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광어에서 Cephalexin의 잔류 소실에 관한 연구

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Residue Depletion of Cephalexin in the Flounder (*Paralichthys olivaceus*)

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Abstract: The objective of this study was to investigate the residue depletion of cephalexin in the flounder (*Paralichthys olivaceus*) after multiple oral administrations and to establish the appropriate withdrawal time for edible tissues. A highly sensitive and specific method for the determination of cephalexin in the serum of flounder by LC/MS was developed and validated. Mean recoveries from serum were 87.2% (ranged from 81.2% to 94.5%) for cepalexin. Recovery and precision met the criteria for the guideline of residual analysis of veterinary drugs by the National Veterinary Research and Quarantine Service (NVRQS) in Korea. The limit of detection and limit of quantitation of cephalexin were 10 ng/ml and 50 ng/ml, respectively. Residual levels of cephalexin in muscle samples were estimated with 95% tolerance limit and 95% confidence to fall below the MRL after a withdrawal time of 4 days and 5 days for the 40 and 160 mg/kg/day, respectively.

Key words: cephalexin, LC/MS, oral administration, flounder, withdrawal time

Introduction

Cephalexin, an oral cephalosporin, is an effective and less expensive alternative to aminopenicillins in combination with penicillinase-resistant penicillins or β-lactamase inhibitors for the treatment of many infections in animals and dose not usually cause gastrointestinal side effects [4, 15, 17, 18, 20]. It is useful in a variety of non-specific infections caused by staphylococci, streptococci and some anaerobic bacteria [4, 17, 18]. Additionally, cephalexin is susceptible to enterobacteriaceae which is the major agent of bacterial fish diseases including edwardsiellosis [4, 14].

Although several routes of administration are possible for treatment of framed fish, group-based treatments such as bath treatment and feed-medication are practically reasonable in consideration of the currently used systems of husbandary involving maintenance of animals in very large groups and different circumstance from mammalians [11]. Due to available oral formulations, cephalexin could be applied in aquaculture.

There is now a strict legislative framework controlling the use of antibiotics in fish, with the aim of minimizing the risk to human health associated with consumption of their residue. Therefore, to ensure human food safety the

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European Union (EU) has set tolerance levels for these compounds as maximum residue limits (MRLs). Recently, MRLs have been established for several antibiotics in fish, but no MRL set for cephalexin [7]. There are no studies on the kinetics and residue depletion of cephalexin in fish. The objective of this study was to investigate the residue depletion of cephalexin in the flounder (*Paralichthys olivaceus*) after multiple oral administrations and to establish appropriate withdrawal time for edible tissues.

Methods and Materials

Animals

Healthy flounders (*Paralichthys olivaceus*) weighing about 1 kg were obtained from a commercial farm and were housed in 150 L aquaria with a continuous flow of aerated seawater. Water temperature and salinity were 15±1℃ and 33‰, respectively. Experimentation began after an acclimatization period of at least 1 week. Cephalexin was given for 3 days as oral administration at a dose rate of 40 mg/kg BW or 160 mg/kg BW *ter in die* (t.i.d.) by oral gavages.

Sampling procedure

After 3-day multiple oral administrations, each of six per group fish was sampled at 0, 1, 3, 5 and 7 day. Blood was drawn from the caudal artery of each fish deeply anaesthetized with tricaine methane sulphonate (MS222, Woojin B&G, Korea). Fish were then killed by concussion and samples of muscle were taken. Blood samples were centrifuged at 2,700 g for 10 min within 1 h of sampling and the serum was collected and stored at -20℃ until assayed. Tissue samples were packaged and frozen at -20℃ pending analysis.

Analytical method

Serum and tissue cephalexin concentrations were analyzed on a Hewlett-Packard 1100 series LC/MSD system. Separation was achieved on the Nova-Pak C_{18} reverse phase column (4 ㎛, 3.9 mm×150 mm I.D., Waters, USA). Flow rate was operated at 0.4 ml/min. The mobile phase consisted of 0.1% acetic acid in water (A) and acetonitrile (B). Gradient runs were programmed as follows: 30% A for 4 min, increase from 70% to 100% B in 8 min, 100% B for 2 min, re-equilibration with 30% A for 5 min, until the next sample injection.

The extraction of cephalexin was carried out as follows: each 1 g of sample was added to 2 ml of distilled water and homogenized and then shaken for 10 min. The homogenized sample was added with 2 ml of 0.05% acetic acid in methanol for deprotenization and then shaken for 5 min. The samples were centrifuged at 1,300 g for 10 min, the supernatant being transferred into other tubes and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 ml of methanol and aliquot of 10 ㎕ was injected after filtration

Data analysis

Concentrations of cepahlexin in serum and muscle are expressed as mean±SD. The withdrawal time was estimated by linear regression analysis of the log-tansformed tissue concentrations and determined at the time when the upper one-sided tolerance limit, with a confidence of 95%, was below the MRLs.

