the whole profile with the best possible superimposition on the original pharmacokinetic profile. Further data smoothing was performed as needed. The data were then imported to a spreadsheet (MS Excel 2007, Microsoft, USA) to calculate the differential function as

$$\frac{dC_I}{dt} = \lim_{t\to 0}\frac{\Delta C_I}{\Delta t} \approx \frac{(C_{I,n+1} - C_{I,n})}{(t_{n+1} - t_n)}$$

The numerical data were fitted with differential equations of the proposed physiological model using commercial software (Systat for WINDOWS V. 12, Systat Software). In addition, as OTC entered the hepatopancreas via the foregut in oral administration, the profile of the drug in the foregut was simulated based on first-order kinetics using the initial drug concentration of the medicated feed and the absorption constant taken from the literature (0.2986 h^{-1}: Faroongsarng *et al.* 2007) in order to complete the nonlinear regression.

Results

Physiological-based model pharmacokinetics of oxytetracycline in Pacific white shrimp

An attempt to simultaneously fit the OTC levels in the haemolymph (C_B), muscle (C_M) and hepatopancreas (C_L) with time after intra-sinus and oral administrations was made. Instead of the several parameters shown in Eqs. (1)–(4), the equations were simplified as

$$\frac{dC_B}{dt} = -\alpha_1 C_B + \beta_1 C_M + \gamma_1 C_L \qquad (1\text{prime};)$$

$$\frac{dC_M}{dt} = \alpha_2 C_B - \beta_2 C_M \qquad (2\text{prime};)$$

and

$$\frac{dC_L}{dt} = \alpha_3 C_B - \gamma_2 C_L \qquad (3\text{prime};)$$

Table 2 Estimated value of parameters after OTC (oxytetracycline) intra-sinus administration at 10 μg g^{-1} body weight dose into white shrimps using Eqs. (1′)–(3′)

Pharmacokinetic profile	Parameter estimate (SE)			
	α_i	β_i	γ_i	R^{2*}
C_B†	0.552 (0.023)	0.396 (0.049)	0.036 (0.007)	0.9373
C_M‡	0.164 (0.013)	0.198 (0.019)	–	0.8266
C_L§	0.412 (0.053)	–	0.105 (0.017)	0.6571

*Coefficient of multiple determination square: $R^2 = 1 - (SSE/SSTO)$, where SSE and SSTO are the sum square error and the sum square total of multiple regression according to Eqs. (1′)–(3′).
†OTC in haemolymph profile fitted into Eq. (1′).
‡OTC in muscle profile fitted into Eq. (2′).
§OTC in hepatopancreas profile fitted into Eq. (3′).

for intra-sinus administration or

$$\frac{dC_L}{dt} = \alpha_3 C_B - \gamma_2 C_L + K_G C_G \qquad (4\text{prime};)$$

for oral administration

$$\alpha_1 = \frac{Q_B}{V_B}, \beta_1 = \frac{Q_M}{R_M V_B}, \gamma_1 = \frac{Q_L}{R_L V_B}$$

where

$$\alpha_2 = \frac{Q_M}{V_M}, \beta_2 = \frac{Q_M}{R_M V_M}$$

$$\alpha_3 = \frac{Q_L}{V_L}, \gamma_2 = \frac{Q_L + Cl_L}{R_L V_L}$$

The interpolated data for pharmacokinetic profiles were numerically fitted into Eqs. (1′), (2′) and (3′) and (1′), (2′) and (4′), yielding α_i, β_i, γ_i as well as K_G and their standard errors of estimation (SE), as tabulated in Tables 2 and 3. Figures 3 and 4 show the intra-sinus and orally administered OTC pharmacokinetic profiles according to the simultaneous regressions of Tables 2 and 3, respectively, compared with actual data coordinates.

The squares of coefficients of multiple determinations R^2 were in the range of 0.6568–0.9904 (Tables 2 and 3). The equations were the best fits into the intra-sinus administered haemolymph and orally administered hepatopancreas profiles with R^2 values of 0.9373 and 0.9904, respectively, while the R^2 values of the muscle profiles were the lowest (intra-sinus administered R^2 of 0.6571 and orally administered R^2 of 0.6568).

Table 3 Estimated values of parameters after OTC (oxytetracycline) oral administration at 50 μg g^{-1} body weight dose into white shrimps using Eqs. (1′), (2′), and (3′)

	Parameter estimate (SE)				
Pharmacokinetic profile	α_i	β_i	γ_i	K_G	R^{2*}
C_B†	0.161 (0.020)	0.035 (0.015)	0.009 (0.001)	–	0.9007
C_M‡	0.047 (0.006)	0.052 (0.006)	–	–	0.6568
C_L§	6.781 (0.410)	–	1.021 (0.028)	194.29 (3.2)	0.9904

*Coefficient of multiple determination square: $R^2 = 1 - (SSE/SSTO)$, where SSE and SSTO are the sum square error and sum square total of multiple regression according to Eqs. (1′), (2′), and (3′).
†OTC in hemolymph profile fitted into Eq. (1′).
‡OTC in muscle profile fitted into Eq. (2′).
§OTC in hepatopancreas profile fitted into Eq. (3′).

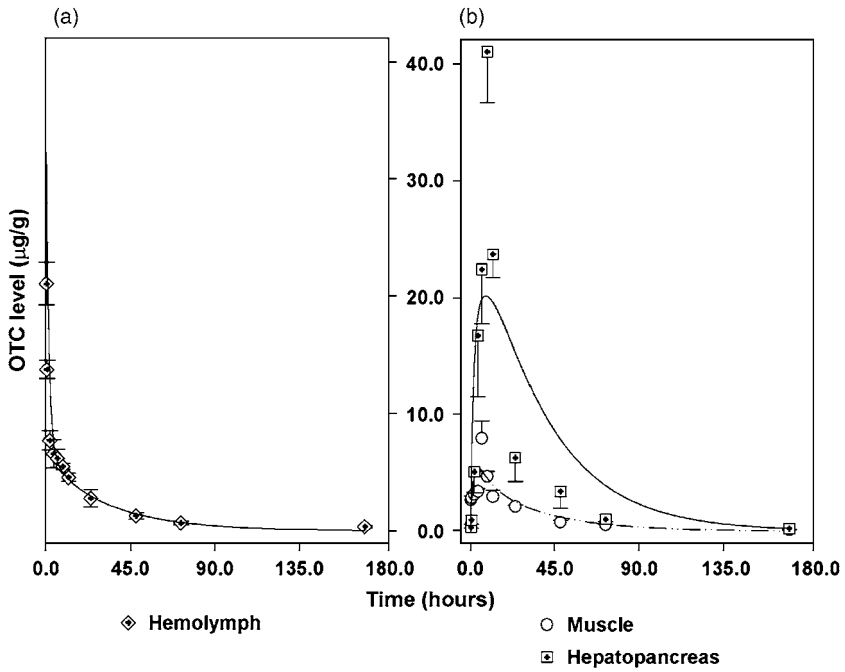

Figure 3 Pharmacokinetic profiles of intra-sinus administered oxytetracycline (OTC) in Pacific white shrimps (a) OTC level in haemolymph (b) OTC levels in muscle and in hepatopancreas. The lines represent the profiles according to the physiological model predictions [Eqs. (1′)–(3′)].

Discussion

It is observed that the proposed model may be applicable to the simultaneous pharmacokinetic profiles of OTC administered to *P. vannamei* of the inter-moult C-D_0 stage (Tables 2 and 3 and Figs 3 and 4). As can be seen in Tables 2 and 3, the R^2 values of the muscle profiles were relatively low. On the one hand, from a statistical point of view, it is common for systemic errors of entrance compartments to be lower than those of peripheral ones when it comes to simultaneous regression, which in turn, might also cause the R^2 values of associated muscle profiles to be lower.

On the other hand, unlike the typical vertebrate in which the blood circulation is well defined as a closed system, the circulatory system of Decapod crustaceans is classed as either 'open' (Dall *et al.* 1991) or 'partially closed' (McGaw 2005). The shrimp cardiovascular system can be subdivided into three parts: (1) the heart, (2) blood or haemolymph and (3) the distribution pathway or arterial system (Guadagnoli, Tobita & Reiber 2007). The haemolymph leaves the

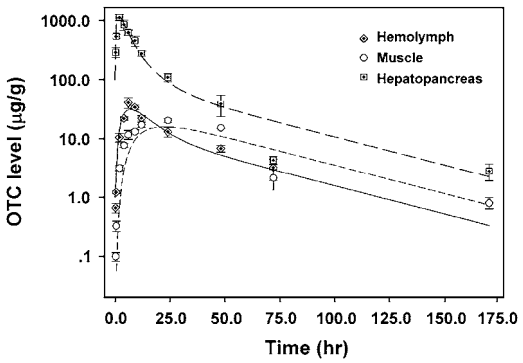

Figure 4 Pharmacokinetic profiles of orally administered oxytetracycline (OTC) in Pacific white shrimp compared between actual data coordinates and predicted lines according to the model of Eqs. (1′), (2′), and (4′).

heart via the arterial system. The arteries then subdivide into arterioles, which perfuse all areas of the body. The arterioles divide into fine capillary-like vessels, most blind ending ('open'), but some finishing in complete capillary beds ('closed'), e.g. in the areas of the antennal gland and supraesophageal ganglion. After passing through these capillaries, the haemolymph drains into a series of sinuses that guide it to the gills to become reoxygenated, and finally to complete the system the oxygenated haemolymph enters the branchio-cardiac veins to the pericardial sinus and then to the heart to begin the circulation again. Thus, OTC kinetics in shrimp body could be modelled only to some extent because the model assumption is flow limited whereas shrimp's circulatory system included not only haemolymph flow but also perfusion.

Relative affinity of drug for haemolymph and tissue

By comparison, for the coefficients of Eqs. (1) with (1′), (2) with (2′), (3) with (3′) and (4) with (4′), the calculation of the relative affinities of drug for haemolymph and tissue (R_I) at equilibrium was possible. Unfortunately, other parameters such as flow rates (Q_I), volumes of distribution (V_I) and hepatopancreatic clearances (Cl_L) could not be explicitly solved because of an insufficient number of equations compared with the number of parameters being calculated. A prior study performed on vertebrates used the measured blood flow to the organs to estimate Q_I in order to cope with the insufficiency (Tsuji, Yoshikawa, Nishide, Minami, Kimura, Nakashima, Terasaki, Miyamoto, Nightingale & Yamana 1983). However, the method used in that study could be used with an animal having a closed circulatory system because the distribution of the drug is assumed to be governed by the rate of blood flow only. Nevertheless, the blood flow measurement for shrimp might not be as appropriate as for vertebrates due to the fact that the crustacean circulatory system is totally different.

It was found that the R_M and R_L values for intra-sinus and oral administrations were 0.828 and 6.277, and 0.904 and 14.374 respectively. Based on the model, R_I is defined as the partition coefficient for the distribution of OTC from blood into tissue where the drug in blood leaving the tissue achieves rapid equilibrium with the drug within the tissue (Welling 1986), i.e., $R_I = \frac{C_I}{C_o}$, where C_I and C_o are the total concentrations in tissues and in blood leaving the tissues respectively. As the R_M of intra-sinus administration is comparable to that of oral administration (intra-sinus R_M of 0.828 vs. oral R_M of 0.904), it is deduced that OTC distributes in shrimp muscle in an essentially identical manner regardless of the route of administration, i.e., the drug passively distributed from the haemolymph to muscular tissues. The accumulation of OTC in muscle is less likely for R_M, being slightly lower than unity, i.e., the total concentration is less than or, at best, equal to that in the haemolymph. Thus, with an appropriate period of time after stopping dosing, OTC would be disposed out of the shrimp body, resulting in the edible portion being free from drug residue and safe for consumption. Previous studies with different doses and dosing regimens (single dose of 10 $\mu g\,g^{-1}$ body weight with intra-sinus administration: Chiayvareesajja *et al.* 2006) and multiple doses (OTC level of 5 $g\,kg^{-1}$ feed with natural feed orally for 14 days: Gomez-jimenez, Espinosa-plascencia, Valenzuela-villa & Bermudez-almada 2008) suggested an appropriate withdrawal time of 7–14 days. As the tolerance of 2 ppm for OTC in an aquaculture product has been codified previously (FDA/CVM, 2007), it is thus demonstrated in the current study by Fig. 4 that at least 7 days could be sufficient for withdrawal time, which is comparable to previous findings.

The analysis of oxytetracycline distribution in the hepatopancreas

It was found in the case of intra-sinus administration that the total concentration of OTC in the hepatopancreas was approximately sixfold higher than that in the haemolymph ($R_{L,is}$ of 6.277). This finding is consistent with that of Chiayvareesajja *et al.* (2006), i.e.,

a significant amount of OTC in the haemolymph was redistributed into the hepatopancreas. Moreover, in oral administration, it was observed that the hepatopancreatic level was the highest compared among the biological matrices tested, which was also consistent with previous reports (Faroongsarng *et al.* 2007; Gomez-Jimenez *et al.* 2008). The hepatopancreatic OTC is not only redistributed from the haemolymph but also directly absorbed from the foregut. This could yield a total concentration of OTC in the organ considerably greater than that in the haemolymph, reflecting an even higher value of R_L ($R_{L,oral}$ of 14.374). In addition, previous studies dealing with OTC bioavailability assessment in this and related species showed highly variable results, i.e., the oral bioavailability varied from 43.2% (*Penaeus japonicas*: Uno, 2004) to 92.2% (*Litopenaeus setiferus*: Reed *et al.* 2006). Those reports made their assessment based on well-established vertebrate pharmacokinetics. As shrimp hepatopancreatic functions include both drug absorption and disposition, the kinetics of the drug may be somewhat different from vertebrates, where such functions are performed by a separate organ. Thus, the previously reported bioavailability variations may be attributed to the variability of not only the extent of absorption but also drug redistribution from the blood to the hepatopancreas.

USP veterinary pharmaceutical information monographs recommend OTC to be used as an infectious treatment in such aquaculture products as catfish and salmonaid (USP, 2003). But infectious disease management might be problematic in shrimp farms as disease progression is rapid and it is difficult to administer the drug due to the fact that infected shrimps' feeding behaviour is limited. The role of antibiotics as a disease-preventing agent in shrimp farms was very first experimentally demonstrated in *Penaeus aztecus*, where it was recommended that the medicated feed containing 0.5–1.0% OTC could prevent the bacterial infection (Corliss, Lightner & Zeian-eldin 1977). Recently, Center for Veterinary Medicine, US FDA approved a few antibiotics including OTC, florfenicol, sulfadimethoxine/trimethoprim and sulfamerazine for use as medicated feeds for disease control in aquaculture (FDA/CVM, 2007). However, the dose level used in the current study was far higher than that in normal practice. As a result, the maximum concentration in the hepatopancreas was approximately fivefold higher than that reported by Gomez-Jimenez *et al.* (2008), which simulated therapeutic use of OTC in an aquaculture-like environment. The current study utilized forced feeding, in which the pre-designed dose could be completely introduced into animals, whereas that of Gomez-Jimenez *et al.* (2008) allowed shrimp feed naturally, in which only some portion of the drug entered shrimp body depending on the feeding behaviour, and the medicated feed used in the current study contained OTC at a concentration of 20 g kg^{-1} feed, which was fourfold higher than that used by Gomez-Jimenez *et al.* (2008).

