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Validation of a UPLC/MS/MS Method for Determination of Compound Amoxicillin and Tylosin in Swine Plasma and its Application in a Pharmacokinetic Study

Xue Gao[1], Gang Xu[1], Yong Liu[2], Jianqing Chen[1], Yang Cui[1], Guangdong Cheng[1], Qingbo Yan[3] and Yanhua Li*

[1]Department of Animal Pharmacy, College of Veterinary Medicine, Northeast Agricultural University, No.59 Mucai Road, Harbin - 150 030, China.
[2]Heilongjiang Entry-Exit Inspection and Quarantine Bureau, No. 9 Ganshui Road, Harbin - 150 001 China.
[3]College of Veterinary Medicine, Northeast Agricultural University, No.59 Mucai Road, Harbin - 150 030, China.

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An ultra-high-performance liquid chromatograph-tandem mass spectrometry (UPLC/MS/MS) method was developed for the determination of amoxicillin sodium and tylosin tartrate in swine plasma. Amoxicillin sodium and tylosin tartrate were orally administrated to swine, and the pharmacokinetics were evaluated in plasma samples by UPLC/MS/MS after solid phase extraction. The linear range for amoxicillin sodium was 0.06–1 ¼Àg/mL (r^2 = 0.9975) and that for tylosin tartrate was 0.5–50 ng/mL (r^2 = 0.9994). The limit of quantitation and limit of detection for amoxicillin sodium were 0.2 ¼Àg/mL and 0.06 ng/mL, respectively, and the corresponding values for tylosin tartrate were 1.7 ng/mL and 0.5 ng/mL. The intra- and inter-day coefficients of variation were <9.8 % for amoxicillin sodium and <13.5 % for tylosin tartrate. The absolute recoveries of amoxicillin sodium and tylosin tartrate from swine plasma were 67 % and 72 %, respectively. The method showed excellent specificity, accuracy, precision, recovery, and stability. The pharmacokinetics of amoxicillin sodium (15 mg/kg) and tylosin tartrate (10 mg/kg) in healthy swine after administration of a single or compound dose were evaluated. Tylosin influenced the pharmacokinetics of amoxicillin, but amoxicillin did not affect tylosin.

Key words: UPLC/MS/MS; Pharmacokinetics; Amoxicillin sodium; Tylosin tartrate; Compound Amoxicillin and Tylosin.

Tylosin (Fig. 1a) is a macrolide antibiotic that is isolated from *Streptomyces spp.*, and is used for treating bacterial infections and mycoplasmosis caused by Gram-positive bacteria and mycoplasma[1]. Tylosin is widely used to treat and prevent respiratory diseases and enteric infections in cattle, sheep, swine, and poultry[2]. It is used to reduce liver abscesses in feedlot cattle and treat swine streptococcosis, colibacillosis, atrophic rhinitis, mastitis, metritis, spirochetosis, and caprine pleuropneumonia, and has been used in chickens[3]. Some studies have investigated the pharmacokinetics of tylosin in pigs[4]. Wang et al. established a liquid chromatography/electrospray ionization-tandem mass spectrometry (LC-ESI/MS/MS) method for five different macrolide antibiotics with recoveries of 95. 4 to 98.8 % and sensitivity (signal-to-noise ratio = 3:1) <1.0 µg/kg[5]. Amoxicillin (Fig. 1b, AMO) is a broad-spectrum antibiotic that shows good absorption, and is recommended by the World Health Organization as a β-lactam oral antibiotic[6]. AMO can inhibit the synthesis of

* To whom all correspondence should be addressed. Tel.: +86 139 4609 9563; Fax: +86 0451 5519 0363; E-mail: liyanhua1970@163.com

peptidoglycans in cell walls. AMO is a valuable antibiotic in pigs[7] and bovines[8, 9] to treat a variety of bacterial infections in the digestive and respiratory systems. Investigations of the pharmacokinetics of AMO for many administration routes have shown that it is almost fully absorbed.

There are many methods available for AMO testing, including microbiological assays[10], spectrophotometric methods[11-13], FI-chemiluminescence[14] and HPLC methods. AMO contains a hydroxylamine benzyl group, which has a characteristic ultraviolet absorption. Wibawa established a fast, selective, and sensitive HPLC fluorometric method to analyze the AMO content (<1 µg/mL) in rat serum, gastric acid, and stomach tissue[15].