Results

A highly sensitive and specific method for the determination of cephalexin in the serum of flounder by LC/MS was developed and validated. The suspected peak of cepahlexin was shown at about 6.0 min and increased in proportion to concentrations. The linear regression line for cephalexin in the range of 0.05 ㎍/g~50 ㎍/g showed high correlation coefficients (r) of 0.99. Recovery and precision met certain criteria for the guideline of residual analysis of veterinary drugs by the National Veterinary Research and Quarantine Service (NVRQS). The limit of detection and limit of quantitation of cephalexin were 10 ng/ml and 50 ng/ml, respectively. Mean recoveries from serum were 87.2% (ranged from 81.2% to 94.5%) for cepalexin. The method has been successfully applied to determine residue concentrations of cephalexin in the flounder.

Residue concentrations were associated with administered doses (Table 1). At the termination of treatment, cephalexin was found in serum and muscle for both dose groups. Residue concentrations of cephalexin were lower than its LOQ in serum and muscle from both dose groups after the withdrawal time of 7 days. Residual levels of cephalexin in muscle samples were estimated with 95% tolerance limit

Table 1. Residue concentration of cephalexin in flounders at different time points 3 days after multiple oral administrations at a dose rate of 40 and 160 mg/kg body weight

	Dose gruop	Residue concentration after treatment (μg/g)				
		0 day	1 day	3 day	5 day	7 day
Serum	40 mg/kg BW	25.86± 4.78	3.95±2.51	0.21±0.08	N.D.	N.D.
(n=6)	160 mg/kg BW	140.23±17.50	14.08±8.81	0.55±0.41	0.07±0.05	N.D.
Muscle	40 mg/kg BW	2.04± 0.33	0.64±0.18	N.D.	N.D.	N.D.
(n=6)	160 mg/kg BW	15.26± 1.13	4.91±0.93	0.67±0.14	N.D.	N.D.

and 95% confidence to fall below the MRL after a withdrawal time of 4 days and 5 days for the 40 and 160 mg/kg day dose, respectively. This calculation was performed using the cephalexin concentrations measured in muscle samples from day 1 (earlier time point on the elimination phase) to day 3 (later time point on the elimination phase). Due to no MRL set for cephalexin in fishes, the MRL in the calculation of the withdrawal time for the flounder was extrapolated from those of mammalian species which has been already established for the MRL of cephalexin at 200 μg /kg in the muscle.

Discussion

Although most β-lactam antibiotics are poorly absorbed from the digestive tract, cepalexin is one of available oral cephalosporins. It has a low rate of protein binding in serum due to the hydrophilicity, which enhances its circulation and penetration into interstitial tissue fluid [3, 16, 23]. In general, the only difference in the disposition of drugs in fish and other species appears to be the rate of the metabolism, fish being slower than mammals, particularly when the temperature of the water of their environment is low [7, 11]. The elimination half-lives of cephalexin in mammals were mainly short within about 1 h [3, 9, 23]. In this study, cephalexin in the flounder were slowly depleted and showed greatly higher levels in serum as compared to those in muscle for both dose groups of 40 mg/kg BW and 160 mg/kg BW. Oral cepalosporins such as cephalexin, cephariadine, cefuroxime and cefixime showed high serum levels and poor extravascular distribution [1, 3, 9, 18]. Bioavailability or oral absorption-rate is better with cephalexin and cepharidine from 50% to 90% than with newer compounds, such as cefuroxime from 30% to 40% or cefixime from 20% to 30% [9, 23].

In the present study, the withdrawal time was estimated by the statistical method for the calculation of withdrawal times as adopted by the Committee for Veterinary Medicinal Products [6, 7]. Theoretically, the withdrawal time may be calculated as follows: withdrawal time = 1.44 ln (C_0/MRL) · $t_{1/2}$ [1, 19]. However, officially established withdrawal times must take into account all inter-animal variability and thus statistical processing of these data establishes the withdrawal time for the worst-case scenario [19].

Various methods available for cephalosporin assay have been reported [2, 3, 8, 10, 12, 13, 21, 22, 24]. Thin-layer chromatography, capillary electrophoresis, high-performance lipid chromatography and liquid chromatography/mass spectrometry have been developed to analyze biological samples such as serum or urine [2, 3, 8, 10, 12, 21, 22, 24]. Some methods are lacking in identity confirmation of drugs and require a long time for chromatographic separation and method development [2, 3, 10, 24]. In addition, they were not suitable to determine low levels of cephalexin in the biological fluid [8, 12, 13, 24]. The limit of detection and limit of quantitation of cephalexin were 10 ng/ml and 50 ng/ml, respectively. The LOQ of this method is more sensitive than other HPLC methods previously reported [2, 8, 12, 13, 21, 24].

In conclusion, the optimal withdrawal time of cepahlexin for edible tissues of flounder was suggested to be 4 and 5 days after treatment of cephalexin at a dose rate of 40 and 160 mg/kg, respectively. LC/MS with electrospray is a simple, rapid and effective technique for the determination of cephalexin in flounder tissues.

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