The Journal of Veterinary Medical Science

FULL PAPER

Pharmacology

Synthesis, characterization and *in vitro* release performance of the *pegylated valnemulin* prodrug

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J. Vet. Med. Sci.
80(1): 173–180, 2018
doi: 10.1292/jvms.17-0434

Received: 20 August 2017
Accepted: 15 November 2017
Published online in J-STAGE: 28 November 2017

ABSTRACT. *Valnemulin*, successfully developed by Sandoz in 1984, is a new generation derivative of pleuromutilin related to tiamulin. *Valnemulin* has low water-solubility, a short half-life period, low bioavailability, and instability. The application of *valnemulin* was restricted. Therefore, finding a more moderate delivery system is necessary to improve the shortcomings of *valnemulin*. The purpose of the study was to improve the strong stability and the irritation caused by of *valnemulin* hydrochloride power through *pegylated-valnemulin* prodrug mode. The prepared *pegylated-valnemulin* prodrug was characterized and evaluated by *in vitro* release performance under buffer solutions with pH levels of 7.4 and 3.6. The loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was determined by ultraviolet spectrophotometer and high performance liquid chromatography (HPLC). HPLC with evaporative light scattering detector was applied to determine the amount of PEG-succinic acid. The loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was 6.46%. *PEG-succinic-valnemulin* prodrug demonstrated a satisfactory solubility of *valnemulin* with 523 mg·ml^{-1} and excellent stability verified by the stability experiment. The result of the *in vitro* release test showed that the prepared *PEG-valnemulin* prodrug has controlled release ability and the release rate of *valnemulin* from *PEG-valnemulin* prodrug with a pH of 7.4 was 64.98%, which was higher than that of pH3.6 with release rate of 31.90%. Therefore, the prepared *PEG-succinic-valnemulin* prodrug has great application potential.

KEY WORDS: PEG, prodrug, release, *valnemulin*, *valnemulin hydrochloride*

Valnemulin (structure shown in Fig. 1 (4), chemical name: acetic acid, 2-[[2-[[(2R)-2-amino-3-methyl-1-oxobutyl]amino]-1,1-dimethylethyl]thio]-(3aS,4R,5S,6S,8R,9R,9aR,10R)-6-ethenyldecahydro-5-hydroxy-4,6,9,10-tetramethyl-1-oxo-3a,9-propano-3aH-cyclopentacycloocten-8-yl ester), which was successfully developed by Sandoz in 1984, is a new generation derivative of pleuromutilin related to tiamulin [9, 17]. As a semisynthetic diterpene antibiotic [2], *valnemulin* was initially deemed as an agent of swine dysentery and pneumonia by the European Union (EU) in 1999 because of being effective in the treatment of pig scour [5], ileitis and the prevention of colitis through binding to the 50S ribosomal subunit of bacteria and inhibiting the synthesis of protein [26]. Pharmacokinetic studies of *valnemulin* have shown that *valnemulin* was 100 times more active than *tiamulin* against *Mycoplasma hyopneumoniae* and *Mycoplasma hyosynoviae* and $MIC_{90\%}$ of *valnemulin* against *staphylococcus aureus* was 0.12 μg·ml^{-1} [8, 25, 34, 35]. In addition to effective antimicrobial activity [16], *valnemulin* has many other advantages, such as short withdrawal time, wide distribution, fast absorption, low toxicity, quick discharging body, low residue and use safety [29]. Because of the low water-solubility and instability, the application of *valnemulin* was restricted and *valnemulin* hydrochloride is used clinically instead. Although hydrochlorination of *valnemulin* has improved its water-solubility and stability as an amorphous powder [18], *valnemulin* hydrochloride has great problems in pharmaceutics due to its strong hygroscopic and irritating effect, and other defects, such as a short half-life and low bioavailability of. Therefore, finding a more moderate delivery system is necessary to improve the shortcomings of *valnemulin*.

"Prodrugs" were first presented by Albert in 1985 [1] and are always formed by the linking of the drug to the carrier via covalent or non-covalent bonds without changing the original basic structure of the pharmacologically active drug [21]. Prodrugs

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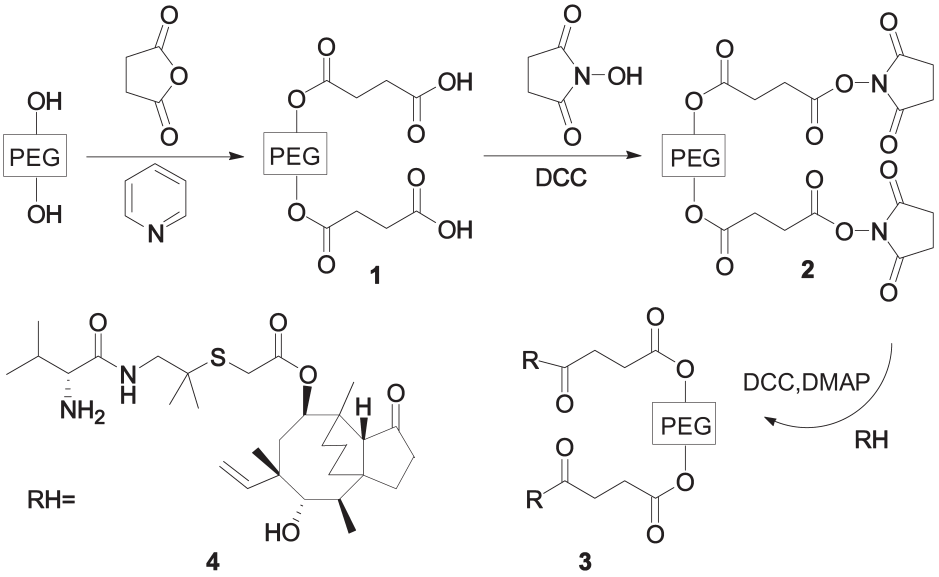

Fig. 1. Synthetic route of PEG-succinic-valnemulin prodrug.

have no activity *in vitro* and take effect by releasing the active drug in the body through enzymolysis or hydrolysis [4, 22]. The carrier can directly link with the drug or if the reactive activity of carrier is low, a linker is needed [14, 15]. A macromolecule carrier is preferred here because modification by small molecules may still lead to the defects of instability and short half-life. Paclitaxel modified by α, β-poly (N-2-hydroxyethyl)-DL-aspartamide is a typical example of a prodrug [2].