Treatment with a combination of anti-microbial agents has become a valid strategy to achieve synergistic antimicrobial activity with mixed bacterial infections and prevent/postpone the emergence of drug resistance[16-18].

This paper reports the optimization and validation of a quantitative ultra-high-performance liquid chromatograph-tandem mass spectrometry (UPLC/MS/MS) method for the determination of the AMO sodium and tylosin tartrate in swine plasma. The pharmacokinetics of compound AMO sodium-tylosin tartrate following oral administration were studied in swine.

MATERIALS AND METHODS

Materials

AMO sodium was purchased from Topology Pharmaceutical Co., Ltd (Zhejiang, China). AMO sodium and tylosin tartrate reference standards were purchased from Dr. Ehrenstorfer GmbH (Augsberg, Germany). Compound AMO sodium-tylosin tartrate was prepared in our laboratory. Chromatography grade acetonitrile, methanol and glacial acetic acid were purchased from Thermo Fisher Scientific (Waltham, MA). Chromatography grade sodium dihydrogen phosphate and ammonium acetate were purchased from Kermel Chemicals Limited (Tianjin, China). All standard samples were stored at –20 °C.

Individual stock solutions of AMO (5 mg/mL) and tylosin (5 mg/mL) were prepared in 1 mL of acetonitrile-acetic acid (95:5, v/v). Working standard solutions (1 mg/L) were prepared by diluting the stock solutions with the initial mobile phase. These solutions were stable for at least 3 months at –20 °C.

Chromatography and Mass spectrometric conditions

Identification and quantification of analytes were carried out using an ACQUITY Ultra Performance LC (UPLC) (Waters, Milford, MA) equipped with a Quattro Micro™ tandem mass spectrometer (MS/MS). A Waters Xbridge C18 column (2.1×50 mm I.Dÿ1.7 µm particle size) was used for the UPLC separation. The column oven temperature was 30 °C, the mobile phase flow rate was 0.25 mL/min, and the injection volume was 5 µL. The mobile phase was acetonitrile and acetic acid (2 %). The gradient elution for amoxicillin sodium was performed as follows: 0.00–0.60 min, acetonitrile- acetic acid (2 %) (5:95, v/v); 0.80–2.00 min, acetonitrile- acetic acid (2 %) (60:40, v/v); 2.50 min, and acetonitrile- acetic acid (2 %) (5:95, v/v). The gradient elution for tylosin tartrate was performed as follows: 0.00–0.40 min, acetonitrile-acetic acid (2 %) (30:70, v/v); 0.80–1.40 min, acetonitrile- acetic acid (2 %) (90:10, v/v); and 2.00 min, acetonitrile- acetic acid (2 %) (30:70, v/v). The mass spectrometer was operated in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The capillary voltage was maintained at 3.0 kV and the multiplier voltage at 650 V. Nitrogen was used as the nebulizing, desolvation, and cone gas. The nebulizing gas flow rate was adjusted to the maximum value, and the desolvation gas flow rate was 500 L/h. The source and desolvation gas temperatures were 110 and 350 °C, respectively. During MS/MS analysis, ultra-high purity argon was used as the collision gas, and the collision chamber was kept 3.8×10-2 Pa. The ions monitored for tylosin tartrate and amoxicillin sodium were m/z 366.3/349.2 and m/z 916.9/772.4, respectively.

Sample preparation

AMO sodium

The blood samples were centrifuged to obtain plasma samples, which were stored at –20 °C until analysis. Before analysis, the samples were thawed at room temperature and 0.5 mL of the plasma was placed in a polypropylene centrifuge tube (15 mL). Acetonitrile (2 mL) was added, and each tube was vortex mixed for 10 s and then placed on a shaker for 10 min. After centrifugation (5 min,

3500 ´g), the supernatant was transferred into another 15 mL polypropylene centrifuge tube, and the solution was extracted with 1 mL of acetonitrile. The organic fraction was then evaporated to dryness at 60 °C under a gentle stream of nitrogen. The residue was dissolved in 1.0 mL of sodium dihydrogen phosphate (pH 8.5; 0.05M) and pretreated by solid phase extraction (SPE) before UPLC/MS/MS analysis. The centrifuge tubes were washed twice with phosphate buffer to remove the sample, and the phosphate buffer was loaded directly onto a SPE (Oasis HLB, Waters) cartridge at a flow rate of 1–2 mL/min. After absorption, the sample was eluted from the SPE cartridge with 5.0 mL of acetonitrile and evaporated to dryness under a stream of nitrogen at 45 °C. The residue was dissolved in 2.0 mL of acetonitrile- acetic acid (2 %) (5:95, v/v) and filtered through a 0.22 µm filtration membrane before UPLC/MS/MS analysis.

