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Capillary electrophoretic determination of thiamphenicol in turkeys serum and its pharmacokinetic application

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Abstract

The pharmacokinetics of thiamphenicol were investigated in 12 healthy turkeys of both sexes, following intravenous (i.v.) and intragastric (p.o.) administration of a single dose of 30 mg/kg body weight (bw). Serum drug concentrations were determined by capillary electrophoresis technique on blood samples collected over 24 h following treatment. The method was statistically validated for its linearity, accuracy, precision and selectivity. The linear range was from 0.2 to 500 μg/ml with correlation coefficients greater than 0.999. The limit of detection of drug was 70 ng/ml, while the quantitative limit was 200 ng/ml, using 0.5 ml sample size. Pharmacokinetic variables of the drug were calculated after both administration routes.

Keywords: Thiamphenicol; Pharmacokinetic study; Turkeys; Validation; CE

1. Introduction

Thiamphenicol (TAP) [D-threo-2,2-dichloro-*N*-β-hydroxy-a-(hydroxymethyl)-*p*-(methyl-sulphonyl)-phenethyl acetamide], is an analogue of chloramphenicol, in which the *p*-nitro group on the benzene ring is replaced by a methylsulphonyl group. It is a broad-spectrum bacteriostatic antibiotic, active against both Gram-positive and Gram-negative pathogens of veterinary importance and especially effective against anaerobic organisms. At a sub-cellular level, TAP inhibits the protein synthesis, joining the ribosomes and thus preventing the binding of the amino acid with peptidyl transferase. In contrast to chloramphenicol, TAP has not been associated with fatal aplastic anemia because the nitro group responsible for induced hematological side effect is absent in TAP [1,2].

The TAP would have the advantage of being less toxic and it appears to be a viable substitute for chloramphenicol in veterinary medicine. Likewise, in vivo activity of TAP is a greater against pathogenic bacteria than other structural analogues and it is also active against some bacteria that are resistant to chloramphenicol [3]. Due to its broad antibacterial spectrum, TAP has the potential to become a valuable antibiotic in the treatment and control of a wide range of respiratory and alimentary tract infections of bacterial origin in livestock of mammals, poultry and recently it has been adopted for the treatment of several infectious diseases in fish [4,5].

The pharmacokinetics investigations of TAP have been demonstrated in different animal species, including cattle [6–8], calves [7,9,10], lambs [7], goats [11], pigs [12] and fish [5]. Furthermore, the pharmacokinetic profile of TAP has been studied, comparing intravenous (i.v.), intramuscular and intragastric (p.o.) administration in sheep [13] and rabbits [14]. Some authors have been compared disposition kinetics only by intravenous and intramuscular routes in dogs [15] and pigs [16]. Furman et al. have been described pharmacokinetics in patients during peritoneal dialysis after intramuscular injections in doses of 10–20 mg TAP/kg of body weight (bw) [17].

This work represents an attempt to determine TAP in serum using a fully validated capillary electrophoretic (CE) method with a rapid, simple and robust quantitative procedure. TAP has strong UV absorption and can be determined directly by capillary electrophoretic method. Likewise, many of the more polar drugs, it can be shake out with acetronitrile is sufficient for the quantitative extraction of TAP from serum samples [18]. The objective of the present study was to investigate and compare the pharmacokinetic of TAP following the intravenous (i.v.) and oral (p.o.) administration of a single dose to turkeys. Although TAP is one of the most widely used antibacterial drugs in vet-

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erinary medicine, the determination of its in serum samples of turkeys by capillary electrophoresis and the kinetics in turkeys are reported for the first time.

2. Experimental

2.1. Reagents

All reagent used during the extraction and analysis were of chromatographic grade and were purchased from Merck (Darmstadt, Germany). Sodium tetraborate decahydrate (pH 9.3; 25 mM) and sodium dihydrogenphosphate (pH 5.7; 20 mM) buffer solutions were prepared according to standard method, using deionized water. Thiamphenicol was obtained from Vetos-Farma (Bielawa, Poland). Pharmaceutical Enterprise (Farm-Impex, Gliwice, Poland) supplied ephedrine hydrochloride used as internal standard.

Individual stock solutions (1 mg/ml) were prepared by dissolving the appropriate amount of each of substances in 10 ml of methanol-deionized water solution (5:95, v/v). The working solutions were also prepared in glass volumetric flasks by appropriate dilution just before use. They were stored in the dark under the refrigeration to avoid possible decomposition.