An analysis of OTC redistributing into the hepatopancreas in oral administration was undertaken in the current study. It was assumed that the amount of OTC in the hepatopancreas was a linear combination of the amount of drug absorption and the drug redistribution. A numerical subtraction of the hepatopancreatic OTC levels simulated according to a reduced model in which the OTC input is solely due to absorption via the foregut, from those of Eq. (4′), could result in a redistribution of OTC in the hepatopancreas, i.e.,

$$C'_L(t) = C_L(t) - C^*_L(t) \quad (5)$$

where $C'_L(t)$ and $C_L(t)$, are the hepatopancreatic levels of OTC redistributed from the haemolymph, and that based on numerical integration of Eq. (4′) respectively. $C^*_L(t)$ is the hepatopancreatic OTC level simulated according to a reduced model in which the drug input is solely due to oral absorption, whose differential equation is illustrated as follows:

$$\frac{dC^*_L}{dt} = -\gamma_2 C_L + K_G C_G \quad (6)$$

As OTC was introduced to the same species, C'_L should exhibit a kinetic pattern identical to the hepatopancreatic level-time profile after intra-sinus administration. In order to verify this, C'_L was plotted against the OTC levels in the hepatopancreas after intra-sinus administration ($C_{L,is}$) and is shown in Fig. 5. As can be seen, C'_L might exhibit a linear relationship with $C_{L,is}$ illustrated by a linear trend line. The line passes the origin and exhibits a slope (S; SE) of 0.114 and 0.013 with a coefficient of correlation (R^2) of 0.922. The inset of Fig. 4 illustrates that C'_L could be successfully superimposed on the pharmacokinetic profile of intra-sinus administered hepatopancreatic OTC. It was noted that C'_L in the inset of Fig. 5 was adjusted by S so as to be comparable with $C_{L,is}$. As intra-sinus (D_{is}) and oral (D_{oral}) doses were 10.0 and 50.2 μg g^{-1} shrimp body weight, respectively, with essentially the same kinetics, it is presumably suggested that an ideal slope of the plot between C'_L and $C_{L,is}$ would simply be the dose ratio ($\frac{D_{is}}{D_{oral}}$), which is 0.1992. The obtained value of S from the trend line in

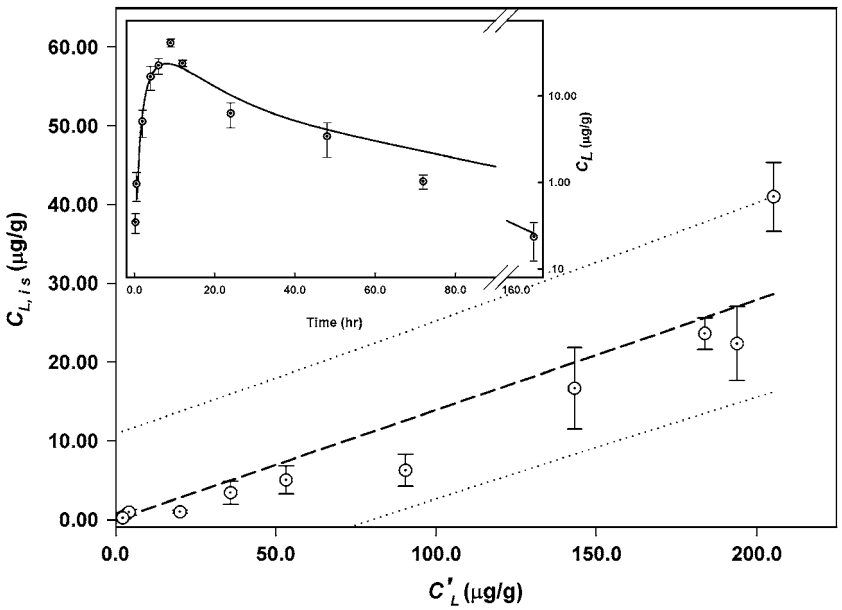

Figure 5 The plot between hepatopancreatic oxytetracycline (OTC) redistributed after oral administration (C'_L) and OTC level after intra-sinus administration ($C_{L,iv}$) showing a linear relationship (broken trend line) as well as 95% confidence prediction intervals (dotted lines). The inset shows the kinetic profile of C'_L after adjusting by slope of the trend line compared with the actual results from intra-sinus administration.

Fig. 5 deviates from the ideal slope by -42.92%, whereas the relative standard error (RSE: SE/mean) of S is $\pm$ 11.40%. It is obvious that the negative deviation is far too high to be solely the result of experimental bias. In other words, there might be another cause or causes. As OTC enters the circulatory system via the hepatopancreas, which has been recognized as a major site of biotransformation of most xenobiotics in crustacean (James & Boyle 1998), the drug may be partially disposed before redistribution. Thus, it is hypothesized that OTC might undergo extensive 'first-pass' elimination, resulting in the loss of C'_L as shown by a significant negative deviation. In addition, the loss might account for approximately 30–40% of the amount of OTC redistributed to the hepatopancreas.

Oxytetracycline disposition via the hepatopancreas

The disposition of OTC in the proposed model is described by hepatopancreatic clearance (Cl_L). As discussed above, the values of Cl_L could not be explicitly stated due to the shortage of number of solving equations. However, it is possible to compare γ_2 coefficients so as to examine the behaviour of drug disposition via the hepatopancreas. Assuming that the volumes of distribution and flow rates were identical for the drug taken by the animals of the same species, the ratio of γ_2 coefficients between oral and intra-sinus administrations would be

$$\frac{\gamma_{2,\text{oral}}}{\gamma_{2,\text{is}}} \approx \frac{Q_L + Cl_{L,\text{oral}}}{Q_L + Cl_{L,\text{is}}} \frac{R_{L,\text{is}}}{R_{L,\text{oral}}} \qquad (7)$$

Should there be a linear pharmacokinetic disposition, the ratio of γ_2 coefficients would approach $\frac{R_{L,\text{is}}}{R_{L,\text{oral}}}$ for the clearance is identical and the first term of the right-hand side of Eq. (7) becomes unity. It is observed that $\frac{\gamma_{2,\text{oral}}}{\gamma_{2,\text{is}}}$ is 9.72 (Tables 2 and 3) whereas $\frac{R_{L,\text{is}}}{R_{L,\text{oral}}}$ is 0.44. This significant discrepancy demonstrates that linear kinetics for OTC disposition is unlikely. Furthermore, it was found that the hepatopancreatic OTC levels at each time interval after oral administration were 8.8-fold greater than at after intra-sinus counterparts (the reciprocal of slope: S of the trend line in Fig. 4) while the oral dose was fivefold the intra-sinus one. It seems that clearance might be dependent on the amount of OTC present. It has been reported previously that the hepatic removal of vertebrates exhibited Machelis–Menten-type kinetics, in which the hepatic drug clearance was non-linearly modelled based on substrate concentration dependence (Liu & Pang, 2006). The hepatopancreatic disposition found

in *P. vannamei* seems to be consistent with the hepatic disposition of vertebrates. Thus, it is deduced that shrimp hepatopancreatic drug disposition kinetics may be non-linear and dependent on the amount of drug present in the organ. It is necessary that further studies be carried out on hepatopancreatic clearance to better understand the mechanism of OTC disposition.

In summary, the proposed model was able to address how OTC distributed and was disposed in tissues of interest, for this particular study: muscle and hepatopancreas. It was deduced that OTC did not accumulate in muscular tissues, which allows a time for deputation before consumption as food, and the model could also predict OTC disposition behaviour via the hepatopancreas.

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A Toxicokinetic Study of Oxytetracycline Antibiotics in Farmed Pacific White Shrimp, Penaeus vannamei

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A Toxicokinetic Study of Oxytetracycline Antibiotics in Farmed Pacific White Shrimp, Penaeus vannamei

Authors and affiliations:

*Damrongsak Faroongsarng

Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand

Sommai Chiayvareesajja

Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai 90112, Thailand

Yongyuth Theapparat

Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand

*Corresponding author: Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand
e-mail:damrongsak.f@psu.ac.th
Phone: +66-74-288-841
FAX: +66-74-428-148

Abstract

Toxicokinetics has demonstrated abnormal signs in drug distribution/disposition without waiting until the drug damages tissues/organs. It is a study of the kinetic assessment of administering high-dose of oxytetracycline (OTC) to white shrimp. Male Penaeus vannamei, in the C-D_0 molting stage, were force fed with medicated feeds at various accurate dose levels including 500, 1,000, and 2,500 mg/kg-body weight (BW). After dosing with different time intervals, hemolymph, muscle, and hepatopancreas were collected, and assayed for OTC by validated HPLC method. The simulated profile based on the maximum recommended dose was tested to approach the systemic level where the drug was anticipated not to cause significant toxic responses. OTC kinetic profiles in the hemolymph were fitted into the flow limited model having r^2 of 0.8341-0.9373. The relative affinities for the muscle and hepatopacreas changed at dose level exceeding 1,000 mg/kg-BW. While hepatopancreatic clearance was non-linearly related with dose, the persistence of OTC in muscle after 2,500 mg/kg-BW-dosing was observed to indicate abnormalities in drug distribution/disposition. It was hypothesized that the pharmacokinetic alteration after extreme dosing was due to induction of functional abnormalities in hepatopancreas. In addition, a single administration of OTC at 1,000 mg/kg BW was anticipated to be a tolerated dose.

Keywords: Oxytetracycline, Toxicokinetics, Relative affinity for tissues, hepatopancreas, muscle, Penaeus vannamei, Pacific white shrimp

Since their discovery in the late 1940s, tetracycline antibiotics have been of interest for use with food producing animals because of their broad spectrum activity with low cost and ability to increase yields especially 'caged 'animals. Oxytetracycline (OTC), one of the tetracycline antibiotics derived from the soil-dwelling bacterium Streptomyces rimosus, has been utilized in shrimp farms worldwide. The United States Pharmacopoeia veterinary pharmaceutical information monographs recommend that OTC be used as an antibiotic for treatment (USP 2003) of shrimp disease. However infectious disease management might be difficult because some infections progress rapidly and there has been a problem in drug administration due to the fact that infected shrimp don't like to feed. The role of OTC as a disease-preventing agent in shrimp farms has been experimentally demonstrated in Penaeus aztecus. It was recommended that the medicated feed containing 5-10 mg/kg OTC could prevent bacterial infection (Corliss et al. 1977). Recently, the Center for Veterinary Medicine, the United States Food and Drug Administration approved a few antibiotics including OTC for use in medicated feeds to control bacterial disease in aquaculture (FDA/CVM 2007).

The maximum dose of OTC has been recommended at 4.5 g/kg feed with a 14-day period of treatment. Although it has been confirmed that shrimp fed with OTC at this level did not show either any significant toxic responses or any negative impact on growth rate, slight histological change in hepatopancreas, i.e., tubule atrophy was observed (Bray et al. 2006). It might be because multi daily dosing caused the drug accumulation up to the level which adversely influenced some specific tissues/organ. Moreover, unfortunately the use of the medicated feed is becoming a problem because of the increase in bacterial resistance and elevation of the minimum inhibitory concentration (MIC) of OTC to microorganisms isolated from shrimp farms. For example, the MIC for Vibrio sp. in Thailand increased to up to 100 mg/L (Chanratchakool et al. 1995) which was significantly greater than that reported in the

U.S. (Mohney et al. 1992). Some reports have found MIC levels as high as 301.0 mg/L (Roque et al. 2001). It may be because this recommended level is just sufficient as it just approaches the lower limit of the effective range suggested by Corliss et al (1977). The farmers might use OTC in high concentrations that exceed this dose level to cope with the bacterial infection. However, of major concern is the effect these high levels of antibiotic might have on human health. Antibiotic residues in shrimp may end up in humans and select for antibiotic resistant human pathogenic bacteria. It might even also cause direct toxic effect to shrimp farmers from handling the drug at such high concentrations (Park et al. 1994). There have been a number of pharmacokinetic studies of OTC in cultured shrimp (For examples: Uno et al. 2006; Reed et al. 2004, 2006; Chiayvareesajja et al. 2006) to assess the changes in OTC levels in the shrimp body with time and to suggest proper handling procedures to minimize contact with antibiotic residues. However, those reports were based on single dosing where the drug level may be considerably lower than usual because OTC might accumulate as the result of multi daily dosing such as occurs in normal practices. Furthermore, OTC might be at toxic levels if the recommended doses are exceeded. To achieve drug levels that are comparable to or exceed accumulated levels as a result of daily dosing practices is difficult but using a single administration at a high dosage level may be an alternative and provide some useful information. While information of the toxicity of OTC as well as its impact on growth of cultured shrimp has been available (Bray et al. 2006), there is very little kinetic information on high doses of OTC. Thus, the current study aims to provide an OTC toxicokinetic assessment, i.e., a kinetic study in a high dosing situation, in farmed Pacific white shrimp.

Materials and Methods

Chemicals

Oxytetracycline (OTC) in its dihydrate form (Sigma, St. Louis, MO, USA) was employed. Chlortetracycline hydrochloride (Sigma, St. Louis, MO, USA) was used as an internal standard for the determination of OTC in hemolymph and muscle. Unless otherwise indicated, other chemicals used were of analytical or HPLC grade.

Experimental Animal and Aquaria Conditions

Sub-adult male Pacific white shrimp, Penaeus vannamei, were preconditioned in the manner described in the previous study (Faroongsarng et al. 2009). Briefly: The animals weighing 14-22 g with a carapace length of 2.30-3.00 cm were collected from a local farm (Pattani, Thailand) and acclimated in a close system with a 3-ton concrete tank filled with continuously oxygenated natural seawater and conditions as per: 28±2 C, 20±2 ppt salinity, pH 7.6±0.5, 200 ppm total hardness, and 12 h of light per day. Shrimps were fed with commercial pellets once a day at a rate of approximately 2-3% body weight. The healthy animals were randomized into 4 groups according to dosage levels of 0 (negative control), 500, 1,000, and 2,500 mg OTC /kg BW. Thirty-six animals were enrolled in each dosage group whereas 12 were in the control group making a total number of 120. No mortality was observed during the study.

Dosage Administrations and Sample Preparations

For dosage levels of 500, and 1,000 mg/kg BW, the medicated feeds were prepared by mixing commercial feed pellets previously ground to powders with OTC, to concentrations of 200, and 400 mg/g. The mixture was then suspended in purified water at 1:1 w/v to form a paste. For a dose of 2,500 mg/kg BW, the drug in powder form was directly mixed with purified water. Each of the medicated feed pastes was packed into a 1-mL syringe fitted with a blunt-end 18G needle, and the tip of the needle was fitted with a silicone tube to protect the shrimp's mouth. The volume of the paste was assigned individually to each of the sampled

animals to meet an accurate dose on the basis of shrimp's body weight. After molting, each of the shrimp samples was kept individually in a 2.5-L glass aquarium to protect the contamination of drug among aquaria. Prior to OTC administration, shrimp were fasted for approximately 2 days so their molt cycle entered the C-D_0 stage (Dall et al. 1991). Each individual animal was forced fed orally with medicated feed at the pre-assigned dosage level until the foregut was full (approximately 0.5% of body weight). Three individual shrimp were sampled for each time interval of 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, and 170 h after oral administration. After administration, feeding was restarted 24 h post-dosing and continued once a day during the study.

After the shrimp were sampled, 1.5-mL hemolymph was removed through the ventral-sinus cavity by using a 5-mL syringe, fitted with a 19-G needle and containing 98% N-ethylmaleimide crystals (~0.01g) as an anticoagulant. Afterwards, the normally edible muscle and hepatopancreas were collected. Samples were frozen and maintained at -70 C prior to OTC extraction. Individual samples were assayed for OTC.

Analytical Procedures

The HPLC system (Agilent 1,200 series binary LC system, Palo Alto, CA, USA) with a photodiode array detector and an analytical column of 5 µm, 4.6X250 mm (Purospher star RP-18 endcapped, Merck, Darmstadt, Germany) was used to determine OTC in shrimp's biological matrices. The mobile phase with a flow rate of 1 mL/min as well as the method of HPLC separation was modified from Li et al (2008).

Frozen hemolymph samples were thawed and vigorously mixed for 15 sec to assure homogeneity. A sample volume of 100 µL was placed in a 1.5-mL Eppendorf tube, spiked with internal standards and diluted 1:1 with purified water, and 20 µL of trifluoroacetic acid

was added to denature the protein residues. The sample was then vigorously mixed for 1 min and centrifuged for 15 min at 13,900 x g at 4 C. A 20-µL aliquot of the resulting clear supernatant was assayed for OTC. Individual samples of frozen muscular and hepatopancreatic tissues were thawed, homogenized and spiked with an internal standard. The homogenates were lyophilized then pulverized to powders and diluted with an equal volume of 0.01 M ethylenediamine tetraacetic acid- McIlvaline buffer. The samples were centrifuged for 15 min at 4,000 x g at 4 C. The supernatant was collected while the precipitate was re-extracted before being discarded. The C18 sorbent solid phase extraction (SPE: 3 mL Sep-Pak Vac C18, Waters, Milford, USA) was employed to accomplish cleanup and simultaneous concentration. The collected supernatant was cleaned by the SPE, pre-conditioned with methanol (3 mL) followed by Milli-Q water (5 mL), and 0.01 M Ethylenediamine tetraacetate-McIlvaline buffer (2 mL), respectively. The samples were then eluted by methanol. The eluant was dried by purging with nitrogen at 40 C and dissolved in the mobile phase of the analytical column ready for OTC determination. The method was validated for specificity, calibration, sensitivity, and precision prior to the actual determination. The validated results are tabulated in Table 1.

Pharmacokinetic Modeling for OTC Administered into P. vannamei in Extreme Doses

The kinetic profiles of OTC in hemolymph after various extreme dosing were modeled by flow limited pharmacokinetics (Faroongsarng et al. 2009). The model speculates about the major distribution of OTC in 2 tissue compartments namely the digestive gland (hepatopancreas) and muscle together with the blood pool as a medium for transport of the drug. As a result, three differential equations describing the changing rates of the drug concentration in blood pool (hemolymph), muscle, and hepatopancreas, respectively, have been proposed (Faroongsarng et al. 2009):

$$V_B \frac{d C_B}{dt} = -Q_B C_B + Q_M \frac{C_M}{R_M} + Q_L \frac{C_L}{R_L} \quad (1)$$

$$V_M \frac{d C_M}{dt} = Q_M C_B - Q_M \frac{C_M}{R_M} \quad (2)$$

$$V_L \frac{d C_L}{dt} = Q_L C_B - [\frac{Q_L + Cl_L}{R_L}] C_L + K_G C_G \quad (3)$$

Where, C_I, V_I, Q_I, and R_I are the drug concentration in compartment I, volume of distribution of compartment I, hemolymph flow rate through compartment I, and the relative affinity of the drug for the tissues of compartment I, respectively. The notations B, M, L, and G represent the hemolymph, muscle, hepatopancreas, and foregut compartments. K_G and Cl_L are the drug uptake constant and hepatopancreatic clearance, respectively. Each of the hemolymph level-time data was fitted into Equation 1 by the software (Pheonix WinNonlin v. 6.1, Pharsight Corporation, St. Louis, MO, USA) to assess the kinetic parameters involved. The obtained parameter estimates were then used as constraints for kinetic fitting of the OTC levels in muscular and hepatopancreatic tissues into Equation 2 and 3 to construct the profiles of the drug in the muscle and hepatopancreas, respectively.