Tylosin tartrate

The blood samples were centrifuged to obtain plasma samples, which were stored at –20 °C until analysis. The samples were then thawed at room temperature, and 0.5 mL of plasma was placed in a 15 mL polypropylene centrifuge tube. Ammonium acetate (pH 4; 0.05 M), 0.5 g of solid NaCl, and acetonitrile (5 mL) were added, and each tube was vortex mixed for 10 s and then placed on a shaker for 10 min. After centrifugation (5 min, 5000 rpm), the supernatant was transferred into another 15 mL polypropylene centrifuge tube and then evaporated to dryness at 50 °C under a gentle stream of nitrogen. Each sample was dissolved in 2.5 mL of ammonium acetate (pH 4; 0.05 M) and loaded onto a Supelclean TM LC-18 SPE cartridge (3 mL/500mg volume, Supelco, Bellefonte, PA) that was equilibrated with 3 mL of methanol and 5 mL of deionized water. The SPE cartridge was dried for at least 30 s under vacuum. The samples were eluted from the cartridges with 5 mL of 5 % ammonia/ methanol solution. The eluate was transferred to a 15 mL centrifuge tube and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved in 1.0 mL of acetonitrile-acetic acid (0.2 %)(30:70, v/v) and filtered through a 0.22 µm filtration membrane before UPLC/MS/MS analysis.

Method validation

Method validation was carried out according to the U.S. Food and Drug Administration (FDA) bioanalytical method validation guide[19]. Blank plasma samples (0.5 g, n = 6) spiked at 5, 50, 200, and 500 µg/mL with the working standard solution were analyzed using the method described above.

The selectivity was determined by testing blank samples from six different swine for interferences. The limit of detection (LOD) was determined as the concentration of the drug giving a signal-to-noise ratio of 3:1, and the limit of quantitation (LOQ) as the lowest concentration of the drug giving a signal-to-noise ratio of 10:1.

Calibration curve

Five-point calibration curves were constructed for AMO sodium (0.06, 0.12, 0.25, and 0.5 µg/mL) and tylosin tartrate (0.5, 1, 5, 10, and 50 ng/mL). The acceptance criterion for each back-calculated standard concentration was that it was within 15 % of the nominal value, and did not exceed 20 % at the LOQ.

Precision and accuracy

Intra- and interday accuracy and precision were determined by replicate analyses of five sets of AMO sodium and tylosin tartrate samples within one day and on five consecutive days, respectively.

Recovery

Quality control (QC) samples at low, middle, and high concentrations (0.06 µg/mL, 0.25 µg/mL, 1 µg/mL for amoxicillin sodium; 0.5 ng/mL, 5 ng/mL, 50 ng/mL for tylosin tartrate) were prepared with blank plasma. The recovery was evaluated according to the peak area ratios of samples spiked with the standard compounds.

Stability

The stability of QC samples in the processed samples during storage at 20°C was studied at three concentrations. The concentration of QC samples after a15-day storage was compared to the initial concentrations as determined for freshly prepared samples. The freezethaw stability was determined after three freeze and thaw cycles. In each cycle, the samples were stored at 20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the sample was refrozen within 24 h.The freeze–thaw cycle was repeated two times and then the samples were analyzed after the third cycle.