PEG is one of the synthetic polymers which are allowed to modify drugs by the Food and Drug Administration (FDA) in the U.S.A. [3]. It has the characteristics of non-toxicity, non-immunity, non-antigenicity, good water solubility and biocompatibility [13, 25, 29, 30,]. Since "pegylation" was first reported by Davis and Abuchowski in the 1970s [7, 19], the pegylation of drugs has been widely studied and great achievements had been made. *Pegylated somavert* and *macugen* have been clinically applied, and *pegylated camptothecin* [6, 24], *irinotecan* and *docetaxel* have been studied [27]. Prodrugs not only improve the solubility and stability of drugs, but also reduce the side effects of drugs [2, 10]. Hence, in our study, pegylated *valnemulin* was designed and synthesized in order to increase the water solubility, prolong the half-life and avoid the hygroscopicty and irritation caused by *valnemulin* hydrochloride.

The *pegylated valnemulin* prodrug was developed via spacer of succinic acid in this paper and the loading rate of *valnemulin* in the *PEG-succinic-valnemulin* prodrug was determined by UV, HPLC-UV and HPLC-ELSD. Previously, the loading rate of PEG conjugate was determined by UV and HPLC-UV because of the good UV absorption of loaded drugs. But *valnemulin* has weak UV absorption, so the content of *valnemulin* is determined under a wavelength of 190–210 nm, according to the European veterinary drug standard. In the synthesis process of *pegylated-valnemulin* prodrug, impurities, such as PEG-succinic acid and PEG, would be residual in the final product. These impurities often affect the detection of loading rate. In our study, the HPLC-ELSD method was first used to determine the content of PEG-succinic acid. The application of the HPLC-ELSD method makes the loading rate of *PEG-succinic-valnemulin* prodrug more accurate, which is important for the later efficacy evaluation study. The prepared *PEG-succinic-valnemulin* prodrug demonstrated satisfactory solubility and stability without irritant, and *in vitro* release performance showed it had a controlled release ability. Furthermore, to date, modified *valnemulin* by PEG-succinyl with better solubility, excellent stability and controlled release ability has not been reported. The results will serve as the foundation for establishing a new challenging pegylated prodrug for *valnemulin*.

MATERIALS AND METHODS

Valnemulin hydrochloride was of analytic grade and purchased from Libang Pharmaceutical Co., Ltd., Xi'an, China. Analytic grade methoxy glycol and PEG2000 were bought from Yongda Chemical Ltd., Tianjian, China. Chemical grade succinic anhydride was obtained from Bodi Chemical Co., Ltd., Tianjian, China. Analytic grade dicyclohexylcarbodiimide (DCC) was purchased from Kaiyang Biotechnology Co., Ltd., Shanghai, China. Analytic grade N-hydroxysuccinimide (NHS) was obtained from Aladdin reagent Co., Ltd., Shanghai, China. N, N-4-dimethylaminopyridine (DMAP) was of analytic grade and bought from Gongjia Chemical Co., Shanghai, China. Pyridine, anhydrous ether, chloroform, ethyl acetate, dichloromethane, dimethyl formamide (DMF), sodium bicarbonate, anhydrous magnesium sulfate, disodium hydrogen phosphate, monopotassium phosphate, phosphoric acid, sodium hydroxide and other chemicals were of analytic grade and purchased from Shijiazhuang Modern Reagent Co., Ltd., Shijiazhuang, China. Water used in HPLC analysis was produced by Wow Haha Food Co., Ltd., Hangzhou, China. HPLC grade acetonitrile was purchased from Oceanpak Alexative Chemical Co., Ltd., Gothenburg, Sweden. Chemical reagents were all used

directly without further purification.

FI-IR spectra were equipped with a FI-IR spectrophotometer (Frontier, PerkinElmer, America) using KBr as the sample holder. Tested samples were scanned from 400 to 4,000 cm^{-1}.

^{1}H-NMR analysis was conducted on an AVANCEDMX-500 (WB) (Bruker, NASDAQ, Billerica, MA, U.S.A.) at room temperature using tetramethylsilane (TMS) as an internal standard and $CDCl_3$ as the solvent.

The melting temperature and other relevant information were measured by DSC (Q600, TA Co., New Castle, DE, U.S.A.) under protection of nitrogen. The amount of sample used was approximately 8 mg, and the scan rate was set to 10°C·min^{-1}.

X-Ray diffraction patterns of the trial samples were determined by a diffractometer (D/MAX-2500, Rigaku Co., Tokyo, Japan) with a copper anode range from 0 to 100° (2θ). The samples were scanned at a scanning speed of 4°·min^{-1}, a voltage of 40 kV and a current of 150 mV.

The total content, including *PEG-succinic-valnemulin* conjugate, PEG, PEG-succinic acid and free *valnemulin* was measured by an ultraviolet spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). The detected maximum absorption wavelength was 210 nm.

The content of free *valnemulin* in PEG-succinic-*valnemulin* prodrug, the contents of PEG and PEG-succinic acid remaining in *PEG-succinic-valnemulin* prodrug and the content of *valnemulin* released from *PEG-succinic-valnemulin* prodrug in the release test were determined by HPLC (e2695, Waters, Milford, America) equipped with an HPLC column of Diamonsil-C18 (4.6 × 250 mm, 5 μm) with the column temperature of 45°C, a flow rate of 1.0 m*l*·min^{-1} and injection volume of 20 μl. The content of free *valnemulin* in *PEG-succinic-valnemulin* prodrug and the content of *valnemulin* released from *PEG-succinic-valnemulin* prodrug in the release test were determined by HPLC with ultraviolet detector (2498, Waters, Milford, CT, U.S.A.) at the detected wavelength of 210 nm. The mobile phase was a mixture of buffer salt and acetonitrile (57:43, v/v). Disodium hydrogen phosphate and monopotassium phosphate were used to prepare the buffer salt solution and pH was adjusted with phosphoric acid to 2.5. The determination of contents of PEG and PEG-succinic acid were performed on HPLC with evaporative light scattering detector (ELSD) (2424, Waters) by using a mobile phase of acetonitrile and water (50:50, v/v). Suitable condition for ELSD were set as follows: Gas pressure of spray, 25 psi; drift tube temperature, 60°C; the atomizer, 60%; gain value, 250.