The Simulation of OTC Pharmacokinetic Profiles after the Recommended Maximum Dose

In order to appraise the available level of the systemic drug after P. vannamei was fed with the recommended maximum dose of 4.5 g OTC/kg feed, a level previously shown not to cause a significant toxic response (Bray et al. 2006), the OTC concentration in the hemolymph-time profile was simulated using commercial software (Pheonix WinNonlin v. 6.1, Pharsight Corporation, St. Louis, MO, USA). The setting was based on an OTC daily dosing according to the dosing regimen of Bray et al (2006), i.e., shrimp was fed at the maximum dose 5 times a day at 08.00, 10.30, 12.00, 14.30, and 16.00 h corresponding to the average feed rate of 155 mg/kg BW/day. A modified two-compartment pharmacokinetic

model was used, and the model parameters were obtained from the literature (Chiayvareesajja et al. 2006).

Results

Figure 1 shows hemolymph level-time profiles after a forced-fed single oral dosing of OTC at various extreme doses including 500, 1,000, and 2,500 mg/kg BW. It was demonstrated that the data were successfully fitted into the flow limited model with an r^2 of between 0.8341 and 0.9373. The estimates of the volumes of the distribution of OTC in the tissues of interest including blood pool (hemolymph: V_B), muscular tissues (V_M), and hepatopancreatic tissues (V_L) as well as the standard errors in parentheses according to the curve fitting of the OTC hemolymph concentration level-time profile after dosing with 500 mg/kg BW dosing were 0.218 (.064), 3.67 (1.20), and 0.304 (.093) mL/g BW, respectively. These volumes were used as the constraints in fitting the data for administration of higher dosages. As a result, the relative affinities for the tissues of interest (e.g. R_M and R_L) with their standard errors in parentheses corresponding to various dose levels were estimated as follows: R_M corresponding to 500, 1,000, and 2,500 mg/kg BW dosing were 0.799 (.110), 0.842 (.115), and 3.28 (.044), respectively. And, R_L corresponding to the same order of dosage levels as above were 21.2 (.013), 27.4 (.012), and 13.2 (.020), respectively.

The plots of the OTC levels in muscular tissues *versus* time were compared among extreme dose administrations as illustrated in Figure 2. With coordinates, the profiles were constructed by force fitting into Equation 2 using the volume parameters estimated from curve fitting after 500 mg/kg BW dosing, and the estimates of R_M- and R_L-parameters as the constraints. These parameters were also used as the constraints in curve fitting into Equation 3 resulting in the hepatopancreatic profiles showed in Figure 3 as well as hepatopancreatic clearance (Cl_L) and drug uptake constant (K_G) of each of the profiles. It was found that the

data were successfully fitted into the model of Equation 2 and 3 with an r^2 of between 0.7267 and 0.9840. The obtained Cl_L as well as K_G was then plotted against dose level (D) (Figure 4). In addition to coordinates shown in Figure 4, the curve illustrating the change of Cl_L as a function of D was constructed by non-linear fitting according to Michaelis–Menten kinetics:

$$Cl_L = \frac{Cl_L^{\max} \cdot D}{K_d + D} \quad (4)$$

Where $Cl_L^{\max}$ and K_d are the clearance of maximum capacity and Michaelis–Menten constant, respectively. The curve was extrapolated to a dose as low as 50 mg/kg BW by taking the data coordinate from literature (Faroongsarng et al. 2009) to participate in curve fitting. It was found that the data were successfully fitted into Equation 4 with r^2 of 0.8090 yielding the estimates of $Cl_L^{\max}$ and K_d with their standard errors in parentheses of 0.34 (0.04) mL/h/g and 83.7 (67.0) mg/kg BW, respectively.

Figure 5 compares the actual hemolymph concentration-time profile after the shrimp were orally administered at a dose of OTC of 1,000 mg/kg BW with that of the simulated systemic OTC levels during the duration of the first 6-days of treatment according to the multiple dosing scenarios described earlier. It is noted that the arrows identified the times of dosing to within a day. It was anticipated from the simulation that OTC accumulated with increasing fluctuations in the drug level due to multiple doses where the dosing interval was shorter than the elimination half-life. It was expected that the level attained a steady state after 3-4 days with an average level C_{av} of approximately 60 μg/mL having the daily-interval area under the curve with an AUC of 1,500 (μg/mL)h. In addition, non-compartmental analysis of the pharmacokinetic profile after dosage of 1,000 mg/kg BW (NCA: Pheonix WinNonlin)

revealed a maximum concentration C_{max} of 73.12 µg/mL, the time to reach C_{max} (t_{max}) of 6 h, and an AUC of, 3,085 (µg/mL)h.

Discussion

In a toxicity study of a drug, the animals are subjected to chronic expose to the drug in increasingly extreme doses with a sufficiently long period of time to allow for observations of the histological changes as well as any toxic responses. This type of study is laborious and time consuming, and the data might be lost due to survival problems. In pharmacodynamic principles, the extents of therapeutic/toxic responses are related to the systemic drug level. Thus, the drug level or more particularly the so-called "toxicokinetic study": the kinetic study of the systemic drug level after an extreme dose may be an alternative method used to assess the drug toxicity so as not to encounter the difficulties described above. However, the toxicity of the drug not only depends on the level but the length of the exposure time. A toxicokinetic study might provide only the drug toxicity potential without any observable toxic responses. In the current study, three levels of extreme doses of OTC including 500, 1,000, and 2,500 mg/kg BW were accurately given to white shrimp in a single dose fashion. Since shrimp were exposed to the drug over a short duration compared to the multiple dosing used in classical toxicity studies, no mortality was observed.

The model fitting yielded an estimate of the shrimp's body volume into which the OTC was distributed of 4.19 mL/g BW (summation of V_B, V_M, and V_L). The value seemed questionable as it has been claimed that the used model was physiologically based (Faroongsarng et al. 2009). The total physiological volume should be close to unity since the density of the shrimp's body is at least a little more than 1. However, the extracellular volume of the related species, which is mostly hemolymph was found to vary between 0.15 and 0.35 mL/g BW (Smith and Dall, 1982). The estimate of V_B of 0.218 mL/g BW in the current study

was essentially comparable to this physiological volume. In contrast, the estimated V_L was as high as 0.304 mL/g BW while it has been reported that the hepatopancreas was less than 3% of the total wet weight; that was about 0.03 mL/kg BW (Barclay et al. 1983). The organ has been regarded as the principle site of absorption and elimination of OTC where the drug was found in significant amounts compared to other tissues (Chiayvareesajja et al. 2006; Faroongsarng et al. 2007). A large amount of OTC may be present in a tissue-bound form to allow the apparent volume to far exceed the real physiological volume of the organ. The estimate of V_M was also apparently discrepant (V_M of 3.67 mL/g BW) as it far exceeded the volume of the shrimp's body. As there were no other tissue components in the used model, the muscle compartment might not only represent muscular tissues but the rest of the peripheral ones. For instance, an amount of OTC has been reported in shrimp shell (Uno et al. 2006). Thus, other peripheral tissues which include the shrimp shell might account for a significant portion of the V_M. In addition, the estimated volume may deviate considerably from the real physiological volume as OTC was lost from time to time due to ecdysis. This might cause the estimate of V_M to be far greater than the physiological volume of the shrimp's body.

The Toxicokinetics of OTC in White Shrimp

As seen in Figures 1 and 3, the kinetic profile of OTC after administration of a dose of 2,500 mg/kg BW-dose exhibited somewhat different characteristic from those after administration of 500 and 1,000 mg/kg BW. Besides common patterns in which the drug level increased, reached a maximum, then decreased, the decreasing portion of the curve of 2,500 mg/kg BW-dose was not parallel with that of the others. It was deduced that the distribution of the drug at the 2,500 mg/kg BW dose level may be different. The relative affinity for tissues (R_M or R_L) is of importance as it addresses the distribution of the drug in the tissues.

By definition, the relative affinity for any tissue is a partition coefficient for the amount of drug in the tissue compared with that in the blood leaving the tissue (Welling, 1986). The higher the drug levels in the tissue, the greater than unity the value is. Faroongsarng et al (2009) estimated an R_M value of 0.904 for a single dose of 50 mg/kg BW orally administered into adult P. vannamei. This may be comparable to that of 500 or 1,000 mg/kg BW in the current study (R_M of 0.799-0.842). Thus, this line of evidence indicates that OTC is distributed in the muscular tissues in an essentially identical manner when the dose level increases up to approximately 1,000 mg/kg BW. However at 2,500 mg/kg BW dose level, the R_M value is far greater than unity. Probably beyond a 1,000 mg/kg BW dose, the shrimp's normal pharmacokinetic function might be altered resulting in an increased R_M to as high as 3.28 in the case of a dose of 2,500 mg/kg BW i.e., the OTC is distributed in the peripheral tissues more than 3 fold as compared to the hemolymph.

The unusual high value of R_M at the dosage level of 2,500 mg/kg BW estimated from the model fitting with the hemolymph data is consistent with its kinetic profile in the muscular tissues. As seen in Fig. 2, the muscular level-time profile of the drug after 2,500 mg/kg BW administration exhibited a different pattern from those of the lower ones, i.e., OTC not only rapidly increased but tended to accumulate in the tissues as the drug level did not instantaneously decrease after reaching its maximum level. The drug remained at a considerably high level in the muscular tissues for more than 7 days (Figure 3). It did not obey the suggested withdrawal time of 7-14 days after treatment had ceased for a safe harvesting practice after normal dosing (Chiayvareesajja et al. 2006; Gomez-Jimenez et al. 2008; Faroongsarng et al. 2009). This finding indicates that there must be caution in using excessively high dosing by OTC or the drug level may exceed the shrimp's capability of its

normal pharmacokinetic function and drug residues may persist in the edible muscle as shown in Figure 2.

Hepatopancreatic OTC Distribution and Clearance Assessment

As described in the Results section, R_L was far greater than unity. OTC tended to concentrate in the hepatopancreas. Several lines of evidence have suggested the important role of this organ in substance uptake and disposition (e.g. Verri et al. 2001; Chiayvareesajja et al. 2006; Faroongsarng et al. 2007). In oral administration, OTC entered the circulatory system via the hepatopancreas in which the drug may undergo significant first-pass elimination (Faroongsarng et al. 2009). The drug in other tissues may redistribute to the hepatopancreas since a major portion of OTC has been found in the organ after intra-sinus administration (Chiayvareesajja et al. 2006). However, the R_L of 2,500 mg/kg BW dose was approximately two-fold less than that of either the 1,000 or 500 mg/kg BW dose, i.e., a significantly lower portion of OTC partitioned in the hepatopancreas whereas K_G which governed the drug uptake seemed not altered with dose (inset of Figure 4). It is thus evident that the hepatopancreatic distribution of the drug changed. Faroongsarng et al (2009) addressed that OTC elimination kinetics via hepatopancreas may be non-linearly dependent on dose which might be consistent with the hepatic disposition of vertebrates, i.e., clearance increased with increasing dose and approached maximum capacity where the drug reached toxic level.

Currently, hepatopancreatic clearance corresponded to dose level was fitted with Michaelis–Menten Equation; a common hepatic elimination kinetics of vertebrates (Figure 4). It was observed from Figure 4 that Cl_L approached the maximum capacity at the dose level of between approximately 500 and 1,000 mg/kg BW. And, the upper level might be corresponded to maximum level before reaching toxicity. It has been reported previously that

one of the underlining toxic responses after white shrimp was exposed to OTC at extreme levels was an observable histological change to the hepatopancreas (both a decline in the lipid reserves and tissue abnormalities: Bray et al. 2006). It is then hypothesized that an extreme dose of OTC, e.g. that exceeding 1,000 mg/kg BW, might significantly alter the hepatopancreatic functions, as Cl_L reached full capacity, for OTC pharmacokinetics resulting in the change of the drug distribution/disposition within the organ. In addition, the hepatopancreatic malfunction might have a negative impact on the unusual prolonged OTC levels in the hemolymph and muscle as it is difficult for the drug to be redistributed from those tissues to the hepatopancreas for further disposal.

An Approach to Systemic Concentration Levels for OTC at the Maximum Recommended Dose and a Single Tolerated Dose

Although the results of the systemic OTC level data with toxicity studies done on white shrimp (e.g. Bray et al. 2006) were not available, they could be approached with the aid of pharmacokinetic simulation. In the current study, the systemic OTC level-time profile was simulated, based on the maximum daily dosing and a modified 2-compartment pharmacokinetic model of the drug in animals of identical species in which the pharmacokinetic model parameters were available in the literature (Chiayvareesajja et al. 2006) as well as oral bioavailability information (Faroongsarng et al. 2007). To P. vannamei, Uno et al (2010) forced fed three consecutive doses of OTC at 50 mg/kg BW level with 8-hour interval corresponded to the average daily dose of 150 mg/kg BW/day. The resultant average level, C_{av} was 53 μg/mL whereas the simulation with the average daily dose of 155 mg/kg BW/day yielded C_{av} approximately 60 μg/mL. It was found that the simulated C_{av} was consistent with that from previous experiment. It could confirm the validity of pharmacokinetic simulation reported in the current study. As seen in Figure 5, it is found that the OTC concentration level after a 1,000 mg/kg BW single-dose administration was

comparable to that which was simulated at the maximum recommended dose. By analogy, it may be deduced that a single administration of 1,000 mg/kg BW of OTC is expected not to cause a significant toxic response to white shrimp. It is consistent with the findings in the current study that OTC pharmacokinetics normally applies until the dose level is increased up to approximately 1,000 mg/kg BW. Or in other words, a single administration at this dose level may be considered as a dose that white shrimp can tolerate.

Tetracycline antibiotics have been classified as AUC driven agents based on drug exposure (Craig, 2002). The pharmacodynamic parameter that needed to be considered for the success of treating an infectious agent was AUC/MIC rather than a C_{av} *vs*. MIC comparison (Agwuh et al. 2006). A single administration of OTC at 1,000 mg/kg BW dose offered a 2-fold higher AUC compared to that of the maximum recommended dosing on a daily basis (AUC of 3,085 *vs.* 1,500 (µg/mL)h) with a considerably shorter time to reach a maximum level. The combination between a high dose with single administration and normal doses might produce more benefit for better treatment. Not only from the pharamcodynamic point of view but also a high but tolerant single dose instantaneously causes the drug level to reach a steady state for a maximum therapeutic effect. This is analogous to a human's dosage regime for antibiotics where a starting dose followed by a maintenance daily dose is given. However, it is cautious that OTC in this level might begin to cause adverse effects on shrimp hepatopancreas even the toxic responses did not explicitly illustrate (Bray et al. 2006).

In summary, a toxicokinetic study of an extreme dose of OTC has demonstrated signs of the abnormalities in distribution and disposition of the drug without waiting until the drug damages tissues/organs. The pharmacokinetics of OTC in the tissues of interest was altered with only a single exposure. As the abnormal OTC level-time profiles might be affected via

an adverse impact of the drug on the shrimp's hepatopancreas, a further study on the organ should be done to have more insight into the toxicokinetics of OTC in this specific organ.

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TABLE 1 Summary of the validation parameters for the HPLC method of OTC determination in cultured shrimps' hemolymph, hepatopancreas, and muscle.

Parameter	Hemolymph	Hepatopancreas	Muscle
Specificity/Selectivity[1]	+	+	+
Linearity (r^2)[2]	0.9964	0.9961	0.9997
LLOQ (μg/mL, μg/g)[3]	0.05	0.05	0.05
Recovery (%)	89.85-103.97	89.90-101.89	90.69-103.45
Precision (%RSD)[4]	4.25	5.84	3.68
Stability[5]	93.32	93.84	93.67

[1]All chromatograms showed a good resolution for the drug and internal standard peaks.
[2]The linearity is expressed as the square of the correlation coefficient.
[3]Lower limit of quantitation.
[4]The precision is expressed as a relative standard deviation on a percentage basis.
[5]The stability is a mean of the percentage of OTC remaining after a 3-month storage at -70 C.