Pharmacokinetic study in swine

The UPLC/MS/MS procedure was applied to a pharmacokinetic study of compound AMO sodium and tylosin tartrate. Fifteen healthy

hybrid swine with an average body weight of 21.3±4.5 kg were obtained from the College of Veterinary Medicine (Harbin, China). The 15 animals were randomly divided into three groups. Group I was orally administered AMO sodium (15mg/kg), Group II was orally administered tylosin tartrate (10 mg/kg), and Group III was orally administered compound AMO sodium (15 mg/kg) and tylosin tartrate (10 mg/kg). Blank blood samples were collected from the precaval vein before administration, and at 0.33, 0.67, 1, 1.5, 2, 3, 4, 5, 6, and 8 h after administration for Group I and Group II and 0.16, 0.33, 0.5, 0.75, 1, 2, 3, 4, 5, 6, and 8 h after administration for Group III. Plasma was separated and stored at –40 °C until analysis after centrifugation at 3000 rpm for 10 min.

Pharmacokinetic analysis of data

Concentration-time curves were constructed for each group and the pharmacokinetics parameters were obtained using 3P97 software (Chinese Pharmacological Society, Beijing, China).

RESULTS AND DISCUSSION

Development of SPE procedures

Over 80% of the analysis time was spent on sample preparation. SPE had gradually replaced classical liquid–liquid extraction. However, AMO is strongly polar, and it was difficult to retain it in the Sep-Pak, Supelco, and Oasis SPE cartridges. It was completely retained on Oasis HLB-SPE cartridges, and these were used for extraction and purification in the present study.

Macrolides tended to be protonated and dissolve well in acidic aqueous solutions.

Table 1. Pharmacokinetic parameters of AMO in pigs following oral administration of AMO sodium (15 mg/kg) and compound AMO sodium (15 mg/kg)-tylosin tartrate (10 mg/kg)

Parameters	Unit	Amoxicillin sodium	Co-Amoxicillin sodium
A	µg/mL	1.31±0.17	14.82±8.17
±	1/h	2.15±1.71	0.93±0.19
B	µg/mL	4.78±1.26	9.11±14.65
²	1/h	0.89±0.06	0.71±0.33
K_a	1/h	1.33±0.05	1.29±0.22
Lagtime	H	0.29±0.01	0.11±0.06
V/F	(mg/kg)/(µg/mL)	7.73±0.41	3.18±0.50
$T_{1/2±}$	H	0.47±0.38	0.78±0.19
$T_{1/2²}$	H	0.78±0.06	1.31±0.96
$T_{1/2ka}$	H	0.53±0.02	0.55±0.10
K_{21}	1/h	2.23±1.82	0.75±0.33
K_{10}	1/h	0.87±0.85	0.88±0.19
K_{12}	1/h	-0.06±0.08	0.01±0.03
AUC	(µg/mL)*h	2.24±0.11	5.81±2.14
CL_s	mg/kg/h/(µg/mL)	6.71±0.34	2.85±0.94
T_p	H	0.92±0.06	0.95±0.16
C_{max}	µg/mL	0.88±0.01	2.13±0.44

Table 2. Pharmacokinetic parameters of tylosin tartrate after administration alone and in combination with AMO sodium

Parameters	Unit	Single administration(n=5)	Co-administration with amoxicillin sodium(n=5)		
t_{max}	H	0.37±0.09	4.55±0.93	0.33±0.07	4.87±1.87
C_{max}	Ng/mL	27.41±10.13	42.49±20.65	33.56±13.84	27.42±13.37
ke	1/h	0.17±0.07	0.19±0.05		
AUC_{0-8}	(ng/mL)*h	156.27±35.63	94.50±23.95		
$AUC_{0-"}$	(ng/mL)*h	162.09±29.76	124.03±23.56		
$t_{1/2}$	H	4.08±0.67	3.65±0.74		