PEG-valnemulin prodrug was prepared by using succinic acid as the linking arm and PEG 2000 as the carrier.

PEG-succinic acid (Compound 1) and PEG-succinic active ester (Compound 2) were synthesized according to literature [31].

Compound 2(1.20 g, 0.5 mmol) was dissolved in 25 m*l* anhydrous ethanol and cooled down to 0~5°C. *Valnemulin* (1.13 g, 2 mmol), DCC (0.41 g, 2 mmol) and DMAP (0.25 g, 2 mmol) were added. The above solution was kept at 0~5°C for 1 hr at room temperature for 24 hr. The resultant white suspension was filtrated. Extra DCC of the filtrate was consumed by adding dilute HCl (1 mol·l^{-1}) aqueous solution. After stirring for 30 min, the above solution was extracted by dichloromethane (15 m*l* × 3) and dichloromethane was separated and collected. The organic phase was washed by saturated brine, dried by anhydrous magnesium sulfate and filtrated. The filtrate was concentrated under vacuo. The residue was recrystallized by anhydrous ether (10 m*l* × 3) at −5 ± 2°C. The resultant white solid was filtrated to afford compound 3 with the yield of 56.3%. ^{1}H-NMR (500 MHz, δ ppm): 0.90 (-CH_3), 0.94 (-CH_3), 1.23–1.15 (-CH_3, -CH_2-), 1.53 (-CH_3), 1.78–1.62 (-CH-, -CH_2-), 2.19–2.06 (-CH_2-), 2.34–2.27 (-CH-, -CH_2-), 3.14 (-CH-, -CH_2-), 3.25–3.16 (-CH-), 3.35–3.31 (-CH-), 3.57–3.72 (-CH_2- of PEG), 5.21 (-CH-), 5.30 (-CH-), 5.73 (-CH-), 6.45 (-CH-). The ^{1}H-NMR patterns of PEG-succinic-valnemulin prodrug are shown in Fig. 2.

The loading rate of *valnemulin* in *PEG-valnemulin* prodrug was determined by UV and HPLC. The content determined by UV always was the total content, which maybe included *PEG-valnemulin* prodrug, PEG, PEG-succinic acid and free *valnemulin*. The contents of PEG, PEG-succinic acid and free *valnemulin* were determined respectively by HPLC-ELSA. The measuring processes of loading rate were as follows. *Valnemulin* was dissolved in 95% ethanol to prepare the samples with different concentrations of *valnemulin*. The samples were scanned by UV spectragraph to give absorbances, and the standard curve of *valnemulin* (Y=4.02015X-5.55687 × 10^{-4}, R^2=0.99987) was established based on the relation of the different concentrations to absorbance with the good linear concentration of 84.4–211 μg·ml^{-1}. A suitable concentration of *PEG-valnemulin* prodrug was measured according to the standard curve of *valnemulin* and the total content in the prodrug was successfully obtained by the standard curve. *Valnemulin hydrochloride* was dissolved in acetonitrile and water (43:57, v/v) to prepare different concentrations of *valnemulin hydrochloride*. The standard curve (Y=5137.21725X-1032.2518, R^2=0.99991) was obtained with the linear concentration of 1.44–144 μg·ml^{-1}. The content of free *valnemulin* in *PEG-succinic-valnemulin* prodrug could be determined by the standard curve. PEG and PEG-succinic acid were dissolved separately in acetonitrile and water (50:50, v/v). The standard curve of PEG (Y=1.51595X+7.04985, R^2=0.99982, 9.15–146.4 μg·ml^{-1}) and standard curve of PEG-succinic acid (Y=1.3639X+7.2144, R^2=0.99959, 8.55~171 μg·ml^{-1}) were obtained by natural logarithm of the concentrations and peak areas. The loading rate of *valnemulin* in *PEG-valnemulin* prodrug (*LR*) was obtained by formula (a), where *TC* represents the total content of *valnemulin* prodrug, *V* is the content of free *valnemulin* in *valnemulin* prodrug, *P* is the content of unreacted PEG in *valnemulin* prodrug and *PS* represents the content of PEG-succinic acid in *valnemulin* prodrug.

$$LR = TC - V - P - PS \quad \text{(a)}$$

Based on the reported measuring method of water solubility [33], a slight change was made and the measuring process of water solubility (S) was as follows. An amount of *PEG-valnemulin* conjugates (m_o/g) was dissolved in a volume of water (V/*l*) and stirred for 12 hr at 37 ± 0.5°C. Then the insoluble *PEG-valnemulin* prodrug was filtered after centrifuging for 5 min at 3,000 rpm. The filtered *PEG-succinic-valnemulin* prodrug was freeze-dried for 48 hr and weighed (m_t/g). The solubility of *PEG-succinic-valnemulin* prodrug could be determined according to formula (b). A contrast test was carried out using *valnemulin* as a reference substance. *Valnemulin* was freshly prepared by alkalization of *valnemulin* hydrochloride and recrystallization twice.