Figure captions

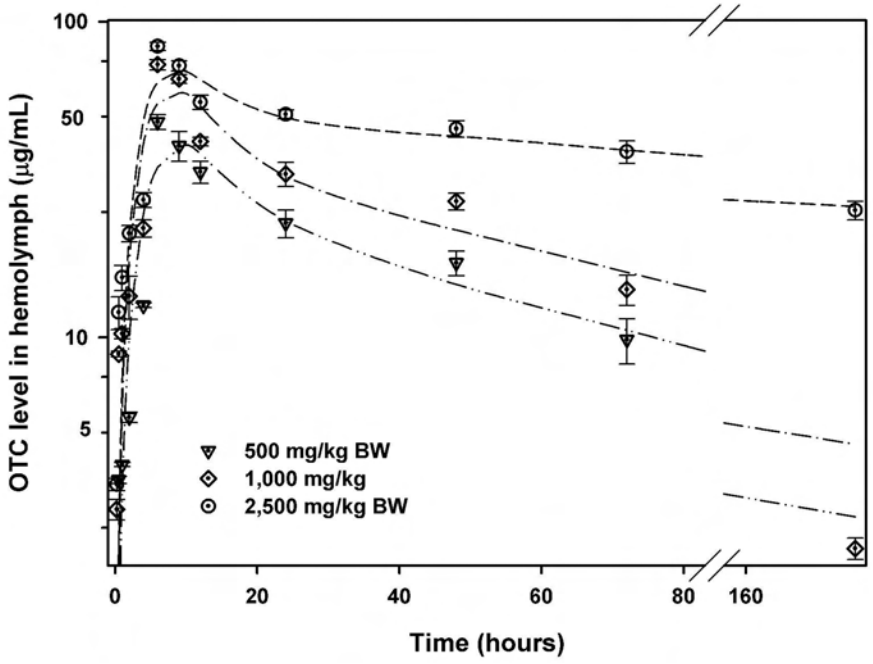
FIGURE 1 The kinetic profiles of OTC in hemolymph after a variety of high-dose oral administrations into *P. vannamei*. The curves of the individual profiles were based on the flow limited model fitting according to Equation 1.

FIGURE 2 The kinetic profiles of OTC in muscular tissues after various high-dose oral administrations into *P. vannamei*. The curves of the individual profiles were based on the flow limited model fitting according to Equation 2.

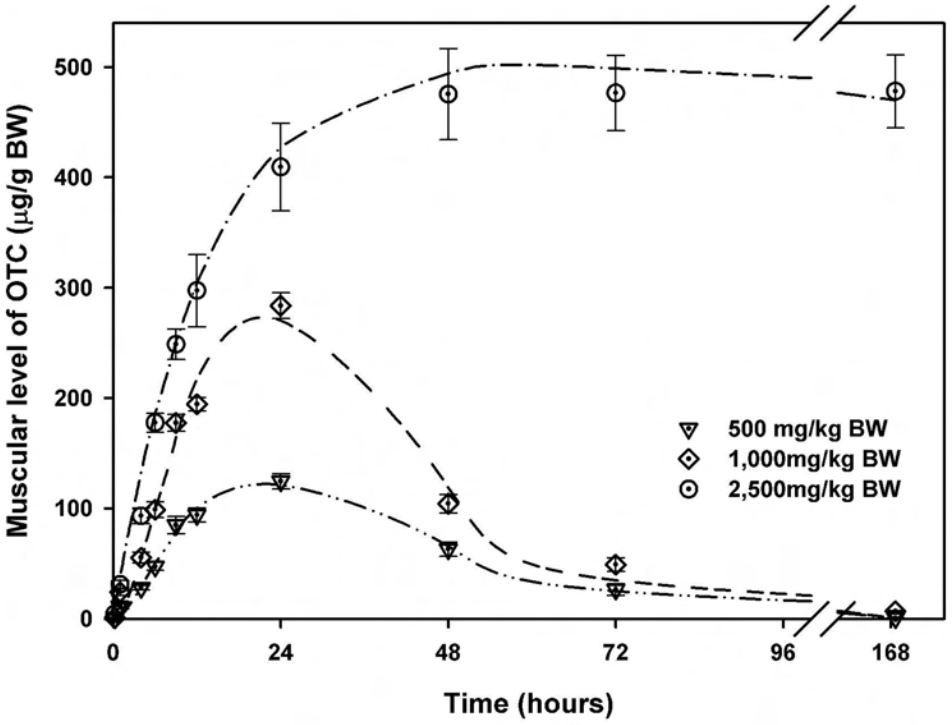
FIGURE 3 The kinetic profiles in hepatopancreas following various high-dose oral administrations into *P. vannamei*. The curves of the individual profiles were based on the flow limited model fitting according to Equation 3.

FIGURE 4 The plot of hepatopancreatic clearance (Cl_L) and OTC uptake constant (K_G: in the inset of the Figure) *versus* administered dose (D). The fitted curve was based on Michaelis–Menten kinetics (see text for details). It was extrapolated, as a dash-line, to a dose as low as 50 mg/kg BW by taking the data coordinate from literature (Faroongsarng et al. 2009) to participate in curve fitting.

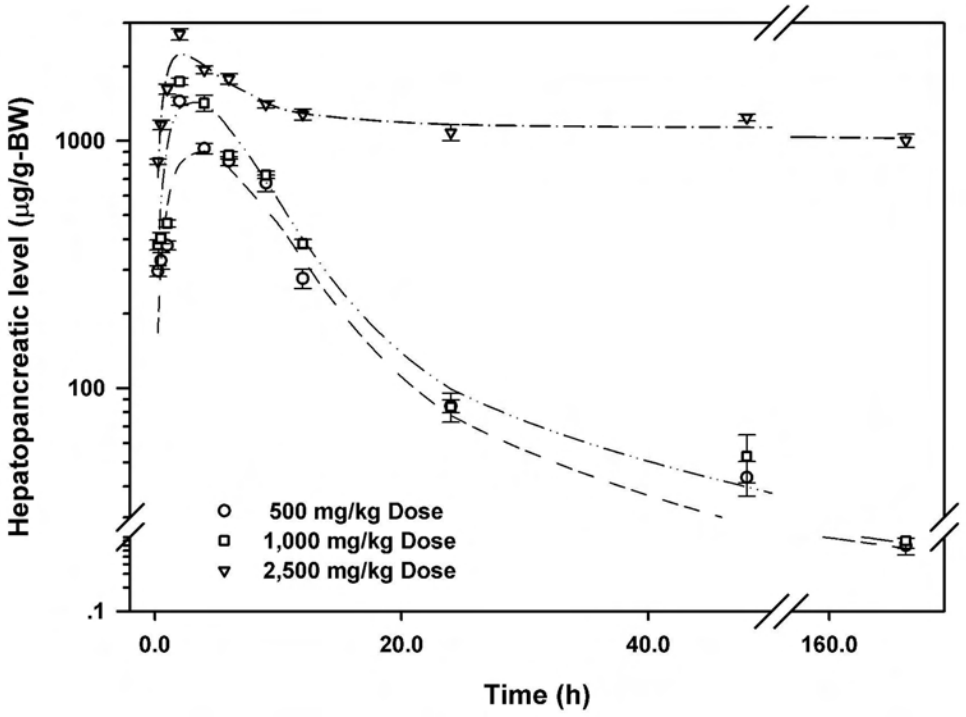
FIGURE 5 The plot of OTC concentration in hemolymph against time after a forced-fed single oral administration of OTC at a dose of 1,000 mg/kg BW into *P. vannamei* (circle with solid line) compared with that of multiple dosing of first 6-day treatment duration simulated according to the medicated feeding protocol of Bray et al (2006) at the maximum therapeutic dose of 4.5 g/kg feed into the animals of the same species.



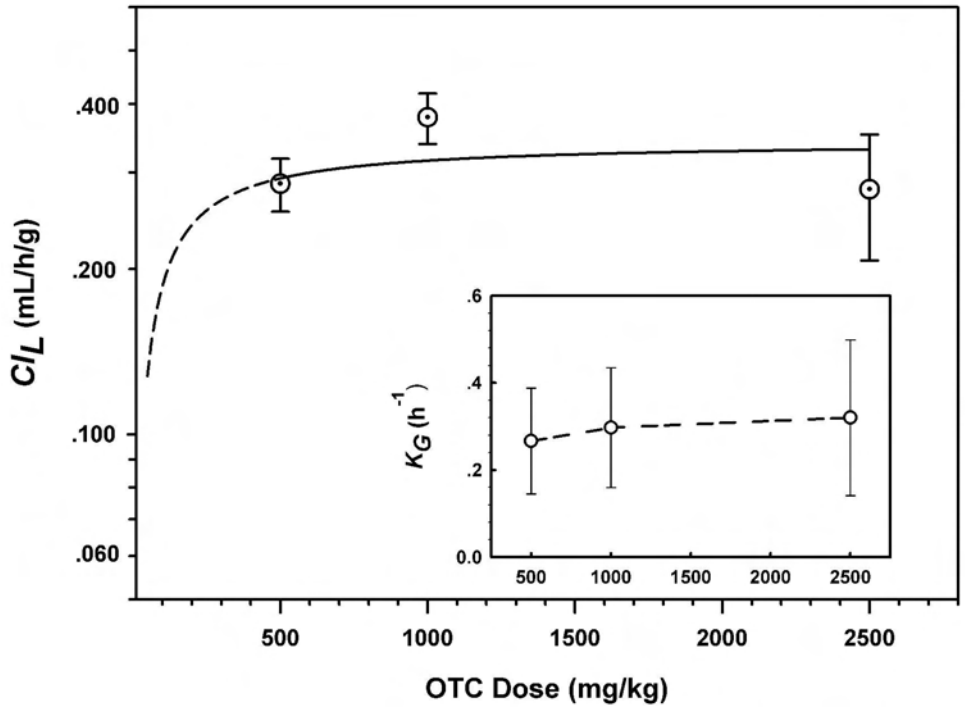
The kinetic profiles of OTC in hemolymph after a variety of high-dose oral administrations into P. vannamei. The curves of the individual profiles were based on the flow limited model fitting according to Equation 1.
165x119mm (600 x 600 DPI)



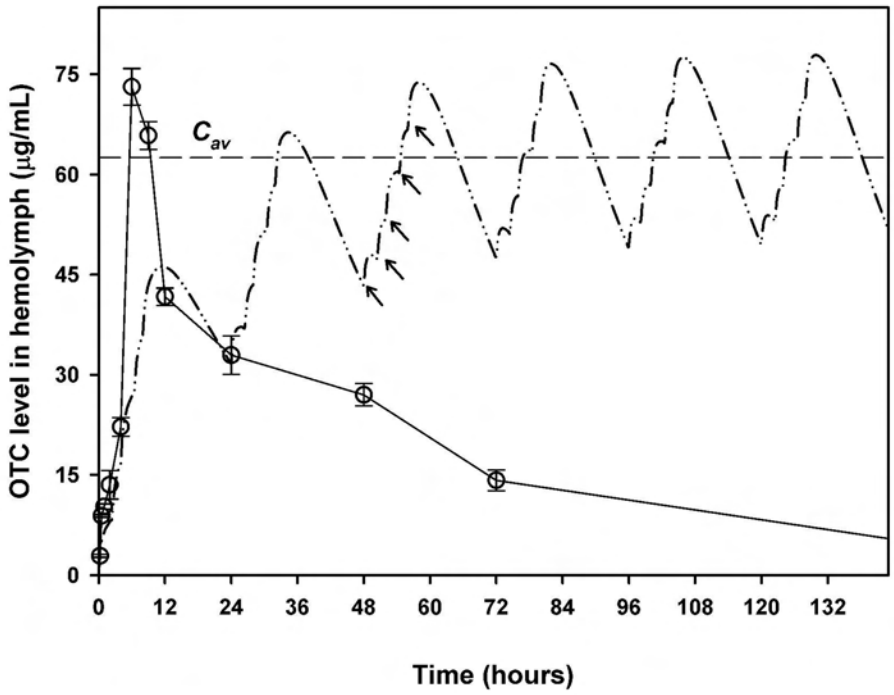
The kinetic profiles of OTC in muscular tissues after various high-dose oral administrations into P. vannamei. The curves of the individual profiles were based on the flow limited model fitting according to Equation 2.
150x119mm (600 x 600 DPI)



The kinetic profiles in hepatopancreas following various high-dose oral administrations into P. vannamei. The curves of the individual profiles were based on the flow limited model fitting according to Equation 3.
153x119mm (600 x 600 DPI)



The plot of hepatopancreatic clearance (ClL) and OTC uptake constant (KG: in the inset of the Figure) versus administered dose (D). The fitted curve was based on Michaelis–Menten kinetics (see text for details). It was extrapolated, as a dash-line, to a dose as low as 50 mg/kg BW by taking the data coordinate from literature (Faroongsarng et al. 2009) to participate in curve fitting.
152x119mm (600 x 600 DPI)



The plot of OTC concentration in hemolymph against time after a forced-fed single oral administration of OTC at a dose of 1,000 mg/kg BW into P. vannamei (circle with solid line) compared with that of multiple dosing of first 6-day treatment duration simulated according to the medicated feeding protocol of Bray et al (2006) at the maximum therapeutic dose of 4.5 g/kg feed into the animals of the same species.
158x144mm (600 x 600 DPI)

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Aquaculture Research

Complete analysis of the distribution kinetics of the oxytetracycline antibiotic in the exoskeleton of farmed pacific white shrimp, Litopenaeus vannamei

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Keywords:	oxytetracycline, exoskeleton, pharmacokinetics, Litopenaeus vannamei, Pacific white shrimp

Complete analysis of the distribution kinetics of the oxytetracycline antibiotic in the exoskeleton of farmed pacific white shrimp, *Litopenaeus vannamei*

Authors and affiliations:

*Damrongsak Faroongsarng

Drug Delivery System Excellent Center, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand

Sommai Chiayvareesajja

Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai 90112, Thailand

Yongyuth Theapparat

Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand

*Corresponding author: Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai 90112, Thailand
e-mail:damrongsak.f@psu.ac.th
Phone: +66-74-288-841
FAX: +66-74-428-148

Running Head: Distribution of OTC in white shrimp's exoskeleton

Complete analysis of the distribution kinetics of the oxytetracycline antibiotic in the exoskeleton of farmed pacific white shrimp, *Litopenaeus vannamei*

Abstract

Oxytetracycline (OTC) in shrimp shells may be dispersed to the environment as shrimp shred old cuticle in growout ponds. The study aims to assess the kinetics of OTC accumulated in shrimp shell. Sub-adult male *Litopenaeus vannamei* in the C-D_0 molting stage, were force fed with medicated feeds at various accurate dose levels that included 50, 500, and 1,000 mg/kg-body weight (BW). In addition to hemolymph, hepatopancreas and muscle were serially collected for 50 mg/kg-BW-dose group while cuticle was sampled for higher dose levels. All were assayed for OTC by a validated HPLC method. Mineral contents in shell samples of 500 mg/kg-BW-dose were also determined. The bioavailability markedly decreased with increasing dose due to incomplete dissolution and/or mild dysfunction in absorption. 2.69% and 2.25% of administered doses ended up in the shell after dosing with 500 and 1,000 mg/kg-BW respectively. OTC data after a dose of 50 mg/kg-BW was fitted into a 3-compartment model with an added shell compartment with r^2 of 0.9920. The model was successfully extrapolated to predict OTC distribution in shell at higher doses. In addition, there was evidence that OTC may disturb the biomineralization process via complex formation with calcium and magnesium lowering the exoskeleton mineral contents.

Keywords: oxytetracycline, pharmacokinetics, exoskeleton, shrimp shell, *Litopenaeus vannamei*, pacific white shrimp

Introduction

Oxytetracycline (OTC) belongs to a group of tetracycline antibiotics derived from a soil-dwelling bacterium named *Streptomyces rimosus*. This antibiotic has been approved by the US FDA for use in aquatic farmed animals including lobster, catfish, and salmonids (FDA/CVM, 2008). The antibiotic has been commonly utilized in shrimp farms worldwide since it is effective against *Vibrio* sp. which are the most common causes of bacterial infections in the aquaculture of penaeid shrimp. OTC has for long been an investigational New Animal Drug in the U.S, that may be permitted for use, but only in specific conditions (Reed, et al., 2006). There have been several investigations to determine a proper wash-out time that will minimize the possibility that humans have exposure to antibiotic residues present in shrimp tissues (e.g. Chiayvareesaja et al, 2006; Reed et al, 2006; Uno et al, 2006; and Weifen et al, 2004). However many countries simply prescribe a minimum residual level in aquatic animals. To name some: The U.S., the E.U., and Japan set values at 0.2, 0.1, and 0.2 μg/g for the edible portions of the aquatic animals respectively (Tu et al, 2010). There is evidence that OTC is present in the shrimp shell (Uno et al, 2006) even after receiving a dose of as low as 10 mg/kg body weight (BW) (Uno et al, 2010). In the process of growth, the shrimp shell is shred from time to time and these old exoskeletons that could contain the antibiotic remain in the culture ponds and become dispersed in the environment. In addition, an abnormal soft exoskeleton was observed in Pacific white shrimp, *Litopenaeus vannamei* fed with the recommended maximum therapeutic dose of 4.5 g-OTC/kg feed five times daily (Bray et al, 2006). It may be a specific characteristic of this antibiotic that the drug forms a complex with calcium/magnesium ions and this might reduce the amount of available calcium/magnesium and cause shrimp to produce this abnormal exoskeleton during molting. It is therefore important to establish if the OTC fed to shrimp does accumulate in the

exoskeleton A distribution profile of OTC in the shrimp shell has been reported but without a full kinetic description (Uno et al, 2006; 2010). This current study aims to assess the kinetics of OTC accumulation in shrimp shell. An attempt is made to model the kinetics of accumulation and distribution of OTC in the shrimp shell.