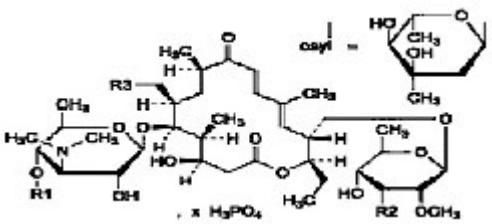

Fig. 1(a). Tylosin

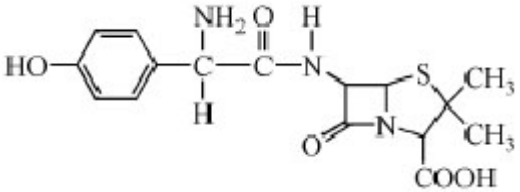

Fig. 1(b). AMO

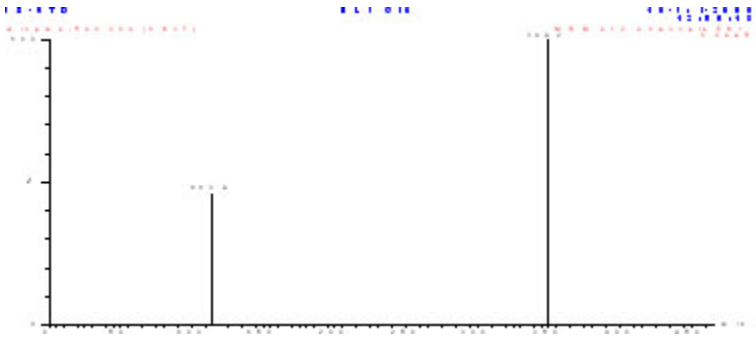

Fig. 2. Positive ESI mass spectrum of [M+H]+ of AMO sodium

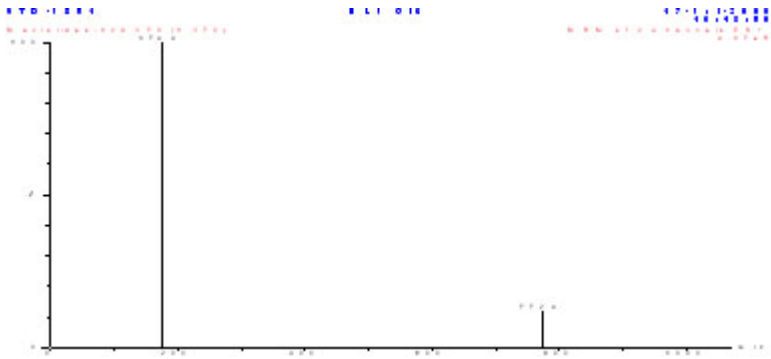

Fig. 3. Positive ESI mass spectrum of [M+H]+ of tylosin tartrate

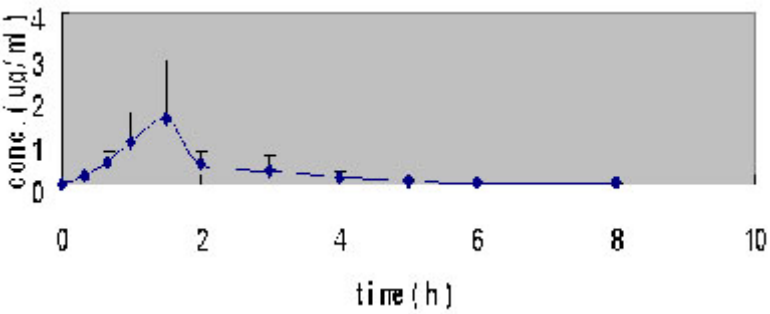

Fig. 4. Concentration-time curve for AMO in pig plasma following oral administration (15 mg/kg). Each data point is shows the mean±S.D. (n = 5)

Therefore, use of an acidic buffer will increase the recovery of tylosin, and simultaneously decreased matrix interference. High recoveries have been reported with 0.1–0.3 % (v/v) metaphosphoric acid in the methanol and acetonitrile. Horie et al. simultaneously determined five macrolides in meat using metaphosphoric acid (0.3 %)–methanol (7:3, v/v) for extraction, and achieved recoveries at the 1.0 µg/g level of 70.8–90.4 %[20]. However, glycosidic bonds could be hydrolyzed in highly acidic environments, and lactones ring could open in alkaline environments. Therefore, the pH of the phosphate buffer needs to be adjusted for the specific extraction. In the present study, 2 mL of