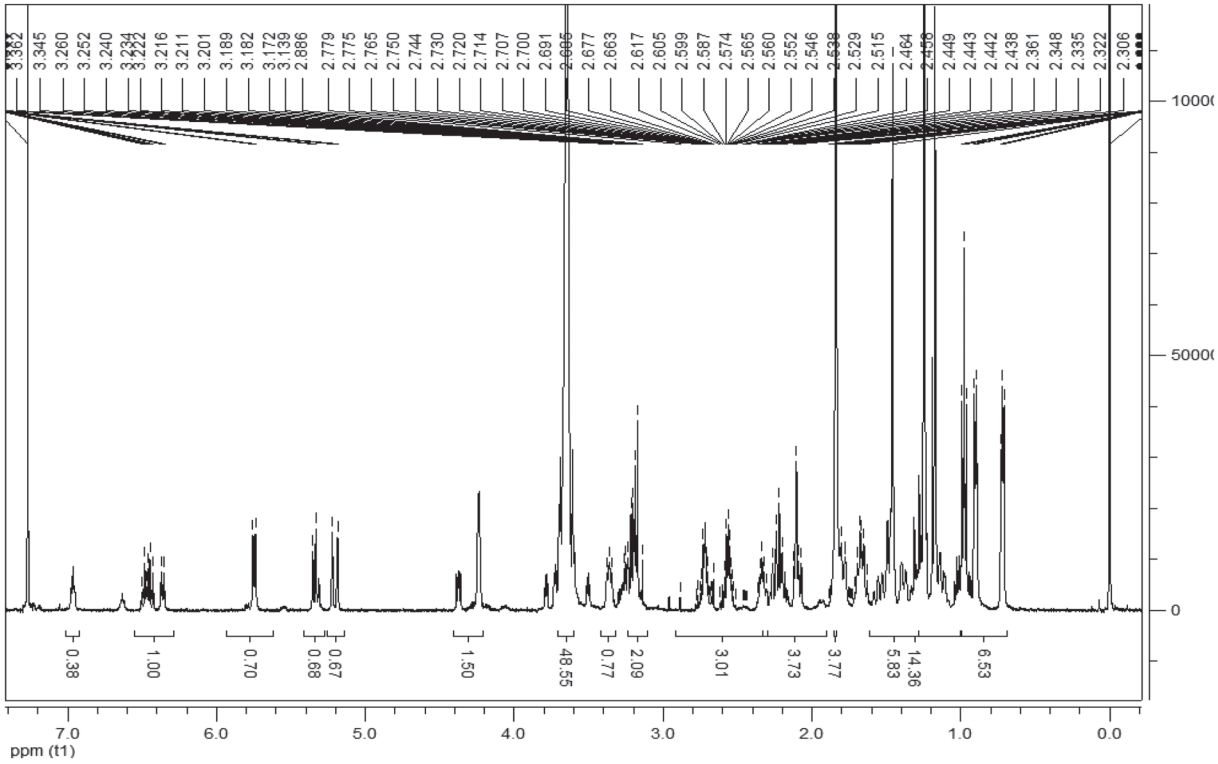

Fig. 2. ^{1}H-NMR of PEG-succinic-valnemulin prodrug.

$$S = \frac{m_0 - m_1}{V} \qquad \text{(b)}$$

On the basis of the 2015 Edition of Chinese Pharmacopoeia, a stability experiment of *PEG-succinic-valnemulin* prodrug and *valnemulin* with high humidity (92.5%, saturated potassium nitrate solution), high temperature (60°C) and high light (4,500 ± 500 lx) was performed.

An amount of *PEG-succinic-valnemulin* prodrug and *valnemulin* were tiled into the vials. Leaving the stoppers off the vials, the vials were put into the enclosed dryer with saturated potassium nitrate solution. The samples were weighed at fixed intervals of 0, 5 and 10 days, and recorded.

An amount of *PEG-succinic-valnemulin* prodrug and *valnemulin* were placed into the vials, separately. The stoppers of the vials were opened and the vials were put in the electro-heating standing-temperature cultivator at the temperature of 60°C. The samples were taken out at the fixed intervals of 0, 5 and 10 days and the content of *valnemulin* in the samples was measured by HPLC.

An amount of *PEG-succinic-valnemulin* prodrug and *valnemulin* were added to the vials. Leaving the stoppers off the vials, the vials were irradiated with the light intensity of 4,500 ± 500 lx. The samples were taken out at the fixed intervals of 0, 5, and 10 days and the content of *valnemulin* in the samples was measured by HPLC. The stability experiment was repeated three times [28].

Release tests of *PEG-succinic-valnemulin* prodrug and *valnemulin* (freshly prepared) were performed under simulation of intestinal condition (pH 7.4) and stomachic condition (pH 3.6) [23]. *PEG-succinic-valnemulin* conjugates (10 mg) and *valnemulin* (10 mg) were put into vials. Two kinds of buffer solution (10 m*l*) were respectively added to the vials. Sealed vials were placed into a shaking basket at 37 ± 0.5°C with the rotational speed of 50 rpm·min^{-1}. At intervals, samples were taken out of the vials and the concentration in the samples was measured by HPLC to observe controlled release performance of *PEG-valnemulin* prodrug and *valnemulin in vitro*. The controlled release *in vitro* experiments were carried out in triplicate.

RESULTS

The XRD patterns are shown in Fig. 3, including for PEG, *valnemulin*, *PEG-succinic-valnemulin* prodrug and the physical mixture of *valnemulin* and PEG. The amount of *valnemulin* in the physical mixture of *valnemulin* and PEG was determined according to the loading rate of *PEG-valnemulin* prodrug, thus, the mixture ratio of PEG and *valnemulin* was of 93.54:6.46 (m/m). Different crystalline structures show representative sharp peaks at different angles. As presented in Fig. 3, *valnemulin* is shows one marked broad peak at 12.9° and that means *valnemulin* has a fine, amorphous crystal form. PEG shows two marked peaks at 19.3° and 23.5°. The mixture of *valnemulin* and PEG shows three marked peaks at 12.9°, 19.2° and 23.4°, which strongly suggest *valnemulin* and PEG in the physical mixture retain their specific crystalline structures. But the prepared *PEG-valnemulin* prodrug

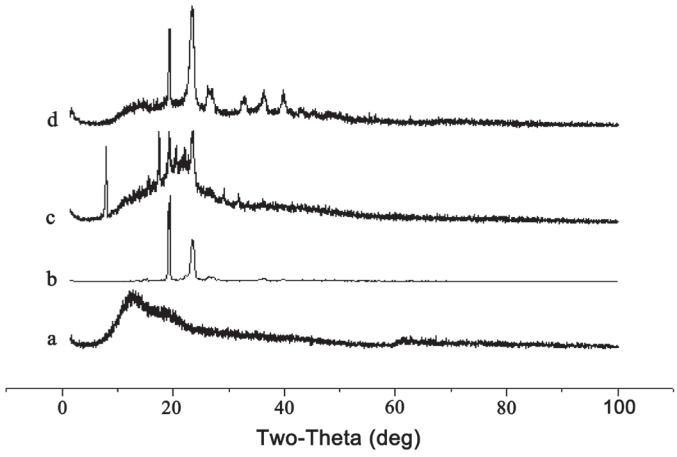

Fig. 3. XRD spectrum.