Materials and Methods

Chemicals

Oxytetracycline (OTC) dihydrate (Sigma, St. Louis, MO, USA) was utilized as the antibiotic under study. Chlortetracycline hydrochloride (Sigma, St. Louis, MO, USA) was used as an internal standard for the determination of OTC by HPLC in the shrimp biological matrices, including the shrimp shell. Unless otherwise indicated, other chemicals used were of analytical or HPLC grade.

Experimental Animals and Aquaria Conditions

Sub-adult male Pacific white shrimp, *L. vannamei*, weighing 14-22 g with a carapace length of 2.30-3.00 cm were collected from a local farm (Damrong Farm, Pattani, Thailand) and preconditioned by acclimating in a 3-ton concrete tank filled with continuously oxygenated natural seawater and conditions as per: 28±2 ^{o}C, 20±2 ppt salinity, pH 7.6±0.5, 200 ppm total hardness, and 12 h of light per day. Shrimp were fed with commercial pellets once a day at a rate of approximately 2-3% of body weight. The healthy shrimp were randomly divided into 4 groups with dosage levels of 0 (control), 50, 500, and 1,000 mg OTC /kg-BW respectively.

Dosage Administrations and Sample Preparations

The medicated feeds were prepared by mixing commercial feed pellets previously ground to powders with OTC, to provide concentrations of 20, 200, and 400 mg/g. The mixture was then suspended in purified water at 1:1 w/v to form a paste. Each medicated feed paste was packed

into a 1-mL syringe fitted with a blunt-end 18G needle, and the tip of the needle was fitted with a silicone tube to protect the shrimp's mouth. The volume of the paste was calculated individually for each of the sampled animals in order to meet an accurate dose on the basis of the shrimp's body weight. After molting when the animals were in the C-D_0 stage, each of the shrimp samples was kept individually in a 2.5-L glass aquarium to prevent contamination of the antibiotic between different aquaria. Each individual animal was then force- fed orally with the medicated feed to fully fill the foregut (approximately 0.5% of body weight). After administration, normal feeding was restarted 24 h post-dosing and continued once a day during the study. No mortality was observed during the study. Three individual shrimp were sampled for each time interval of 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, and 170 h after the oral administration of the antibiotic.

After the shrimp were sampled, 1.5-mL of hemolymph was removed through the ventral-sinus cavity by using a 5-mL syringe, fitted with a 19-G needle and containing 98% N-ethylmaleimide crystals (~0.01g) as an anticoagulant. For shrimp administered with 50 mg antibiotic /kg-BW, the muscle and hepatopancreas were collected later. For animals dosed with 500 and 1,000 mg/kg-BW, shell samples which were a cuticle mixture of abdominal shell, swimmerets, and telson were collected. All samples were frozen and maintained at -70 C prior to OTC extraction. Individual samples were assayed for OTC levels in all the shrimp biological matrices.

Analytical Procedures

The HPLC system (Agilent 1,200 series binary LC system, Palo Alto, CA, USA) with a photodiode array detector and an analytical column of 5 μm, 4.6X250 mm (Purospher star RP-18 end-capped, Merck, Darmstadt, Germany) was used to determine OTC in the shrimp biological

matrices. The mobile phase with a flow rate of 1 mL/min as well as the method of HPLC separation was modified from Li et al (2008).

Prior to the drug determination, the frozen hemolymph, muscle, and hepatopancreatic samples were prepared to make them suitable for the analytical procedure in the manner previously described (Faroongsarng et al, 2011). OTC was extracted from shell samples as follows: The samples were frozen-dried and gently pulverized to powders. The powders were then accurately weighed and chlortetracycline hydrochloride used as an internal standard was accurately added to a concentration level of 20 μg/g. An equal volume of 0.01 M ethylenediamine tetraacetic acid- McIlvaline buffer was added to the powdered sample and after mixing was centrifuged for 15 min at 4,000 x g at 4 C. The supernatant was collected while the precipitate was re-extracted before being discarded. The collected supernatants were cleaned by C18 sorbent solid phase extraction (SPE: 3 mL Sep-Pak Vac C18, Waters, Milford, USA), pre-conditioned with methanol (3 mL) followed consecutively by Milli-Q water (5 mL), and 0.01 M Ethylenediamine tetraacetate-McIlvaline buffer (2 mL). The samples were then eluted by methanol. The methanol solutions were dried by purging with nitrogen at 40 C and dissolved in the mobile phase of the analytical column.

While the analytical procedure used for OTC in hemolymph, muscle, and hepatopancreas had been previously validated (Faroongsarng et al, 2011), validation for the determination of OTC in shrimp shell was as follows: the mean recovery was 93.58% with the repeatability expressed as a relative standard deviation of 2.88%, the limit of quantitation was 0.05 μg/g whereas the linearity of the method with an r^2 of 0.9998 was observed up to a concentration of 100 μg/g, after a 3-month storage period at -70 C, 99.13% of the drug remained in the samples

with a mean recovery of 92.76%, and the reproducibility expressed as a relative standard deviation was 4.55%.

Determination of mineral contents in shrimp shell

The mineral contents including calcium, Ca and magnesium, Mg were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES: Perkin-Elmer, Model optima 4300 DV, Norwalk, CT, USA) according to the method of AOAC (1999). The equipment was operated in the following conditions: Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.5 and 0.8 L/min, respectively. Sample flow rate was set at 1.5 ml/min whereas the wavelengths for the analyses of Ca and Mg were 317.9 and 285.2 nm, respectively.

The serially collected shrimp exoskeleton after the animal was administered with OTC at 500 mg/kg-BW was subjected to determine the mineral contents. A shell sample was weighed, and frozen-dried. The dry sample (0.2 g) was ignited to ash at 700 C for 6 h. The ash was transferred to a volumetric flask and the volume was added to 10 mL with 10% nitric acid. The solution was then subjected to ICP-OES analysis. Three replications were done for each of collected samples. The concentration of each mineral was calculated and expressed as mg/g sample.

Pharmacokinetic analyses

The non-compartment pharmacokinetics of the systemic concentrations of OTC of each of the dosage levels were explored using commercial software (Pheonix WinNonlin v. 6.1, Pharsight Corporation, St. Louis, MO, USA). For each dosage level together with the area under the curve, (AUC) from non-compartmental analysis; the oral bioavailability, F was calculated using the AUC after shrimp of the identical species was dosed intra-sinusly with 10 mg OTC/kg-BW, as a reference, as previously reported by Chiayvareesajja et al (2006). The OTC

concentrations in hemolymph, muscle, and hepatopancreas with time data after dosing with 50 mg/kg-BW were then simultaneously fitted into the model modified from the first-pass 3-compartment open pharmacokinetic model previously proposed by Faroongsarng, et al (2007). Figure 1 illustrates the modified model utilized. Notice that a first order rate process, k_{34} where the drug distributes from a peripheral muscular compartment (compartment 3 in Figure 1) to the shell compartment (compartment 4) has been added to the model. The estimate of k_{34} was extrapolated to the shell distribution profiles after OTC was dosed at 500 and 1,000 mg/kg-BW levels by the simulation using those particular dosage levels with *F*s. The simulated profiles were compared with the OTC levels found in the shell to examine the degree of predictability of the proposed kinetic model.

Results

The non-compartment pharmacokinetic parameters in addition to *F*, for each OTC dose are listed in Table 1. After oral administration of 50 mg/kg-BW to white shrimp, the concurrent non-linear fitting of three drug concentration-time profiles represent the central compartment (hemolymph: compartment 1) and peripheral ones (hepatopancreas: compartment 2 and muscle: compartment 3) was successful with r^2 value of 0.9920. Table 2 lists the estimates of volume of the central compartment, V_1 and fractional rate constants, k_{ij} where subscripts i and j are compartments in which the antibiotic movement is from and to, respectively, whereas Figure 2 shows the simulated profiles compared to actual data. The plots of the distribution of OTC in the shell against time for doses of 500 and 1,000 mg/kg-BW are illustrated in Figure 3. The area under the shell concentration-time curves from the beginning to last sampling interval, AUC_0^t where *t* was 170 h, using the trapezoidal calculation were found to be 2290.0 and 3830.3 (μg/g)h for the profiles of doses of 500 and 1,000 mg/kg-BW respectively. Figure 4 shows the simulated

158 profile of OTC in the shell using the estimates of parameters listed in Table 2 with a dose level
159 of 500 mg/kg-BW as well as its *F*-value (Table 1) compared with the actual coordinates, i.e., the
160 OTC in shell levels after shrimp was orally administered with the same dose. It is noted that the
161 comparison of 1,000 mg/kg-BW dose demonstrates a similar pattern.

162 The variations of mineral contents in *L. vannamei*'s exoskeleton with time after oral
163 administration of OTC at a dose level of 500 mg/kg-BW are illustrated in Figure 5. In addition, it
164 was found that the contents of calcium and magnesium in control exoskeleton with standard
165 deviations in parentheses were 50.91 (2.89) and 1.79 (0.98) mg/g, respectively.

166 **Discussion**

167 To achieve a drug level that is comparable to an accumulated level as a result of daily
168 dosing practices, a single administration at a high dosage level may be an alternative process to
169 use. For instance, a dose of 1,000 mg/kg-BW yielded a systemic concentration of OTC
170 equivalent to the recommended maximum therapeutic level (Faroongsarng et al, 2011). This was
171 selected together with a dose of 50 mg/kg-BW as a unit dose used in normal practice. A dose of
172 500 mg/kg-BW that should produce the systemic level in between these two amounts was also
173 chosen. As seen in Table 1, the time to achieve a maximum level, t_{max}s and clearances of the
174 terminal phase, *Cl*s were practically invariant between the dosages given. Thus, it is deduced that
175 the systemic OTC kinetics in shrimp remained unaltered up to a dose level as high as 1,000
176 mg/kg-BW. However, the volumes of distribution, V_λ and half-lives, $t_{0.5}^{\lambda}$ of the terminal phase
177 were slightly different compared between the dosages (Table 1). These parameters exhibit the
178 limitations on the kinetic interpretation as V_λ is dependent on the dose, *AUC*, and *F* while $t_{0.5}^{\lambda}$
179 is a complicated parameter that could not address the characteristics of the distribution of the

antibiotic and elimination intrinsically (Sahin and Benet, 2008). A slight but non-significant increase in the mean transit time, $T_{transit}$ with increasing dose of administration was observed. As the calculation is based on the moment curves, extreme doses of OTC that result in considerably high concentration levels might carry a minor artifact whereas the drug kinetics may still be essentially identical.

In contrast, the oral bioavailability of OTC was significantly decreased with increasing dosage levels (*F* in Table 1). This is because the increased amount of the drug dose may exceed its solubility making a portion of the drug unavailable for absorption. In addition, the drug uptake might be saturated as the hepatopancreas, normally regarded as a major site of absorption (Faroongsarng et al, 2007) either approaches its full capacity or becomes partially dysfunctional. There is some evidence that slight histological changes e.g. tubule atrophy occurred with exposure to high levels of OTC (Bray et al, 2006).

The model development

As seen in the model illustrated by Figure 1, there are now 4 compartments as an extra shell compartment was added, i.e., the 3-compartment model turns to four. It has been demonstrated previously that the systemic OTC level declined in a tri-phasic manner after intra-sinus administration to white shrimp even over a time period of as long as 2 weeks (Chiayvareesajja et al, 2006). Or in other words, the OTC kinetics in shrimp should be best described by a 3-compartment model. So in order to retain the tri-phasic distribution pattern of the systemic antibiotic, a fourth peripheral compartment can be added in the model only if it was identified as being separate from existing compartments as well as the OTC being irreversibly transported into it. In addition, exuviations represent a net loss especially from the shell materials such as chitin and minerals. Thus, an apparent unidirectional transport of OTC into the shrimp

shell is hypothesized. There may be two possible sources for OTC in the shrimp shell: from the hemolymph and from peripheral tissues- mainly muscle. Hemolymph serves as the source for blood circulation that also supplies essential substances such as oxygen, nutrients, hormones, and cytokines as well as compounds dissolved in it including the antibiotic. However according to the restrictions imposed by the model, transport of the antibiotic via the hemolymph into the shell being a first order process from the shrimp's open circulatory system is unlikely as OTC does not distribute across the boundaries between compartments. However the epidermis and integumentary epithelium, the peripheral tissues that connect the muscle to the exoskeleton, is a part of the sampled muscular tissues, and might play a role to provide supplies of the substances for cuticle (Dall et al, 1990). OTC might be transported from these cells that differentiate from the epidermis during the D stage of the molting cycle into a new cuticle that will then possibly make a peripheral compartment to be the source of OTC for any new cuticle. Thus, the additional shell compartment that obtains its OTC by a first order rate process, k_{34} where the drug distributes from a peripheral muscular compartment was added to the model (Figure 1).

OTC distribution in the shrimp shell

The OTC distributions in the shrimp shell after doses of 500 and 1,000 mg/kg-BW is illustrated in Figure 3. This shows a consistent pattern with those previously reported (Uno et al, 2010), i.e., OTC in the shell not only increased with time after dosing but tended to persist throughout the study period as the drug level did not decrease after reaching its maximum level. This characteristic pattern confirms the proposed one-way transport of the drug into the exoskeleton. Since the total sampling time interval was within the inter-molt period observed in *L. vannamei* (10-12 days: Feng et al, 2008), the curves did not show any discontinuity due to ecdysis. The average OTC present in the shell calculated as AUC_0^t divided by the total time

interval (170 h) for shrimp dosed with 500 and 1,000 mg/kg-BW are 13.5 and 22.5 μg/g, respectively, and corresponds to 2.69% and 2.25% of the administered doses. That is about 20% of the available systemic drug (Bioavailability, *F* in Table 1). As OTC is sufficiently stable with a degradation half-life of the free drug in aqueous solution of more than a week (Connors et al, 1986), the drug residue might accumulate in the environment. It might be associated with an increase in bacterial resistance as well as an elevation of the minimum inhibitory concentration of OTC to bacteria dwelling in shrimp farms (for examples: Chanratchakool et al. 1995; and Roque et al. 2001) Potentially this might result in hazards for human health through difficulties of controlling human bacterial diseases derived from shrimp farm environments

The OTC in the biological matrices that represent the central (hemolymph) and peripheral (hepatopancreas and muscular tissues) compartments were simultaneously fitted into the model with r^2 value as high as 0.9920 yielding the estimated parameters tabulated in Table 2. All parameter estimates are statistically significant from null. As also demonstrated in Figure 2, it is thus deduced that the current proposed model is successful in describing the kinetics of OTC in the biological matrices after the antibiotic was administered orally to white shrimp at a level of 50 mg/kg-BW. It can be seen from Table 2 that the parameters of the current model are totally different from those of the original model (the first-pass three-compartment open model) as most of the estimates of fractional rate constants are not comparable to those reported previously (Faroongsarng et al, 2007). It allows for the prediction of the OTC levels in the shrimp shell using its fractional rate constant k_{34} with standard error in parenthesis being estimated as 0.0371 (0.0075) h^{-1}.

As shown in Figure 4, the distribution of OTC in the shell was simulated using the estimates of kinetic parameters from a lower dose is consistently comparable to the actual level

of OTC in the shell with time, after a dose of 500 mg/kg-BW. The model successfully predicted the actual data to a great extent as the simulated curve superimposed the coordinates especially after 24 hours following dosing. However as seen in the inset of Figure 4, there is some discrepancy within the very first 24 hours: The actual OTC levels seemed to be slightly greater than those calculated by the simulation. An analysis of the discrepancy was undertaken in the current study. Each of the actual concentrations of OTC in the shell, C_4 had the corresponding simulated one $C_4^{/}$ subtracted to provide the discrepant quantity namely: δ, i.e., $\delta = C_4 - C_4^{/}$. Then for each of corresponding time intervals up to 24 hours, δ was plotted against the concentration of OTC in the hemolymph, C_1 as illustrated in Figure 6. It was observed that δ is linearly propotional to C_1 which may be described as:

$$\delta = 0.05 \cdot C_1 \quad (r^2: 0.8017) \qquad (1)$$

Although the correlation was not high, it did tend to indicate that the antibiotic in the hemolymph may be an initial source for OTC in the shrimp shell in addition to that from the peripheral muscular compartment. Penaeid shrimp's circulatory system is classed as an open system having capillary-like vessels with mostly blind endings where the hemolymph soaks the peripheral tissues directly (Dall et al., 1991). Some of the dissolved drug in the hemolymph may be partitioned to the shrimp shell as the shell is bathed with hemolymph. It becomes distributed between the two phases in a definite concentration ratio which may be estimated by the slope of the linear relationship shown in Equation 1, i.e., the ratio of 0.05. It appears that the OTC in the hemolymph contributed to the shell level when the systemic concentration was high enough to allow for a sufficient amount of the drug to partition as the ratio was moderately low. This event occurred very soon after the OTC was administered since the systemic OTC peaked at about 6

hours (t_{max} in Table 1) while the OTC contributed from the peripheral compartment was not yet fully in operation.