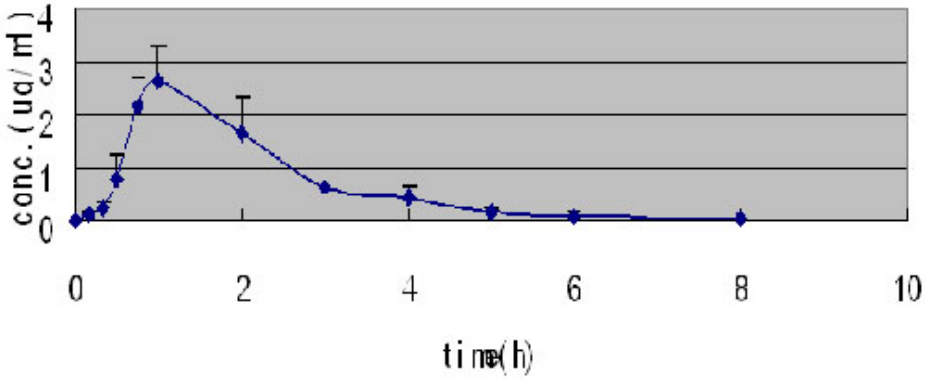

Fig. 5. Concentration-time curve for AMO in pig plasma following oral administration of compound AMO sodium(15 mg/kg)-tylosin tartrate(10 mg/kg). Each data point represents the mean±S.D. (n = 5)

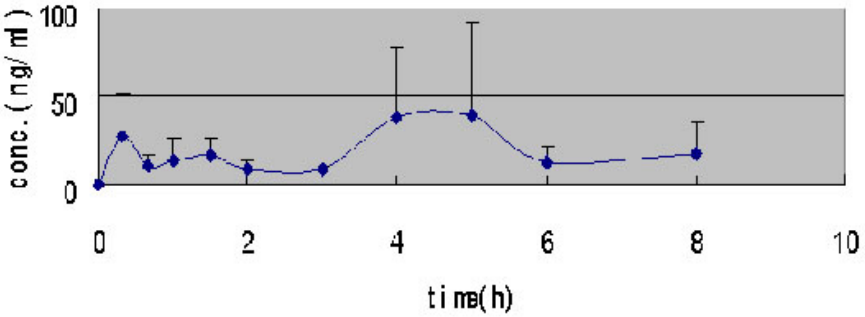

Fig. 6. Concentration-time curve of tylosin in pig plasma following oral administration (10 mg/kg). Each data point represents the mean±S.D. (n = 5)

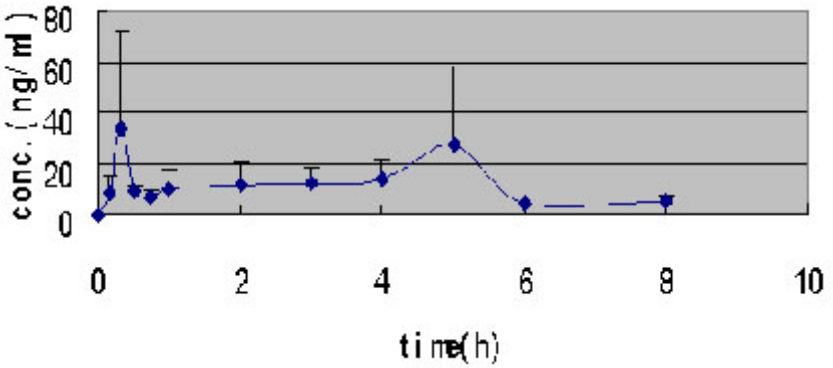

Fig. 7. Concentration-time curve of tylosin in pig plasma following oral administration of AMO sodium (15 mg/kg)-tylosin tartrate (10 mg/kg). Each data point represents the mean±S.D. (n = 5)

ammonium acetate (pH 4; 0.05 M), 0.5 g of NaCl and 5 mL of acetonitrile were used to extract the samples.

Optimization of the UPLC/MS/MS conditions

The mass/charge (m/z) ratio of the protonated ion [M+H]+ of AMO sodium was 366.3 and that of tylosin tartrate was 916.9. Fig. 2 and Fig. 3 showed the positive ESI mass spectra for AMO sodium and tylosin tartrate. The peaks at m/z 349.2 and 772.4 were the strongest and had the least background noise, respectively, and were chosen for MRM for quantitative analysis.

Method validation

Assay specificity

The specificity of the method was evaluated by analyzing blank plasma samples. These results showed that endogenous substances in the plasma did not interfere with AMO sodium and tylosin tartrate.

Linearity and calibration curves

The linear ranges of AMO sodium and tylosin tartrate calibration curves were 0.06–1 µg/mL and 0.5–50 ng/mL, respectively. The regression equations were y = 3153.57x–80.2037 for AMO sodium and y = 257.29x + 118.601 for tylosin tartrate with regression coefficients of R2>0.99.