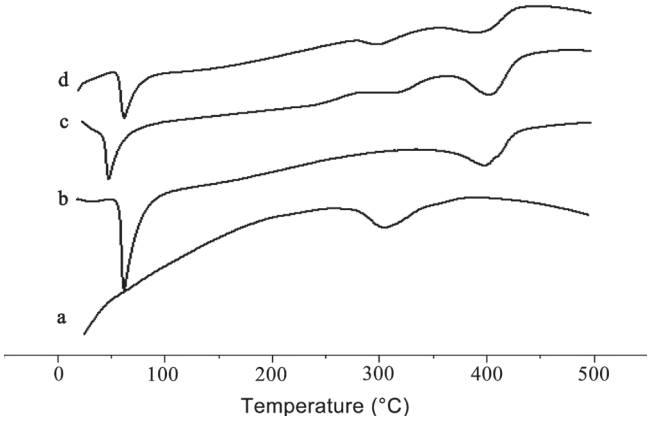

Fig. 4. DSC spectrum.

exhibits a completely different XRD result with two marked peaks appear at 7.9° and 17.6°. Obviously, because of the formed *PEG-valnemulin* prodrug having a different crystalline structure, this leads to different typical peaks from PEG and *valnemulin*. It is also illustrated that the prepared *PEG-valnemulin* prodrug has a crystal form.

Analysis of DSC reveals some information about substances, including specific heat capacity, reaction heat and purity. As shown in Fig. 4, the characteristic wide endothermic peak of *valnemulin* is at 303.41°C. The melting point and decomposition point of PEG appear at 61.46 and 397.99°C, respectively. Based on the curve of the mixture of PEG and *valnemulin*, three endothermic peaks are observed at 62.00, 300.78 and 395.70°C. In comparison, two different peaks of *PEG-valnemulin* prodrug appear 47.41 and 316.62°C. The peak of 316.62°C, which is different from *valnemulin* and the physical mixture no matter what the peak pattern and endothermic temperature, may be caused by the laden *valnemulin* in *PEG-succinic-valnemulin* prodrug. As is known, *valnemulin* itself has poor heat stability, hence, even if it was laden in PEG, decomposition of *valnemulin* still cannot be avoided at temperatures over 300°C. The different endothermic temperature and peak pattern clearly indicate that the laden *valnemulin* in *PEG-valnemulin* prodrug exists not in the free style, but a new kind of compound has formed.

Table 1. Water solubility of PEG-succinic-valnemulin prodrug and valnemulin (n=3)

Determinant	Soubility/mg·ml^{-1}
PEG conjugates	523.5 ± 1.3
Valnemulin	2 ± 0.5

Improvement of water solubility of *valnemulin* is an aim of this study because good water solubility of a drug can result in good absorption and distribution. The result of the test for water solubility of PEG-succinic-valnemulin prodrug and valnemulin was shown in Table 1.

To obtain preliminary information for the potential use of *pegylated-valnemulin* and to observe the performance of PEG prodrug as a delivery system for controlled release of *valnemulin*, tests of in vitro release were studied in buffer solutions of pH3.6 and pH7.4 (simulating the conditions of stomach and intestine, respectively) in a period of 72 hr. The result is showed in Fig. 5. Degradative performance of valnemulin is showed in Fig. 6.

DISCUSSION

Loading rate is a significant therapeutic property and is an important criterion for conjugate evaluation [34]. Thus, establishing an exact measuring method of loading rate is important for the determination of suitable dosage and safety of use. As reported, the loading rate of prodrug was calculated by formula (c), and was determined only by the difference between the amount measured using UV with ultraviolet absorption of drug and that measured using HPLC-UV; the absorption of PEG and PEG carboxylic acid was not taken into account. It is reasonable for most drugs because they always have clear and strong ultraviolet absorption, therefore, their specific ultraviolet characteristic peak can be used to distinguish between PEG and PEG carboxylic acid, thus, accurate contents of the drug can be determined from standard curve of drug. But those drugs with weak ultraviolet absorption drugs, like *valnemulin*, with similar ultraviolet absorption to PEG at 210 nm, the reported method is not appropriate for determination of the loading rate of the prodrug. In the process of loading rate determination of *PEG-succinic-valnemulin* prodrug, if the reported method is applied, the influence of PEG and PEG-succinic acid (they both have weak ultraviolet absorption, close to 210 nm) leads to an inaccurate loading rate. Hence, it is urgent to find a new method to get the accurate loading rate of valnemulin in *PEG-succinic-valnemulin* prodrug. The method of SFC-ELSD has been reported to successfully determine the contents of compounds with weak ultraviolet absorptions such as fatty acids, saccharides and plasticizers [11]. The amount of free PEG in pegylated interferon alpha-2α injection has also been measured by HPLC-ELSD [12, 20]. But the HPLC-ELSD analytic method has not been reported in the determination of the content of PEG and PEG-succinic acid in peglyted prodrug, or in the loading rate

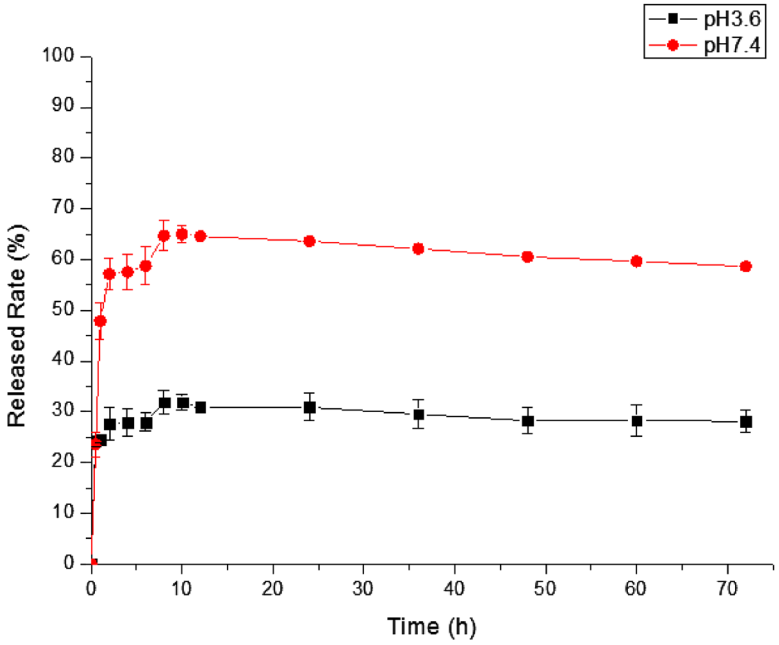

Fig. 5. Release of valnemulin from PEG-succinic-valnemulin prodrug.