The exoskeleton mineral contents after OTC administration

The mineral contents in exoskeleton varied among shrimp species. For example, Ca content in shell of *Parapenaeopsis stylifera*, *Pandulus borealis*, and *Trachypena curvirostris* were 190, 33.7, and 30.4 mg/g whereas that of Mg were 10, 1.4, and 1 mg/g, respectively (Percot et al, 2003, and Heu et al, 2003). In the present study, the content of Ca in exoskeleton of *L. vannamei* in a control group (50.91 mg/g) was comparable to *P. indicus* where its Ca content in the shell varied from 29.32 mg/g in the swimmerets to 165.09 mg/g in posterior regions of the abdominal cuticle (Vijayan and Diwan, 1996) while the Mg content was far lower (1.79: *L. vannamei vs*. 11.94 mg/g: *P. indicus*). As showed in Figure 5, the exoskeleton mineral contents markedly decreased immediately after OTC administration, flattened off, and decreased in the end. The decrease in mineral contents may not be due to an effect of the different molting stages as the animal under study was standardized with the inter-molt stages of C and C-D transition where shrimp usually exhibit a hard shell. Because these cations involve with biomineralization which harden the exoskeleton, the decrease in Ca and Mg contents in shell may be consistent with the previous observation of excessive exoskeleton softness in the shrimp of identical specie treated with extreme OTC dose levels (Bray et al, 2006). It has been demonstrated that the mobilization of these cations, especially Ca, between cuticle and storage tissues occurred via hemolymph (Cheng et al, 2002). And, OTC has been known to exhibit strong chelating properties with divalent ions. Hemolymph Ca and Mg may form complexes with systemic OTC and disturb shell metabolism by making the mobilized soluble minerals unavailable. As a result, decrease in the exoskeleton mineral contents was observed in the present study.

In summary, the evidence indicates that there are two possible sources for OTC found in the shrimp shell: hemolymph and peripheral tissues. While the main source is from the peripheral compartment with a first order rate process, it can be obtained directly from the hemolymph via partitioning but in the relatively modest amount. As illustrated in Figure 3, the complete simulated curves using both possible sources satisfactorily described the kinetics of accumulation of OTC in the shrimp shell after the drug were orally administered at doses of 500 and 1,000 mg/kg-BW.

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The authors appreciate the assistance of Dr. Ausa Chandumpai of the Department of Biochemistry, Faculty of Science, Prince of Songkla University for OTC determinations, and the Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University for providing the facilities for shrimp samples. Sincere thanks go to Damrong Farm, Pattani province, for the animals under study provision and to Dr. Brian Hodgson for English language revision. The authors are also very much grateful to the Thailand Research Fund for financial support.

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Parameter	50 mg.kg-BW Dose	500 mg.kg-BW Dose	1,000 mg.kg-BW Dose
$C_{\max}$ (µg/ml)	41.4	48.1	73.1
$t_{\max}$ (h)	6.0	6.0	6.0
[1] λ (h^{-1})	.014	.022	.019
[2] $t_{0.5}^{\lambda}$ (h)	49.5	32.0	36.0
[3] AUC_0^t (µg h/ml)	1035.0	1853.5	2742.8
[4] AUC_0^{∞} (µg h/ml)	1093.5	1908.1	2864.3
[5] V_{λ} (ml/g-BW)	2.63	1.71	1.91
[6] Cl (ml/h)	.037	.037	.038
[7] $T_{transit}$ (h)	49.2	51.3	54.5
[8] F (%)	80.61	14.08	10.54

1. Rate constant of terminal phase
2. Half-life of terminal phase of the blood level profile
3. Area under the curve from time zero to the time samples were last collected
4. Area under the curve from time zero to infinite time
5. Volume of distribution of terminal phase
6. Clearance of terminal phase
7. Mean transit time: $= \frac{AUMC_0^{\infty}}{AUC_0^{\infty}}$ where AUMC is area under the moment curve.
8. Bioavailability: $F = \frac{AUC_{oral}^{0\to\infty}}{AUC_{IS}^{0\to\infty}} \cdot \frac{D_{IS}}{D_{oral}}$ X100 where *D*s' are doses given intra-sinus (IS) taken from the literature (Chiayvareesajja et al, 2006) and orally (oral).

Table 1 Data for exploratory pharmacokinetic parameters of systemic OTC values obtained from non-compartmental analysis.

382

Parameter	[2]Estimate (S.E.)	[3]3-compartment model
[1]V_1 (ml/g)	0.2649 (0.1324)	0.2678
k_{02} (hr^{-1})	0.1644 (0.0156)	0.2986
k_{20} (hr^{-1})	0.3582 (0.0361)	0.0388
k_{12} (hr^{-1})	0.0108 (0.0006)	2.4617
k_{21} (hr^{-1})	0.6752 (0.0920)	0.7244
k_{13} (hr^{-1})	0.2809 (0.0550)	0.0899
k_{31} (hr^{-1})	0.1251 (0.0235)	0.0116
k_{34} (hr^{-1})	**0.0371 (0.0075)**	**-**

383 1 Volume of distribution of compartment 1
384 [2] These parameter estimates with standard errors in parentheses were determined according to the model based on
385 the diagram illustrated in Figure 1
386 3 These parameter estimates were taken from the literature (Faroongsarng et al, 2007) after OTC was dosed intra-
387 sinusly to white shrimp.
388 Note: In Fig 1 the subscripts 1, 2, 3, and 4 refer to central (hemolymph), and peripheral (hepatopancreatic, muscular
389 tissue, and shell) compartments, respectively.

390 **Table 2** The estimates of pharmacokinetic parameters after white shrimp was orally administered
391 with 50 mg-OTC/kg-BW according to the model described in Figure 1.

392

393 **List of Figures**

394 **Figure 1** Schematic diagram of the modified first-pass 3-compartment open pharmacokinetic
395 model with additional shell compartment for oxytetracycline oral administration. Compartment
396 1, 2, 3, and 4 are central (hemolymph), digestive (hepatopancreas), peripheral muscle, and shell
397 compartments, respectively. The arrows with fractional rate constants refer to first order
398 processes across compartments whereas a broken arrow refers to the transport process via
399 partitioning.

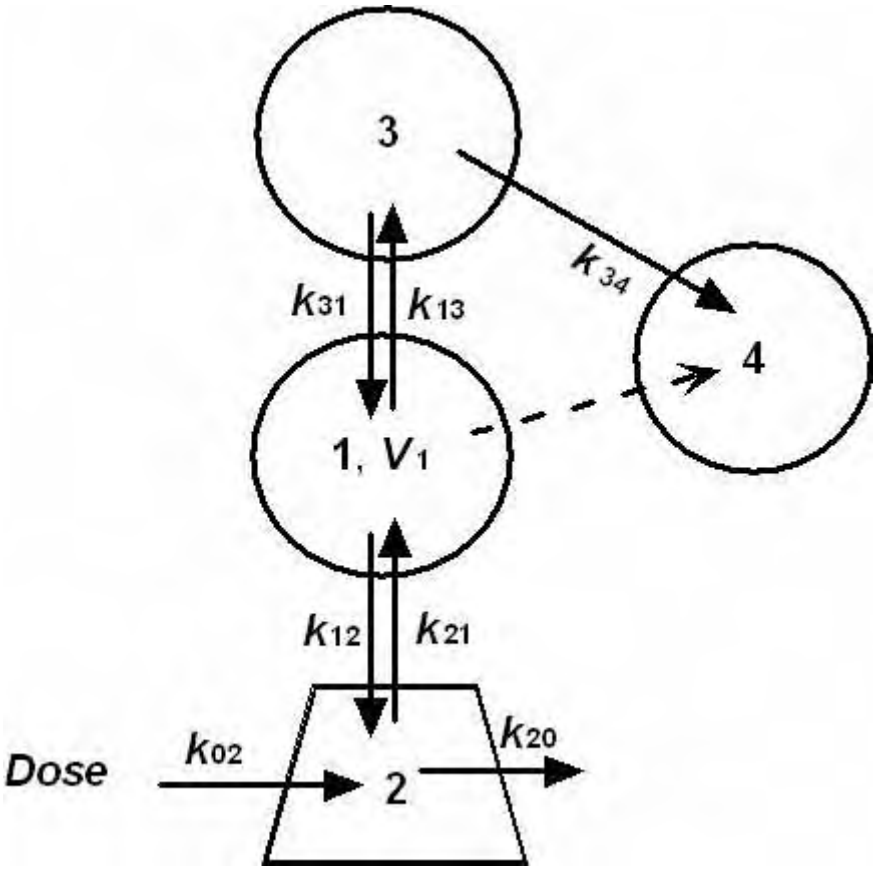
400 **Figure 2** The simulated profiles of OTC in hemolymph (solid line), hepatopancreas (dash-dot-
401 dot line), and muscular tissues (dash-dot line) according to the model of Figure 1 using the
402 parameters listed in Table 2 compared with the actual coordinates. The data were obtained after
403 *L. vannamei* was orally administered with OTC at 50 mg/kg-BW dose level. Each of the data
404 coordinates was the mean of three replicates. The indicators of data variability (error bars) were
405 omitted so as not to interfere with the visualization.

406 **Figure 3** The concentration of the drug in the shrimp shell plotted against time after dosing by
407 oral administration of oxytetracycline into *Litopenaeus vannamei* at dose levels of 500 and 1,000
408 mg/kg-BW. The symbolic coordinates are indicated in the inset to the figure. The broken lines
409 are based on the complete model simulation.

410 **Figure 4** The simulated pharmacokinetic profile of oxytetracycline in the shrimp shell using the
411 estimates of parameters listed in Table 2 with a dose of 500 mg/kg-BW and 14.08%
412 bioavailability compared with the shell levels after *Litopenaeus vannamei* was orally
413 administered with the same dose level. The inset figure illustrates the magnification of the
414 profiles within the first 24 hours post-dose showing some discrepancy between the simulated and
415 actual data.

416 **Figure 5** The variation with time of calcium and magnesium contents in the cuticle of *L.*
417 *vannamei* after OTC at dose level of 500 mg/kg-BW was orally administered to the animal. The
418 arrows indicate the mineral contents in control exoskeleton, i.e., 50.91 and 1.79 mg/g for Ca and
419 Mg, respectively.

420 **Figure 6** The plot of δ parameter against systemic concentration of oxytetracycline, C_1 after
421 *Litopenaeus vannamei* was orally administered with a dose of 500 mg/kg-BW providing
422 evidence for the drug distribution via partitioning from the hemolymph to the shell. The
423 dashlines indicate the 95% confident interval for a linear relationship as presented by the solid
424 trend line.



Schematic diagram of the modified first-pass 3-compartment open pharmacokinetic model with additional shell compartment for oxytetracycline oral administration. Compartment 1, 2, 3, and 4 are central (hemolymph), digestive (hepatopancreas), peripheral muscle, and shell compartments, respectively. The arrows with fractional rate constants refer to first order processes across compartments whereas a broken arrow refers to the transport process via partitioning.
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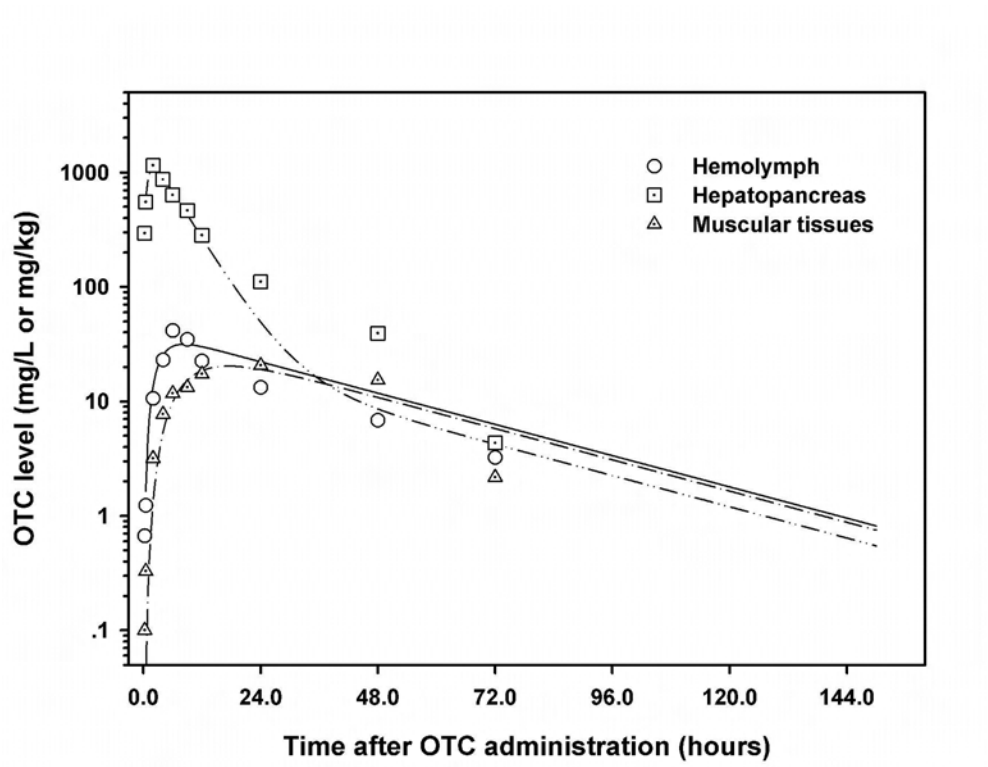
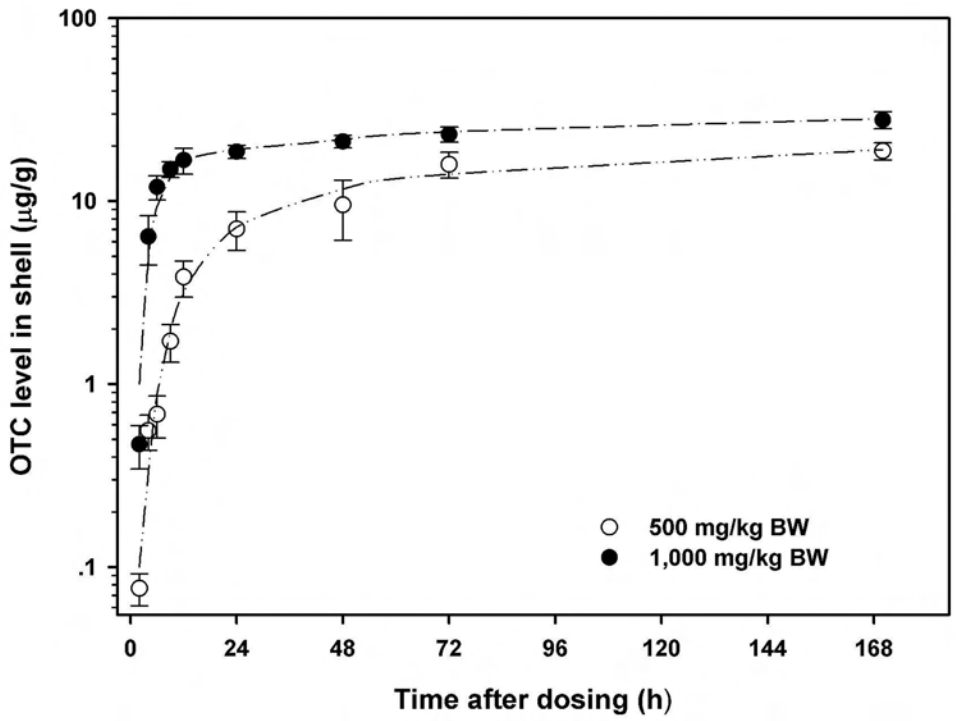
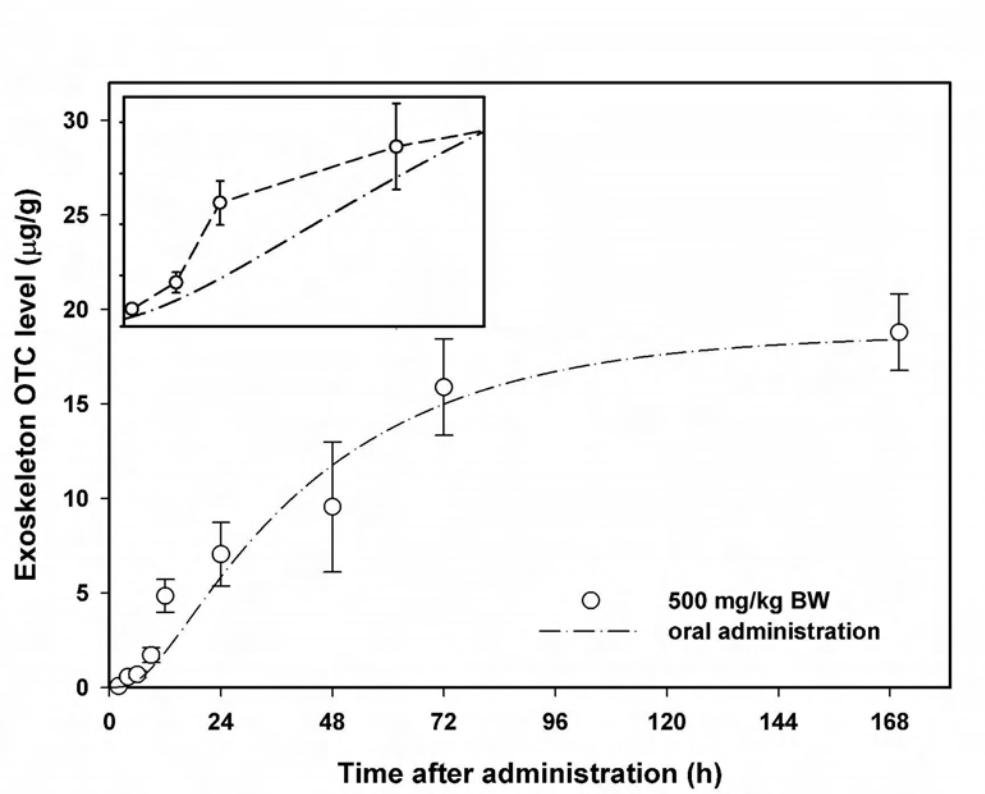