Precision and accuracy

In the intraday assay, the accuracy range was 68.3–75.1 % for AMO sodium and 74.0–83.3 % for tylosin tartrate. The precision expressed as the CV was 6.7–9.8 % for AMO sodium and 8.6–13.5 % for tylosin tartrate. The interday accuracy range was 67.0–73.0 % for AMO sodium, and 72.0–81.6 % for tylosin tartrate. The highest CV for interday precision was 7.5–9.3 % for AMO sodium and 9.7–12.7 % for tylosin tartrate.

Sensitivity

The LDL was 0.06 µg/mL for AMO sodium and 0.5 ng/mL for tylosin tartrate, and the LOQ was 0.2µg/mL for AMO sodium and 1.7 ng/mL for tylosin tartrate.

Recovery

The mean extraction recoveries of AMO and tylosin were determined at low, medium, and high concentrations in plasma. Regardless of the AMO or tylosin concentration, the recovery range was 67–75.1 % for AMO and 72–83.3 % for tylosin.

Pharmacokinetics study of AMO sodium and tylosin tartrate

After orally administration of AMO sodium, tylosin tartrate, and compound AMO sodium-tylosin tartrate to pigs, the blood concentrations of AMO were measured at different times (Figs. 4, 5, 6 and 7). The pharmacokinetic parameters are shown in Table 1 and Table 2.

AMO had an average t1/2± of 0.47 h, and was absorbed from the gastrointestinal tract with an average ($t1/2^2$) of (0.78±0.06) h and CLs of (6.71±0.34) L/kg/h. The parameters for AMO after oral administration of compound AMO-tylosin were first order absorption rate constent (Ka) (1.29±0.22) h, t1/2ka (0.55±0.10) h, $t1/2^2$ (1.31±0.96) h, AUC (5.81±2.14) (µg/mL) h, CLs (2.85±0.94) mg/kg/h/(µg/mL), and peak time (tp) (0.95±0.16) h. Compared to administration of AMO alone, oral administration of compound AMO-tylosin resulted in similar ka, tp, t1/2ka but different AUC, apparent volume of distribution (V/F), $t1/2^2$, and CLs. The results indicated that tylosin tartrate increases the absorption intensity of AMO, but reduces its rate of elimination in pigs.

Compared to administration of a tylosin alone, oral administration of compound AMO-tylosin resulted in insignificant differences in tmax, ke and significant differences in the AUC. These results indicated that AMO does not influence the absorption or elimination of tylosin .

CONCLUSIONS

A sensitive, specific, accurate and precise UPLC–MS-MS method is developed for evaluation of AMO sodium and tylosin tartrate in pig plasma. The calibration curves are linear between 0.06 and 1 µg/mL for AMO and 0.5 and 50 ng/mL for tylosin with R^2>0.99. The extraction recoveries for both drugs are >67 %.

This method is successfully applied to a pharmacokinetic study of AMO sodium and tylosin tartrate in pigs. The pharmacokinetic parameters show that tylosin reduce the rate of elimination and prolonged the action of AMO in pigs.