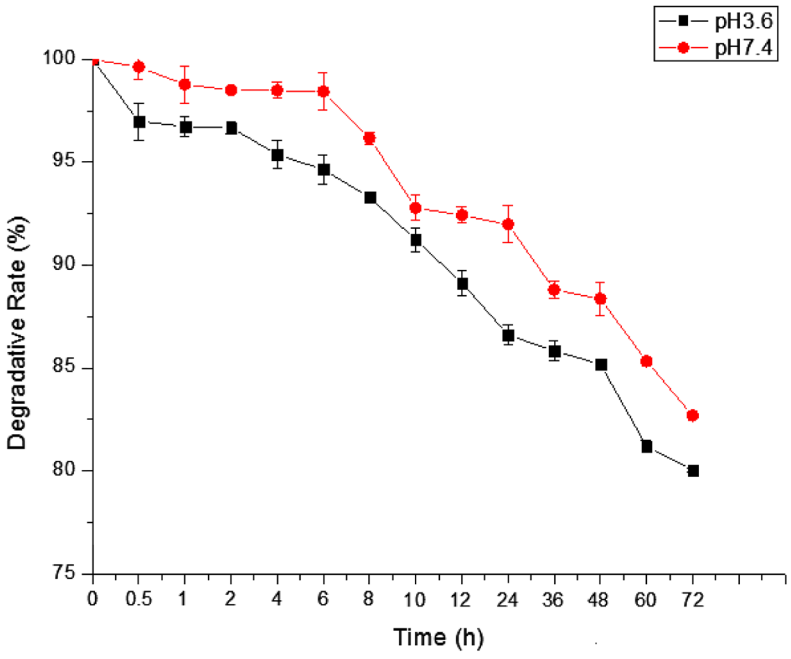

Fig. 6. Degradation of Valnemulin.

Table 2. Stability of PEG-succinic-valnemulin prodrug and valnemulin (n=3)

Groups	Determinant	0 day	5 day	10 day
high humidity	PEG conjugates/g	5.1649 ± 0.12	5.1698 ± 0.10	5.2848 ± 0.09
	Valnemulin/g	5.3677 ± 0.26	5.3681 ± 0.11	5.3876 ± 0.11
high temperature	PEG conjugates/%	63.50 ± 0.24	62.52 ± 0.16	62.08 ± 0.13
	Valnemulin/%	99.50 ± 0.61	92.57 ± 0.12	91.84 ± 0.12
high light	PEG conjugates/%	63.50 ± 0.37	62.46 ± 0.25	62.44 ± 0.21
	Valnemulin/%	99.50 ± 0.52	99.17 ± 0.23	98.44 ± 0.11

determination of PEG prodrug. In this work, the loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was determined by UV, HPLC and HPLC-ELSD. The application of HPLC-ELSD made the resultant loading rate more accurate than before because determination of contents of PEG and PEG-succinic acid become reality. The experimental results demonstrated that the loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was 6.46%. The large molecular structure and spatial hindrance of *valnemulin* may be the reason for the low loading rate. Wrapping and entanglement of PEG also results in a decrease in reactivity.

Valnemulin has poor water solubility, hence, *valnemulin* hydrochloride is used clinically. *PEG-succinic-valnemulin* prodrug demonstrated a great ability to increase the solubility of *valnemulin* up to 523 ± 1.3 mg·ml^{-1}, compared with *valnemulin* hydrochloride with 160 mg·ml^{-1}.

The data of the stability experiment of *PEG-succinic-valnemulin* prodrug and *valnemulin* is shown in Table 2. As shown in Table 2, the weighting of 0.10% on day 5 and 2.32% on day 10 of *PEG-succinic-valnemulin* prodrug in the high humidity experiment can meet the standard with not more than 5% of the sample (2015 edition of Chinese pharmacopoeia appendix XIXC), while the weighting was of 0.0075% on day 5 and 0.37% on day 10 of *valnemulin*. The declining contents in the high temperature experiment of *PEG-succinic-valnemulin* prodrug are 0.98% after 5 days and 1.42% after 10 days and in the high light experiment of 1.04% after 5 days and 1.06% after 10 days, which are less than 5% of the sample and meet the criterion (2015 edition of Chinese pharmacopoeia appendix XIXC). The declining contents of *valnemulin* in the high temperature experiment are of 6.93% after 5 days and 7.66% after 10 days, which are more than 5% of the sample and exceed the criterion, and in the high light experiment of 0.33% after 5 days and 1.06% after 10 days. Meanwhile, the appearance of *PEG-succinic-valnemulin* prodrug and *valnemulin* has no obvious change in the high light experiment. The stability experiment indicated that *PEG-succinic-valnemulin* prodrug was more stable than *valnemulin* and the stability experiment might present suitable storage conditions of the conjugates.

The release mechanism of prodrugs has been reported as being covalent bonds including ester linkage, carboxylic hydrazine and amido bonds between carrier and drugs were hydrolyzed under acidic or alkaline conditions to release drugs [13, 21]. As shown in Fig. 5, the cumulative release rate of *PEG-valnemulin* prodrug is up to 64.98% at pH 7.4 and it is higher than that of pH 3.6 with release rate of 31.90% at 8 hr. The release performance of a prodrug may be controlled by the strength of the covalent bond with the supporter, which is sensitive to pH condition and by the spatial wrapping pattern of conjugates, which influences the inclusion degree of drugs [2, 32]. For the most part, pH not only affects the reactivity of the substrate but also the mode of existence, especially for macromolecules, for example, elongation and crimpness. Therefore, the faster release of *valnemulin* from prodrug in pH7.4 may be caused by the combination of various factors; the reactivity of the formed covalent bond between drug and supporter or the elongation and crimpness of the prepared PEG prodrug. Simultaneously, the low solubility of *valnemulin* may be the other reason, it also may promote hydrolysis because released *valnemulin* from prodrug in pH7.4 is much more easily precipitated from solution than that in pH3.6, resulting in the equilibrium moving in the release direction. This suggests that release of *PEG-valnemulin* prodrug might have low irritability in intestinal conditions rather than stomachic conditions. In the initial stages of release (within 2 hr), the burst effect of the drug still exists whether *PEG-succinic-valnemulin* prodrug is under the condition of pH3.6 or pH7.4. The release rate *in vitro* of *PEG-succinic-valnemulin* prodrug reaches the maximum at 8 hr and subsequently decreases gradually in the next 48 hr. The modest decline of release rate is due to the instability of *valnemulin*. As shown in Fig. 6, the degradative rates of *valnemulin* were 17.28% at pH7.4 and 19.93% at pH3.6 at 72 hr. The hydrolyzed *valnemulin* from *PEG-succinic-valnemulin* prodrug in the early stage degrades with time, and this was proved by HPLC detection. The results demonstrate that *PEG-succinic-valnemulin* prodrug has the ability of controlled release.