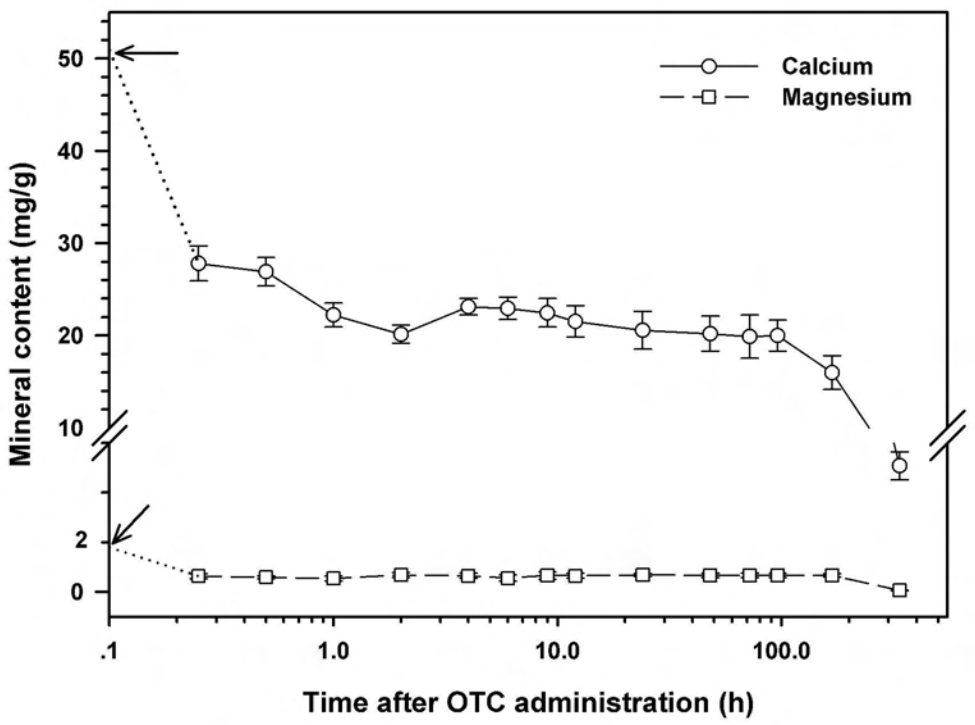
The simulated profiles of OTC in hemolymph (solid line), hepatopancreas (dash-dot-dot line), and muscular tissues (dash-dot line) according to the model of Figure 1 using the parameters listed in Table 2 compared with the actual coordinates. The data were obtained after L. vannamei was orally administered with OTC at 50 mg/kg-BW dose level. Each of the data coordinates was the mean of three replicates. The indicators of data variability (error bars) were omitted so as not to interfere with the visualization.
157x119mm (600 x 600 DPI)



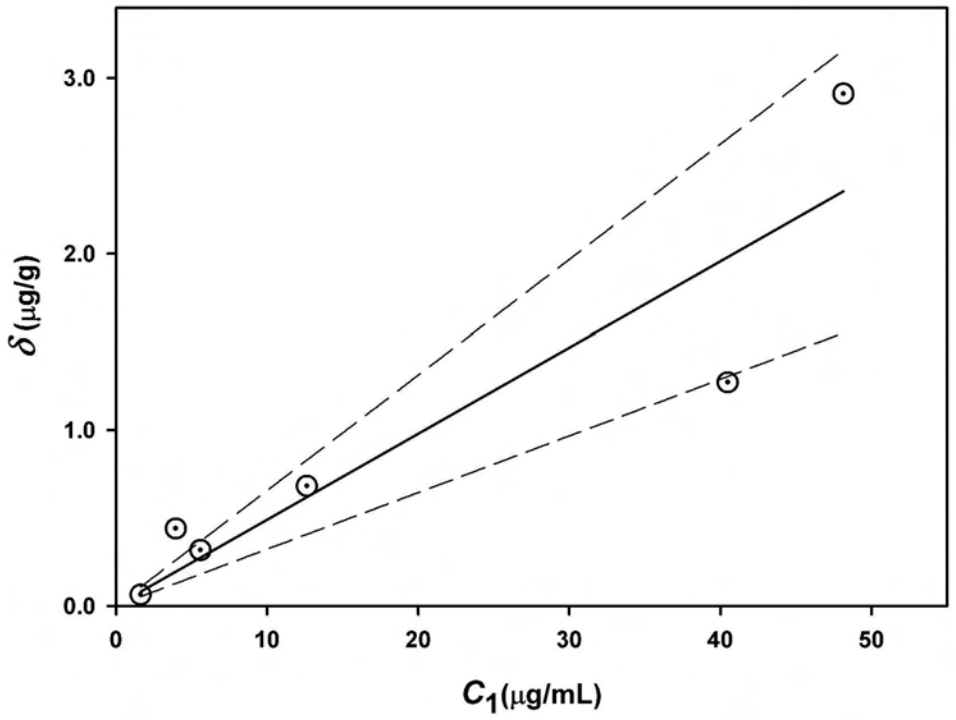
The concentration of the drug in the shrimp shell plotted against time after dosing by oral administration of oxytetracycline into Litopenaeus vannamei at dose levels of 500 and 1,000 mg/kg-BW. The symbolic coordinates are indicated in the inset to the figure. The broken lines are based on the complete model simulation.
150x119mm (600 x 600 DPI)



The simulated pharmacokinetic profile of oxytetracycline in the shrimp shell using the estimates of parameters listed in Table 2 with a dose of 500 mg/kg-BW and 14.08% bioavailability compared with the shell levels after Litopenaeus vannamei was orally administered with the same dose level. The inset figure illustrates the magnification of the profiles within the first 24 hours post-dose showing some discrepancy between the simulated and actual data.
148x118mm (600 x 600 DPI)



The variation with time of calcium and magnesium contents in the cuticle of L. vannamei after OTC at dose level of 500 mg/kg-BW was orally administered to the animal. The arrows indicate the mineral contents in control exoskeleton, i.e., 50.91 and 1.79 mg/g for Ca and Mg, respectively.
149x119mm (600 x 600 DPI)



The plot of δ parameter against systemic concentration of oxytetracycline, after Litopenaeus vannamei was orally administered with a dose of 500 mg/kg-BW providing evidence for the drug distribution via partitioning from the hemolymph to the shell. The dashlines indicate the 95% confident interval for a linear relationship as presented by the solid trend line.
150x120mm (600 x 600 DPI)

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Book proposal

Tentative Book Title: Pharmacokinetics of Antibiotics in Penaeid Shrimp Aquaculture

Author:
Damrongsak Faroongsarng, Ph.D.

Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences
Prince of Songkla University, Hat Yai, Thailand 90112
e-mail: damrongsak.f@psu.ac.th
Phone: +66-74-288-841
FAX: +66-74-428-148

Education: Ph.D. (Industrial & Physical Pharmacy, Purdue University, USA.) 1993

Publications (from 2000 to present):

Faroongsarng, D*., Chiayvareesajja, S., and Theapparat, Y. A Toxicokinetic Study of Oxytetracycline Antibiotics in Farmed Pacific White Shrimp *Penaeus vannamei*. *Journal of the World Aquaculture Society*, Accept, 2011. **Impact factor: 0.78**

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*Corresponding Author

Proposal:

Since 1994, world aquaculture production more than doubled and now constitutes an important food supply. Countries in Asia such as China, India, Vietnam, Thailand, Indonesia, and Bangladesh have been the major producers and beneficiaries (Heuer et al, 2009). It has been predicted that aquaculture would account for 39% of total global seafood production by 2015 (Sapkota et al, 2008). The improper use of antibiotics to handle the disease outbreaks in aquaculture especially penaeid shrimp products has been a problematic issue. For instance, a survey done in 2000 showed that large proportion of shrimp farmers in Thailand used at least 13 different antibiotics in shrimp aquaculture management including tetracycline and its derivatives, several members of quinolones/fluoroquinolones, sulphonamides, chloramphenicol, and gentamycin (Holmstrom et al, 2003). As these antibiotics might contaminate products, the intermittent and chronic exposure of human to relatively low “residue” concentrations of antibiotics is of concern. The residues may destine for consumption resulting in the development of the antibiotic resistance in human pathogenic bacteria (Park et al, 1994). The risks associated with these harmful effects have resulted in the bans on the use of certain antibiotics in aquatic animal food production (Tu et al, 2010). As a consequence, for example in 2001, Thai frozen shrimp product was rejected by E.U. due to the chloramphenicol residue resulting in the massive loss of revenue. The event was considered as a crisis on frozen food export leading to the governmental defensive measures such as 100% inspection for chloramphenicol residue prior to export, and prohibiting control of the imports of 16 antibiotics and related chemicals such as chloramphenicol, nitrofurans, fluoroquinolones, and sulfonamides (Somjadelertcharern, 2002).

The crisis as well as the government measures hit the author’s attention as the prohibiting import of such drugs caused the impact on the shortage of not only animal but human

pharmaceuticals. “Proper use” of antibiotics in farm management rather than “ban to use” may be the answer of this problem. It is because the drug might be available in black market even though they were commercially banned. But, to know “the proper use”, understanding how the drugs were biologically handled by the animal is required. The so-called *pharmacokinetics* which is the study of the time course of drug absorption, distribution, biotransformation/ metabolism, and elimination to optimize the design of the drug medication program can be very well fitted into the need. Thus, the purpose of the book is to convey the information regarding to antibiotic pharmacokinetics in penaeid shrimps to the readers who have little background of pharmacokinetics. The primary markets are the scientists and graduate students in the research area of aquaculture whereas regulatory agencies and farmers who manage shrimp aquaculture are secondary ones.

This book does not intend to cover every detail but to give some concepts currently applied to the study of pharmacokinetics in penaeid shrimps. Reviews and critical discussions of previous works are provided. It is expected that the book may contribute to the research area of penaeid shrimp pharmacokinetics which could help establishing the proper handling of antibiotics so as to ease the problematic excessive use in shrimp farms.

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Tu, H.T., Silvestre, F., Phuong, N.T., and Kestemont, P. 2010. Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses. Environ Toxicol Chem. 29(4): 929-938.

Key feature:

The primary concern about the excessive use of antibiotics in shrimp farms has been its adverse impact on human health via resistant pathogenic microorganisms and through direct damage due to antibiotic residues. It has brought the pharmacokinetic study to shrimp aquaculture for better understanding how the animal biologically handled these substances. Several investigators adopted human pharmacokinetic models to describe their data despite the fact that the biology of Penaeidae is totally different from the advanced vertebrates. To what extent that the models can describe shrimp pharmacokinetics is questionable. Thus, this area of study is challenging. The book intends to review with critical discussion as well as to give some theoretical concepts applicable to the study of pharmacokinetics in penaeid shrimps. The scope

will cover some prospective views of shrimp biology for pharmacokinetic experiment, the antibiotics' pharmacokinetic modeling and absorption analyses. Brief description of antibiotics in use/used to be in use in shrimp aquaculture is also provided.

Preparing the Manuscript:

a. The manuscript will be approximately 85 printed pages (450 words per page) excluding figures and tables.

b. It is estimated that 10 line diagrams, 30 black and white graphic illustrations, and 15 tables may be in the book.

c. As most of the chapters have been already written, it should take approximately 4 months to complete the manuscript once contract.

Table of Contents (tentative):

In shrimp pharmacokinetic studies, most of them utilized a single dose setting whereas farmers always repeatedly dose to their cultured animal. This chapter will give the idea how single dose pharmacokinetic parameters extrapolate to everyday multiple dosing practice.

Chapter 8 Toxicokinetics of antibiotic in Penaeid shrimp: An Oxytetracycline case

Toxicokinetics, a kinetic study of a drug substance in extreme dose level, could demonstrate signs of the abnormalities in distribution and elimination of the drug without waiting until the drug damages tissues/organs. This chapter will cover the toxicokinetic issue of an example of a common antibiotic used in Penaeid shrimp culture.

Competition:

Unlike human pharmacokinetics which has been well established, few shrimp pharmacokinetic studies have been done. It is because shrimp is not a model animal for human drug research and development. To my knowledge, there is no book of the related title available whereas approximately 2,000 books about human pharmacokinetics were found (http://www.amazon.com). Only a single remotely related book entitled Xenobiotics in Fish (D.J. Smith et al eds. Springer, 1999) may be counted as a competition.

Reviewers:

The potential reviewer:

K. Uno, Laboratory of food safety, Department of Sciences for Living, Aichi Konan College, Konan, Aichi 483-8086 Japan. E-mail: uno@konan.ac.jp

3 The biology of post-larval Penaeid shrimps: A prospect for pharmacokinetic study

Penaeid shrimp belong to the crustacean class Malacostraca, order Decapoda. They have been important in aquaculture since the mid 1970s and at present provide approximately 30% of the shrimp supplied to the world markets. *Penaeus monodon*, *P.* (*Litopenaeus*) *vannamai*, *P. indicus*, *P. merguiensis*, and *P. chinensis* are the major cultured species (Rönnbäck, 2001). The viability of cultured shrimp production is dependent on the balance between – i) environment quality, ii) prevention of disease, and iii) the health of the cultured species. To prevent disease and promote the culturing and health of shrimp, xenobiotics, antibiotics, and other chemicals have been utilized in shrimp farms. Just as with other life forms and organisms, the body of the shrimp functions as "a biological machine" to take up, distribute, metabolize, and eliminate given substances. However, to maintain the quality of the farm environment, the

residues from the disease-preventing agents as well as the health promoters should be minimized. Moreover, these agents, especially antimicrobials/antibiotics, should not be found in any portions that may be consumed by man so as not to be harmful to human health. An understanding of the biology of Penaeid shrimp is necessary to provide background information to aid studies such as the pharmacokinetics and bioavailability of disease-preventing agents associated with the cultured species. The aim of this chapter is to begin with a biological overview of Penaeid shrimps. The variability of the results from pharmacokinetic studies of the antibiotics of interest in Penaeid shrimps according to their physiological fluctuations is also discussed.

Morphology and circulatory system

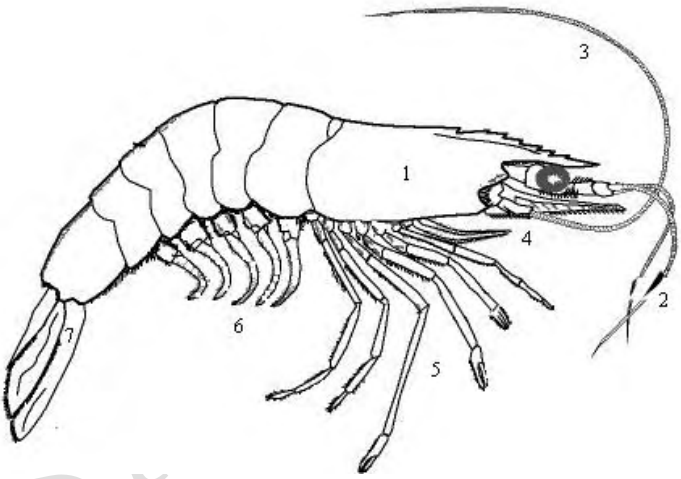

Figure 1 External morphology of a Penaeid shrimp, the numbers positioning various parts of the body are referred to in the text.

The physical morphology of a Penaeid shrimp is illustrated in Figure1. Like most crustaceans, their body is composed of three parts, a head (cephalon), a thorax, and an abdomen. These parts of the body bear a number of appendages that contribute to various functions including movement, food intake, respiration, and reproduction. The head and the thorax are not distinct structures and are always referred to as the cephalothorax and are shielded within a calcified chitinous carapace (1 in Figure 1). The cephalothorax bears the first antennae (2: minor antennae or antennules), the second antennae (3: major antennae), maxillae (4), and

five pairs of pereiopods (5: walking legs). The abdomen consists of six segments and each of the first five segments bears a pair of appendages called pleopods (6: swimming legs) whereas the last one bears the uropod (7: tail fan). Unlike the typical vertebrate in which the blood circulation is well-defined as a closed system, the circulatory system of Penaeid shrimps is classed as either "open" (Dall et al., 1991) or "partially closed" (McGaw, 2005). A crustacean's cardiovascular system can be subdivided into 3 parts: 1) the heart, 2) blood or hemolymph, and 3) a distribution pathway or arterial system (Guadagnoli et al., 2007). Blood leaves the heart via the arterial system. The arteries then subdivide into arterioles that perfuse the blood to all areas of the body. The arterioles split into fine capillary-like vessels, most blind ending ("open"), but some finish in complete capillary beds ("closed"), e.g. in the areas of the antennal gland and supraesophageal ganglion. After passing through these capillaries, the blood drains into a series of sinuses that guide it to the gills to become reoxygenated, and to complete the system the oxygenated blood enters the branchio-cardiac veins to the pericardial sinus and then to the heart in order to begin circulation again.