2.2. Instrumental parameters

Experiments were carried out on a Beckman P/ACE 2100 system instrument (Fullerton, USA), comprised with high-voltage built—in power supply, a selectable fixed-wavelength UV detector, the Gold software for system controlling and data handling. The temperature was controlled using a fluorocarbon-based cooling fluid ($25.0 \pm 0.1\,^{\circ}C$), and the voltage was maintained at 25 kV. All experiments were performed using an uncoated silica capillary 57 cm (separation distance 50 cm) × 50 μm i.d. The resultant electropherograms were monitored at 200 nm, with a fixed-wavelength detector. Analytes were introduced into the capillary at anode via a 7 s, 3.45 kPa argon pressure injection, whereas the detector was set on the cathode end of the capillary. Between analyses, the capillary was regenerated by treatment with 0.1 M hydrochloric acid (0.5 min), then with 1 M sodium hydroxide solution (1.5 min) and finally with triple distilled water (1.5 min) at high pressure (137.9 kPa). The best results were obtained using a background electrolyte composed with the sodium tetraborate decahydrate (pH 9.3, 25 mM) and sodium dihydrogenphosphate (pH 5.73, 20 mM) buffer solutions.

2.3. Experimental design

Twelve white turkey poults (including six females and six males) between 6.89 and 10.12 kg of body weight were divided into two experimental group. The groups were placed in separate deep bedding pens. During the experimental period, they were housed in identical conditions (at room temperature), according to the requirements of the species. Six birds (three males and three females) were included in experiments with i.v. administration and six with p.o. administration. All birds were collected from a poultry farm—PFO Vetos-Farma (Bielawa, Poland).

All procedures involving birds complied with the local animal ethics regulations were conducted under the dose supervision and guidance of an experienced veterinarian. The animals before the experiment were sexed, marked with numbers and the individual weight of each was determined just before drug administration to establish administration of a precise dose. All birds were in good health which was determined by physical examination and body weight. Commercial diets and water were provided ad libitum. The rations did not contain any other drug or growth promoter. The administered dose of TAP was based on a 30 mg drug/kg body weight dosage for i.v. (into the right brachial vein) as well as for p.o. for each turkey, directly into the crop, using a thin plastic tube attached to a syringe. After administration the blood samples were collected from the left brachial vein not used for drug administration. Blood samples (3 ml) were taken immediately before dosing and at 5 min after i.v. drug injection and at 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h after both i.v. and p.o. administration. The blood samples were directly deposited into microtainer tubes and allowed to clot at room temperature for 2 h, after which the samples were centrifuged at $2500 \times g$ for 15 min. Subsequently the serum was decanted and immediately frozen in plastic tubes and stored at $-20\,^{\circ}C$ until assayed by CE.

2.4. Sample preparation

In brief, serum samples (0.5 ml) were spiked with internal standard and deproteinized with acetonitrile (2 ml). Next the samples were shaken on a rotary mixer for 10 min to complete the deproteinization process and centrifuged at $8000 \times g$. The organic phase was taken to evaporated to dryness in a water bath at $50\,^{\circ}C$, and finally, the residue was suspended in 0.5 ml of buffer solution (2 mM sodium tetraborate decahydrate) for CE analysis.

2.5. Validation of analytical method

Control blank serum (0.5 ml) was obtained from untreated animals. TAP standards were prepared from pooled blank serum of turkeys by adding know amounts of drug to achieve concentrations of 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 μg/ml and with a fixed concentration of internal standard (40 μg/ml). The concentrations used were based on the range expected during pharmacokinetic investigations. The samples were prepared before experiment, stored in the same conditions as samples taken from animals, and were assessed over the current made calibration curve. Calibration curves were established by plotting peak-height ratio (TAP/I.S.) versus TAP concentration (μg/ml). Thanks to the higher precision, the peak-height ratio (TAP/I.S.) instead peak-area ratio was used in this study.

Intra- and inter-day precision and accuracy were assessed from the results of quality controls at four different concentrations (0.5, 2, 20 and 200 μg/ml). The precision as repeatability of the CE method was determined by the total analysis of six replicate samples under the same operating conditions, on the same day, and by the same analyst. The intermediate precision data were obtained by repeating the intra-assay experiment on

6 different days with newly prepared buffer solution and samples. The precision of a CE method of intra- and inter-day was calculated as the coefficient of variation (R.S.D.%), while the accuracy of the method was determined by calculating the percentage deviation observed in the analysis and expressed as the relative error (R.E.).

The limit of detection (LOD) was defined as the lowest concentration of TAP from which it is possible to deduce the presence of drug with reasonable statistical certainty (signal/noise, 3:1). The limit of quantification (LOQ) was investigated in serum samples from 6 different days, and calculated as the smallest measured content of analyte in a sample that may be quantified with specified degree of accuracy (signal/noise, 10:1). For the determination of LOQ, the percentage deviation and R.S.D. values are to be less than 10%.