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REFERENCES

1. Clay, S.A., Liu, Z., Thaler, R., Kennouche,H. Tylosin sorption to silty clay loam soils, swine manure, and sand. *J Environ Sci and Health B*, 2005; **40**(6): 841-850.
2. Prats, C., Korchi, EL. G., Francesch, R. Disposition kinetics of tylosin administered intravenously and intramuscularly to pigs. *Res Vet Sci*, 2002; **73**: 141-144.
3. Kowalski, C., Rolinski, Z., Zan, R. Pharmacokinetics of tylosin in broiler chickens. *Pol J Vet Sci*, 2002; **5**(3): 127-130.
4. Anne, M.J., Bent, H.S. Multi-component analysis of tetracyclines, sulfonamides and tylosin in swine manure by liquid chromatography–tandem mass spectrometry. *Anal Bioanal Chem*, 2006; **384**: 1164-1174.
5. Wang, J. Confirmatory Determination of Six Penicillins in Honey by Liquid Chromatography/ Electrospray Ionization–Tandem Mass Spectrometry. *J AOAC Int*, 2004; **87**: 45-55.
6. Agerso, H., Friis, C. Penetration of amoxycillin into the respiratory tract tissues and secretions in pigs. *Res Vet Sci*, 1998a; **64**: 245-250.
7. Lugoboni, B., Gazzotti, T., Zironi, E., Barbarossa, A., Pagliuca, G. Development and validation of a liquid chromatography/tandem mass spectrometry method for quantitative determination of amoxicillin in bovine muscle. *J Chromatogr B*, 2011; **879**: 1980-1986.
8. Liu, C.J., Wang, H., Jiang, Y.B., Du, Z.X. Rapid and simultaneous determination of amoxicillin, penicillin G, and their major metabolites in bovine milk by ultra-high-performance liquid chromatography–tandem mass spectrometry. *J Chromatogr B*, 2011; **879**: 533-540.
9. Tim, R., Marc, C., Siegrid, D.B., Patrick, D.B., Siska, C. Rapid method for the quantification of amoxicillin and its major metabolites in pig tissues by liquid chromatography-tandem mass spectrometry with emphasis on stability issues. *J Chromatogr B*, 2008; **861**: 108-116.
10. Pierre, L., Catherine, G., Alain, N., Philippe, A., Germaine, A. Comparative assay of amoxicillin by high-performance liquid chromatography and microbiological methods for pharmacokinetic studies in calves. *Int J Pharm*, 1992; **82**(3): 157-164.
11. Mouayed, Q., Hind, H., Anas, M. Spectrophotometric determination of amoxicillin by reaction with N,N-dimethyl-p-phenylenediamine and potassium hexacyano-ferrate(III). *Anal Chim Acta*, 2005; **554**(1-2): 184-189.
12. Theerasak, R., Praneet, O., Tanasait, N., Choedchai, S., Suthep, W. et al, A simple, sensitive and green bienzymatic UV-spectrophotometric assay of amoxicillin formulations. *Enzyme and Microb Tech*, 2010; **46**(3-4): 292-296.
13. Mohamed, G.G. Spectrophotometric determination of ampicillin, dicluxacillin, flucloxacillin and amoxicillin antibiotic drugs: ion-pair formation with molybdenum and thiocyanate. *J Pharmaceut Biomed*, 2001; **24**(4): 561-567.
14. Garcia, M.S., Sanchez-pedreno, C., Albero, M.I., Rodenas, V. Determination of ampicillin or amoxycillin in pharmaceutical samples by flow injection analysis. *J Pharm Biomed Anal*, 1994; **12**(12): 1585-1589.
15. Wibawa, J.I., Fowkes, D., Shaw, P.N. Measurement of amoxicillin in plasma and gastric samples using high-performance liquid chromatography with fluorimetric detection. *J Chromatogr B*, 2002; **774**(2): 141-148.
16. Kim, M.H., Gebru, E., Chang, Z.Q., Choi, J.Y., Hwang, M.H., Kang, E.H., et al Comparative Pharmacokinetics of Tylosin or Florfenicol after a Single Intramuscular Administration at Two Different Doses of Tylosin-Florfenicol Combination in Pigs. *J Vet Med Sci*, 2008; **70**(1): 99-102.
17. Zhanel, G.G, Mayer, M., Laing, N., Adam, H.J. Mutant prevention concentrations of levofloxacin alone and in combination with azithromycin, ceftazidime, colistin (Polymyxin E), meropenem, piperacillin-tazobactam, and tobramycin against Pseudomonas aeruginosa. *Antimicrob Agents Chemother*, 2006; **50**: 2228-2230.
18. He, J., Tang, S., Li, L., Zhang, C., Li, X., Xia, X., et al. Pharmacokinetics of a novel amoxicillin D colistin suspension after intramuscular administration in pigs. *J vet Pharmacol Therap*, 2011; **34**(1): 42-50.
19. U.S. Department of Health and Human Services Food and Drug Administration, *Guidance for Industry: Bioanalytical Method Validation*, Center for Drug Evaluation and Research, Rockville, MD, 2001; pp 1-22.
20. Horie, M., Saito, K., Ishii, R., Yoshida, T., Haramaki, Y., Nakazawa, H. Simultaneous determination of five macrolide antibiotics in meat by high-performance liquid chromatography. *J Chromatogr A*, 1998; **812**(1-2): 295-302.