In summary, the *PEG-succinic-valnemulin* prodrug is expected to improve the water solubility and prolong the half-life period. Prepared *PEG-succinic-valnemulin* prodrug via the spacer of succinic acid was characterized and studied by ^{1}H-NMR, XRD and DSC. The loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was determined by UV, HPLC-UV and HPLC-ELSD and the loading rate of *valnemulin* in *PEG-succinic-valnemulin* prodrug was 6.46%. The *PEG-succinic-valnemulin* prodrug demonstrated a great ability to increase the solubility of *valnemulin* up to 523 ± 1.3 mg·ml^{-1}. The stability of *PEG-succinic-valnemulin* prodrug was good. The release behaviors *in vitro* of *PEG-succinic-valnemulin* prodrug indicated *PEG-valnemulin* prodrug had the controlled release ability and the release rate of *valnemulin* from *PEG-valnemulin* prodrug was 64.98% with pH7.4, which was higher than that of pH3.6 with a release rate of 31.90%. The results showed that the novel *PEG-succinic-valnemulin* prodrug can greatly enhance the solubility, stability and controlled release while further improving the pharmacokinetics of *valnemulin*. Therefore, the prepared *PEG-succinic-valnemulin* prodrug has great potential for application.

CONFLICT OF INTEREST. The authors declare no conflicts of interest in this work.

ACKNOWLEDGMENTS. We express our gratitude to the Hebei Educational Grant Foundation (ZD2015076), the Team Fund of Hebei University of Science and Technology, Hebei Medical and Chemical Engineering Centre and Pharmaceutical Molecular Chemistry Key Laboratory of the Ministry of Technology.

REFERENCES

1. Albert, A. 1958. Chemical aspects of selective toxicity. *Nature* **182**: 421–422. [Medline] [CrossRef]
2. Cavallaro, G., Licciardi, M., Caliceti, P., Salmaso, S. and Giammona, G. 2004. Synthesis, physico-chemical and biological characterization of a paclitaxel macromolecular prodrug. *Eur. J. Pharm. Biopharm.* **58**: 151–159. [Medline] [CrossRef]
3. Chandrashekar, B. N., Swamy, B. E. K., Pandurangachar, M., Shankar, S. S., Gilbert, O., Manjunatha, J. G. and Sherigara, B. S. 2010.