The molting cycle

A shrimp possesses an exoskeleton (cuticle) that is shed periodically. The shedding is called molting or ecdysis in a cycle in which the old cuticle is replaced by a new and larger one. It is an essential process for post-laval growth and development. The molt staging of Penaeid shrimps has been characterized such as that of *P. monodon* (Promwikorn et al, 2004). In the process of molting, the retraction of the epidermal tissue away from the cuticle begins at the pre-molt stage of D_0. The degree of retraction determines the sub-stages of the pre-molt designated as D_1 – D_4 stages. During the retraction, the new cuticle is being formed continuously. The retraction is then completed and a shrimp sheds its cuticle at ecdysis-E stage. Immediately after ecdysis, shrimp enters the post-molt-A stage in which the new cuticle and setae are very soft and delicate. Soon the cuticle is hardened by biomineralization and setal cones develop. This stage is designated as the post-molt-B stage. When the setal cones are fully developed, shrimp enter the intermolt-C stage. During the late C stage, all layers of the new cuticle are completely synthesized. Various physiological activities including ovarian maturation (Fernandez et al, 1997) occur in the intermolt stage after the exoskeleton becomes hard. Shrimp retain their mature cuticle during the intermolt C - D_0 stage until the process restarts. Molting is controlled by the shrimp endocrine system. The

molting hormones are ecdysteroids, the C-27 steroids, predominantly 20-hydroxyecdysone, produced by paired Y-organs in the anterior cephalothorax. The ecdysteroids are present at a low level during the intermolt-C stage, then increase in the hemolymph and reach a maximum in the D_2 –D_3 (pre-molt) stages, before falling prior to molting. This results in a low level of ecdysteroids during ecdysis (stage E) and the post-molt (stages A–B). The periodic fluctuations in the level of ecdysteroids is negatively regulated by the molt-inhibiting hormone (MIH) a peptide hormone from the X-organ sinus gland complex in the eye stalk. The cellular signaling pathways involve cGMP, cAMP, and both may play a role in the MIH-induced suppression of ecdysteroidogenesis (Nakatsuji et al, 2009). The expression of MIH may be molecularly regulated by the growth arrest-specific protein (Gas7), a multifunctional protein involved in the maturation of neurons and release of neurotransmitters (Devaraj and Natarajan, 2006).

Like other crustaceans, shrimp require calcium for the major biomineralization that occurs during molting. A large amount of calcium is transferred through the epithelial cell layers of the gills, gut, antennal glands and integumentary storage depots during the molting cycle, especially for the pre-molt and post-molt stages. However this does not interfere with the cell signaling system in which cytoplasmic calcium needs to be at a low level because the level of calcium serves as an intracellular secondary messenger of hormonal action throughout the body. It has been proposed that there are processes involved in the massive transport of calcium by the epithelial endoplasmic reticulum together with cytoplasmic unstirred layers adjacent to the apical and basolateral plasma membranes where calcium levels may be greater than that in the main cytoplasm. As a result these large quantities of calcium can be moved through these cells without affecting the strictly controlled calcium activities in the main cytoplasm (Ahearn et al, 2004).

Endocrine system and nutrient metabolisms

Crustaceans utilize various neuro-endocrine signaling cascades regulated by several genes, for examples, the receptor HR3 (El Haj et al, 1997), a putative sex determination gene (Olmstead and LeBlanc, 2003), and the hemoglobin gene hb2 (Rider et al, 2005) that controls their physiology. Several peptides including those of the hyperglycemic hormone family (e.g. MIH described above, belongs to this family) and from the androgenic gland, serve as the negatively regulating intermediates between the neurological and terminal signaling pathways.

Ecdysteroids and terpenoids with 20-hydroxyecdysone and methyl farnesoate as the predominant examples respectively are two major classes of these terminal endocrines. In association with peptide hormones, these terminal signaling endocrine molecules may either independently or in concert regulate various growth and development processes, including molting, maturation, reproduction, and metabolism (LeBlanc, 2007). Other endocrines observed in crustacean are vertebrate-type sex steroid hormones, but their role in shrimp reproduction is not yet completely understood (Okumura, 2004). For the readers who are interested in crustacean endocrinology, a good review of this topic with toxicant-mediated endocrine disruption in crustaceans has been presented (LeBlanc, 2007).

In addition to the negative regulation of ecdysteriods and terpenoid syntheses, the hyperglycemic peptide hormones are involved in regulating carbohydrate metabolism to meet the energy needs of the shrimp. Carbohydrate metabolism varies according to the stages of molting (Gaxiola et al, 2005). Shrimps use carbohydrates as precursors for the synthesis of not only glycogen but chitin; a major biopolymeric constituent in their cuticle. Following molting, the ratio of chitin to protein is low resulting in a soft cuticle. This ratio increases as the cuticle matures through its post-molt, intermolt, and pre-molt stages (LeBlanc, 2007). The feeding behavior also varies according to the molting stage. Shrimp does not feed during the post-molt-A stage when its exoskeleton is too soft to support and handle the weight of food (Sanchez-Paz, et al, 2007). It is not until the post-molt-B stage when its cuticle is strong enough, that feeding activity begins. The animal feeds actively during stage C (intermolt) then feeding declines in stage D just before molting (stage E). In addition to hormonal control (van Wormhoudt, 1980) the environmental conditions such as salinity (Gaxiola et al, 2005) affect feeding activity. Feeding is one of the important factors that determine the release of digestive enzymes. The presence of the required nutrients from feeding causes a rise of specific enzyme activities. Enzymes such as amylase, lipase, and proteases as well as trypsin, the most important crustacean enzyme, have activity peaks at various sub-stages within the intermolt and early pre-molt stages (Fernandez et al, 1997; Gaxiola et al, 2005; Carrillo-Farnes et al, 2007) when animals are actively fed.

In general, the primary source of energy for crustaceans is protein rather than carbohydrate or lipid (New, 1976). However, unlike most other crustaceans, that require more than 50% protein diets, shrimps need less protein so long as the energy requirements can be met from carbohydrates (Verri et al, 2001). Proteins on

the other hand are required for the essential physiological processes such as the synthesis of spermatophores and sperm (Ceballos-Vazquez et al, 2003) and osmotic regulation in the shrimps' body (Gaxiola et al, 2005). Carbohydrates may spare dietary protein (Rosas et al, 2000) in shrimp because it can be more readily digested by *P. indicus* (Omondi and Stark, 1995), *P. vannamai* (Omondi and Stark, 1995; Cousin et al, 1996), and *P. monodon* (Shiau and Peng, 1992 when compared to other crustaceans. In addition, an experiment on the effect of short-term starvation in *L. vannamai* (Sanchez-Paz et al, 2007) revealed that shrimps utilize stored glycogen and acylglycerides whereas the protein levels in the plasma and hepatopancreas remain fairly constant. This is an important finding as feed accounts for approximately 30% of the total cost in a semi-intensive shrimp farm and the most expensive food component is protein. Lowering the protein component without significantly decreasing the growth rate of the cultured species may result in a decrease of production costs.

Immunity

The health of the cultured shrimp is mainly dependent on their immunity. Just as for other invertebrates, the crustaceans have been assumed to have no acquired immunity but they do have an innate immune system in which their haemocytes play a central role as host defense organs. Apart from the haemocytes, lymphoid organ is also believed to have an important role in immuno-defense against invading pathogens. Located nearby hepatopancreas and found exclusively only in Penaeid shrimp, it acts as a major phagocytic organ. Recently, insight into this organ has been extensively reviewed for the readers who are particularly interested in this issue (Rusaini and Owens, 2010). In general, the response to an invasion by microorganisms begins with the process of non-self recognition mediated by plasma proteins. The bacterial cell wall components such as β-glucans, lipopolysaccarides, and peptidoglycans bind to these plasma recognition proteins, and later adhere to haemocytes. The cell wall component-plasma recognition protein complexes induce degranulation and the synthesis of melanin (melanization) by activation of the prophenoloxidase activating system (proPO) (Vargas-Albores and Yepiz-Plascencia, 2000). Melanin is responsible for sclerotization as well as wound healing of the cuticle, and the defense reactions, i.e., nodule formation and/or encapsulations (Sritunyalucksana and Söderhäll, 2000). It was found that melanization is closely related to the activation of haemolymph coagulation as this defense mechanism also prevents blood loss upon

wounding. The immediate defense reactions also include phagocytosis and encapsulation of foreign matter, antimicrobial action based on the production of cytotoxic reactive oxygen intermediates, and cell agglutination (Bachére, 2000; Söderhäll, 1999). There are three different types of haemocytes that include hyaline, semigranular and granular cells (Martin and Graves, 1985). Their functions include coagulation, phagocytosis, encapsulation, and the storage and release of proPO (Johansson et al, 2000). Upon immune activation, haemocytes release the clotting factors essential for haemolymph coagulation, lectins for immune recognition and phagocytosis through opsonization (Marques and Barracco, 2000), agglutinating factors and antimicrobial peptides called Penaeidins (Destoumieux et al, 1997). However, there is little information available on the immune effector encoding genes and regulation of the expression of their protein. And the innate defense reactions that involve the synthesis of Penaeidins in crustaceans have been poorly studied (Bachére, 2000).

There is still a question with regard to the possible existence of a peculiar form of acquired immunity. This arose from an observation that the proliferation rate of *P. japanicus* haemocytes increased after a second microorganism challenge (Arala-Chaves and Sequeira, 2000). Shrimp immunity and disease control leading to the good health of shrimps are not thoroughly understood. This area does need further research.

Biotransformation and disposition

The biological activities of Penaeid shrimp are influenced by not only internal but external factors as the animals need to adapt to environmental changes in order to survive. To cope with the many compounds that may be toxic the shrimp need to biochemically modify them to become less toxic and/or appropriate for elimination or disposal. An important physiological process is therefore biotransformation/ metabolism as well as elimination. Chemicals such as xenobiotics and environmental pollutants are metabolized and eliminated from the animal body. The cytochrome P450 enzymes are mainly responsible for this process. These enzymes comprise one of the largest and most versatile protein families (i.e., CYP1, CYP2, and CYP3) that metabolize not only a variety of lipophilic exogenous compounds such as pesticides and polycyclic aromatic compounds but also endogenous signal molecules such as ecdysteroids, and sex steroids. They catalyze various reactions including hydroxylations, epoxidations, oxidations, N-, O-, S-dealkylations, and dehalogenations (Mansuy, 1998). Thus

regulation of these enzyme activities may play a central role in the adaptation of shrimps to environmental pollutants (Rewitz et al, 2006)., The metabolism of a number of antibiotics and antimicrobials utilized in aquaculture, for examples, erythromycin, sulfadimethoxine, ormetoprim, and oxytetracycline have been examined in crustacean species. In some cases, oxidative metabolites of these drugs were detected. However, the role of cytochrome P450 in their biotransformation is mostly implied but not established (James and Boyle, 1998).

The major site of cytochromes P450-dependent biotransformation is the hepatopancreas; a large, bilateral, multilobate diverticulum of the midgut. But some P450-like activities were also found in other organs that may become specifically exposed to certain physiological important substrates by specific routes such as gills, stomach, intestine, and antennal glands (James and Boyle, 1998). In addition to biotransformations, the hepatopancreas is recognized to have multifunctional roles including the release of a large complement of digestive enzymes and emulsifiers to the foregut for dietary digestion, absorption of nutrients and compounds, storage of exoskeleton calcium and phosphate during the molting cycle, and accumulation of nutrient reserves such as glycogen and lipids (Verri et al, 2001). Furthermore, this organ may play an important role in the elimination of drugs as Chiayvareesajja et al (2006) reported that a large amount of oxytetracycline antibiotic was kinetically distributed into and eliminated from the hepatopancreas even though the drug had been directly introduced into the hemolymph of *L. vannamei*.

The influence of shrimp physiology on pharmacokinetic data

Several pharmacokinetic studies in post-laval Penaeid shrimp have revealed a variability of results. Oxytetracycline bioavailability, for example, varied from approximately 40 to 90% among the related species (*P. japonicus*: Uno, 2004; *L. setiferus*: Reed, et al.; 2004 and Reed, et al., 2006; *P. vannamei*: Faroongsarng et al, 2007; *Macrobrachium rosenbergii*: Poapulathep et al., 2008). As well as the different behavior of different species, it has been reported that the estimated parameters even in identical specie can show a very high variability. It is common in a pharmacokinetic study that the levels of the compound of interest in biological matrices such as the hemolymph and muscular tissues at time intervals were determined to obtain a drug level-time “pharmacokinetic” profile. For each of the time intervals, a replication of samples is always carried out. Previous studies often

utilized 2-3 replications (e.g.: Reed et al, 2004; Reed et al, 2006; Gomez-Jimenez et al, 2008), but it seemed that the variability was high. Some reports revealed a standard deviation of as high as ± 32.7% (Reed, et al., 2006). The results might be so high that they were meaningless and not reliable. It may be because the physiological conditions of Penaeid shrimp significantly fluctuate over the time period of the studies resulting in variations of absorption, distribution, biotransformation and elimination of a compound under study. More replications were suggested in order to cope with these variations. Poapulathep et al (2008) utilized eight shrimps for each time interval to determine the muscular tissue kinetics of oxytetracycline in giant freshwater shrimp (*Macrobrachium rosenbergii*) and reported the relative variations of the extent of the drug distribution in the muscle, i.e., area under the pharmacokinetic profiles, of between ±5 and ±20%. Increase in the number of replications could succeed in lowering the resultant variability, but in contrast it could create a hassle as the task of the drug determination is labor intensive. As previously discussed, the physiology of shrimps varies periodically depending on the molting stages. It is postulated that the differences in the molting stages of the animals sampled for each time interval may bring considerable variations to the results. Thus, controlling the molting stage of the animals instead of increasing the number of replications could be an alternative and better remedy. The first report of the use of standardized C-D_0 intermolting shrimps in the study of oxytetracycline oral bioavailability (Faroongsarng et al., 2007) exhibited a standard variation of as low as ±2.5%, i.e., at best, lower than any other reports, but used only three replicates for each sampling interval. Thus it is possible to lower the resultant variability in a Penaeid shrimp pharmacokinetic study by not only increasing the number of replications of sampled animals but also by controlling the stage of molting prior to the drug dosing.

In previous pharmacokinetic studies, the models utilized for Penaeid shrimp did not correspond exactly to the drug kinetics. A pharmacokinetic exploration with non-compartment analysis was done at the beginning. This kind of data treatment could only empirically reveal the kinetic parameters of the drug uptake and elimination. The parameters have little anatomical or physiological significance but do have an environmental one as it could project to what extent the drug left the shrimp bodies to its surroundings. More details of the kinetics of the drug were required to examine the drug distribution in edible portions as the presence of drug residues in the shrimp bodies would be a major concern to human health. Thus, the compartment models from human pharmacokinetic studies have

been adopted (for examples: Uno, 2004; Reed, et al., 2004 and 2006). However, there is a model extrapolation limitation. First, humans possess a closed circulatory system whereas that of a Penaeid shrimp is open or partially closed. Secondly, the organs for absorption, biotransformation, and elimination of drugs in humans are well separated and defined while the hepatopancreas of shrimp seems to have several functions with also regard to pharmacokinetics. Lastly, elimination by the renal pathway is a major source of excretion for most antibiotics in humans but not in shrimp. Thus the compartment models developed for humans could not exactly demonstrate the pharmacokinetics of any particular antibiotic in shrimp because their physiology is totally different. It is suggested that a unique pharmacokinetic model should be developed for Penaeid shrimps rather than borrowing one from humans.

Summary

The post-laval Penaeid shrimp biological conditions fluctuate periodically. The molting cycle is an essential process for growth and development with their physiological activities being regulated by the crustacean endocrine system. Shrimp possess an “open” or “partially closed” circulatory system whereas the hepatopancreas is a major organ for drug and chemical biotransformations and elimination. As a result there should be a unique shrimp pharmacokinetic model developed rather than one adopted from humans In addition, the periodic fluctuation of shrimp physiology may influence the pharmacokinetics of the substances under study. The fluctuation may deliver considerable variability of the pharmacokinetic data. In general, each of the data should have several replications to be confident that the variability is being properly handled, and the variations must be reduced. An alternative remedy, i.e., control of the molting stage, has been suggested. To obtain consistent and stable pharmacokinetic parameters with fewer replications, the animals used in the study should be standardized with regard to their state of molting prior to the experiment.

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