Electrochemical oxidation of dopamine at polyethylene glycol modified carbon paste electrode: A cyclic voltammetric study. *Int. J. Electrochem. Sci.* **5**: 578–592.
4. Chung, Y. and Cho, H. 2004. Preparation of highly water soluble tacrolimus derivatives: poly(ethylene glycol) esters as potential prodrugs. *Arch. Pharm. Res.* **27**: 878–883. [Medline] [CrossRef]
5. Dip, R., Nemet, Z., Schiessl, B., Klein, U. and Strehlau, G. 2015. Efficacy and tolerability of early administration of valnemulin hydrochloride premix on epizootic rabbit enteropathy. *Vet. J.* **204**: 309–314. [Medline] [CrossRef]
6. Gund, M., Khanna, A., Dubash, N., Damre, A., Singh, K. S. and Satyam, A. 2015. Water-soluble prodrugs of paclitaxel containing self-immolative disulfide linkers. *Bioorg. Med. Chem. Lett.* **25**: 122–127. [Medline] [CrossRef]
7. Guo, H., Liu, K., Liu, Y., Fang, B., Liu, M., He, L. and Zeng, Z. 2011. Molecularly imprinted solid-phase extraction for the selective determination of valnemulin in feeds with high performance liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **879**: 181–185. [Medline] [CrossRef]
8. Hannan, P. C., Windsor, H. M. and Ripley, P. H. 1997. *In vitro* susceptibilities of recent field isolates of *Mycoplasma hyopneumoniae* and *Mycoplasma hyosynoviae* to valnemulin (Econor), tiamulin and enrofloxacin and the *in vitro* development of resistance to certain antimicrobial agents in *Mycoplasma hyopneumoniae*. *Res. Vet. Sci.* **63**: 157–160. [Medline] [CrossRef]
9. Henne, W. A., Kularatne, S. A., Hakenjos, J., Carron, J. D. and Henne, K. L. 2013. Synthesis and activity of a folate targeted monodisperse PEG camptothecin conjugate. *Bioorg. Med. Chem. Lett.* **23**: 5810–5813. [Medline] [CrossRef]
10. Huang, Q., Li, J., Xia, L., Xia, X., Duan, P., Shen, J. and Ding, S. 2010. Residue depletion of valnemulin in swine tissues after oral administration. *Anal. Chim. Acta* **664**: 62–67. [Medline] [CrossRef]
11. Lecoeur, M., Simon, N., Sautou, V., Decaudin, B., Vaccher C., ARMED study group 2014. A chemometric approach to elucidate the parameter impact in the hyphenation of evaporative light scattering detector to supercritical fluid chromatography. *J. Chromatogr. A* **1333**: 124–133. [Medline] [CrossRef]
12. Li, M. and Peng, G. 2010. HPLC-ELSD determination of macrogo l4000 in macrogo l4000 powder. *Chin. J. Pharm. Anal.* **30**: 1276–1278.
13. Li, Y. H., Li, X., Han, C. M. and Rao, C. M. 2014. Quantitation of free polyethylene glycol in PEGylated interferon alpha-2α injection by HPLC-ELSD. *Pharm. Biotechnol.* **21**: 353–354.
14. Min, T., Ye, H., Zhang, P., Liu, J., Zhang, C., Shen, W. B., Wang, W. and Shen, L. S. 2016. Water-soluble poly(ethylene glycol) prodrug of pemetrexed: Synthesis, characterization, and preliminary cytotoxicity. *J. Appl. Polym. Sci.* **111**: 444–451. [CrossRef]
15. Mishra, P., Nayak, B. and Dey, R. K. 2016. PEGylation in anti-cancer therpy: An overview. *J. Pharm. Sci-us* **11**: 337–348.
16. Ouyang, J. B., Wang, J. K., Wang, Y. L., Yin, Q. X. and Hao, H. X. 2015. Thermodynamic study on dynamic water and organic vapor sorption on amorphous valnemulin hydrochloride. *Front. Chem. Sci. Eng.* **9**: 94–104. [CrossRef]
17. Ouyang, J. B., Wang, J. K., Hao, H. X., Huang, X., Gao, Y., Bao, Y., Wang, Y. L. and Yin, Q. X. 2014. Determination and correlation of solubility and solution thermodynamics of valnemulin hydrogen tartrate in different pure solvents. *Fluid Phase Equilib.* **372**: 7–14. [CrossRef]
18. Ouyang, J. B., Wang, J. K., Huang, X., Bao, Y., Wang, Y. L., Yin, Q. X., Liu, A. L., Li, X. D. and Hao, H. X. 2015. Solution thermodynamics of valnemulin hydrogen fumarate in different pure solvents. *J. Chem. Thermodyn.* **91**: 73–79. [CrossRef]
19. Pfister, D. and Morbidelli, M. 2014. Process for protein PEGylation. *J. Control. Release* **180**: 134–149. [Medline] [CrossRef]
20. Rodrigues, P. C. A., Roth, T., Fiebig, H. H., Unger, C., Mülhaupt, R. and Kratz, F. 2006. Correlation of the acid-sensitivity of polyethylene glycol daunorubicin conjugates with their *in vitro* antiproliferative activity. *Bioorg. Med. Chem.* **14**: 4110–4117. [Medline] [CrossRef]
21. Sheng, Y., Yang, X., Pal, D. and Mitra, A. K. 2015. Prodrug approach to improve absorption of prednisolone. *Int. J. Pharm.* **487**: 242–249. [Medline] [CrossRef]
22. Stella, V. J. and Himmelstein, K. J. 1980. Prodrugs and site-specific drug delivery. *J. Med. Chem.* **23**: 1275–1282. [Medline] [CrossRef]
23. Suk, J. S., Xu, Q., Kim, N., Hanes, J. and Ensign, L. M. 2016. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv. Drug Deliv. Rev.* **99** Pt A: 28–51. [Medline] [CrossRef]
24. Veronese, F. M. and Pasut, G. 2005. PEGylation, successful approach to drug delivery. *Drug Discov. Today* **10**: 1451–1458. [Medline] [CrossRef]
25. Wang, R., Yuan, L. G., He, L. M., Zhu, L. X., Luo, X. Y., Zhang, C. Y., Yu, J. J., Fang, B. H. and Liu, Y. H. 2011. Pharmacokinetics and bioavailability of valnemulin in broiler chickens. *J. Vet. Pharmacol. Ther.* **34**: 247–251. [Medline] [CrossRef]
26. Wang, X., Ling, Y., Wang, H., Yu, J., Tang, J., Zheng, H., Zhao, X., Wang, D., Chen, G., Qiu, W. and Tao, J. 2012. Novel pleuromutilin derivatives as antibacterial agents: synthesis, biological evaluation and molecular docking studies. *Bioorg. Med. Chem. Lett.* **22**: 6166–6172. [Medline] [CrossRef]
27. Yu, D., Peng, P., Dharap, S. S., Wang, Y., Mehlig, M., Chandna, P., Zhao, H., Filpula, D., Yang, K., Borowski, V., Borchard, G., Zhang, Z. and Minko, T. 2005. Antitumor activity of poly(ethylene glycol)-camptothecin conjugate: the inhibition of tumor growth *in vivo*. *J. Control. Release* **110**: 90–102. [Medline] [CrossRef]
28. Yuan, L. G., Luo, X. Y., Zhu, L. X., Wang, R. and Liu, Y. H. 2011. A physiologically based pharmacokinetic model for valnemulin in rats and extrapolation to pigs. *J. Vet. Pharmacol. Ther.* **34**: 224–231. [Medline] [CrossRef]
29. Zacchigna, M., Di Luca, G., Maurich, V. and Boccù, E. 2002. Syntheses, chemical and enzymatic stability of new poly(ethylene glycol)-acyclovir prodrugs. *Farmaco* **57**: 207–214. [Medline] [CrossRef]
30. Zhang, H., Mi, T., Khan, O. Y., Sheng, Y., Eremin, S. A., Beier, R. C., Zhang, S., Shen, J. and Wang, Z. 2015. Fluorescence polarization immunoassay using IgY antibodies for detection of valnemulin in swine tissue. *Anal. Bioanal. Chem.* **407**: 7843–7848. [Medline] [CrossRef]
31. Zhang, J., Liu, Y. F., Bo, L. and Qiao, C. A. 2011. Synthesis of poly(ethylene glycol)-dopamine conjugate and their controlled drug-release behaviors. *J. Appl. Polym. Sci.* **121**: 1992–1998. [CrossRef]
32. Zhang, J., Fan, X., Liu, Y., Bo, L. and Liu, X. 2007. Synthesis of poly(ethylene glycol)-metaxalone conjugates and study of its controlled release in vitro. *Int. J. Pharm.* **332**: 125–131. [Medline] [CrossRef]
33. Zhang, Y., Song, H., Shang, Z., Chen, A., Huang, D., Zhao, H. and Du, H. 2014. Amino acid-PEGylated resveratrol and its influence on solubility and the controlled release behavior. *Biol. Pharm. Bull.* **37**: 785–793. [Medline] [CrossRef]
34. Zhao, D. H., Yu, Y., Zhou, Y. F., Shi, W., Deng, H. and Liu, Y. H. 2014. Postantibiotic effect and postantibiotic sub-minimum inhibitory concentration effect of valnemulin against *Staphylococcus aureus* isolates from swine and chickens. *Lett. Appl. Microbiol.* **58**: 150–155. [Medline] [CrossRef]
35. Zhao, D. H., Zhou, Y. F., Yu, Y., Shi, W., Yang, X., Xiao, X., Deng, H., Qiao, G. G., Fang, B. H. and Liu, Y. H. 2014. Integration of pharmacokinetic and pharmacodynamic indices of valnemulin in broiler chickens after a single intravenous and intramuscular administration. *Vet. J.* **201**: 109–115. [Medline] [CrossRef]