The method used was selective for the compound analyzed; endogenous interference was not observed on electropherograms. Specificity of the assay was determined on the basis of different serum samples. The stability of investigated compound, stored in four concentrations (0.5, 2, 20 and 200 μg/ml), after each of three freeze-thaw cycles during 2 months, have been also controlled. To measure absolute recovery, various concentrations of TAP and fixed concentration of I.S. were added to serum and the samples were extracted as described above. The percentage recovery was determined by comparing the peak height of TAP and I.S. extracted from samples with peak height obtained by direct injection of standard solutions.

2.6. Pharmacokinetic and statistical analysis

The pharmacokinetic parameters for TAP were determined using the time course of drug in serum by a non-compartmental analysis with the aid of the program WinNonLin (version 4.01). Various parameters such as area under curve (AUC), peak plasma concentration (C_{max}), time to reach the peak (T_{max}), elimination rate constant (K_{el}), elimination half-life ($T_{1/2}$), the total mean residence time (MRT) and absorption efficiency were determined for each bird by standard methods. $AUC_{(0-t)}$ was determined by linear trapezoidal rule. $AUC_{(0-\infty)}$ was calculated as $AUC_{(0-t)} + C_{last}/K_{el}$, where C_{last} is the last measurable concentration. All calculated values are given as means ± S.D. The bioavailability (F) of orally administred of TAP was calculated by dividing the area under the serum concentration-time curve ($AUC_{0-\infty}$) after oral administration by $AUC_{(0-\infty)}$ after i.v. injection. The volume of distribution was obtained from the following expression: dose/($AUC_{0-\infty} \times K$). The statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by multiple comparisons between data for turkeys. A difference at $P < 0.05$ was taken as being significant.

3. Results and discussion

3.1. Assay validation

The development method was validated for the determination of TAP in serum samples according to accuracy, limit of detection (LD), limit of quantification (LQ), stability and recovery. The linearity of the method was confirmed with precision and inaccuracy below 10% over a concentration range from 0.2 to 500 μg/ml of TAP. The mean (±S.D.) regression equation for six replicated calibration curves constructed using 0.5 ml of turkey serum samples was: $y = 0.1458\ (\pm 0.0008)x + 0.025\ (\pm 0.126)$ ($n = 11$) and correlation coefficients (r^2) was 0.9998. From this experiment, the LD of the electrophoretic method was estimated to be 70 ng/ml ($n = 6$), while the LQ was 200 ng/ml.

The selectivity of the assay was investigated by processing and analyzing blanks prepared from at least six independent lots of control turkeys serum. It is demonstrated by the absence of endogenous substances, in the drug free matrices, that could interfere with the quantification of TAP. Typical electrophoregrams of blank serum sample (A) and serum obtained from animal number one with concentration of TAP 20 μg/ml and ephedrine hydrochloride I.S. 20 μg/ml (B) are shown in Fig. 1. TAP is detected with a migration time (t_m) of 4.7 min and does not interfere with ephedrine hydrochloride I.S. (t_m 2.95 min) and with peaks of endogenous constituents from serum and reagents.

The precision and accuracy of the method was evaluated as the intra- and inter-day R.S.D. values of the measured peak height standards at quality control (QC) concentrations (0.5, 2, 20 and 200 μg/ml). The R.S.D. values from TAP for intra- and inter-day precision were found to be 0.8–5.8% and 1.5–6.1%, respectively, whereas the R.E. values were showed from −2.99 to 1.01%, and from −4.71 to 1.69%, respectively (Table 1). The precision and accuracy investigations showed acceptable values for both intra- and inter-day studies for TAP in turkey serum,

Table 1
Intra- and inter-day precision and accuracy and absolute recovery of measurement of TAP in turkeys serum

Nominal concentration (μg/ml)	Concentration found (μg/ml ± S.D.)	Precision R.S.D. (%)	Accuracy (% RE)	Absolute recovery (%)
Intra-day				
0.5	0.50 ± 0.03	5.8	1.01	79.5
2	2.08 ± 0.03	4.3	−2.99	79.9
20	20.27 ± 0.72	3.6	−1.36	81.1
200	206.17 ± 1.98	0.8	−2.91	81.6
Inter-day				
0.5	0.49 ± 0.03	6.1	1.69	78.8
2	2.09 ± 0.09	4.7	−4.71	79.5
20	20.37 ± 0.82	4.0	−1.86	81.6
200	206.81 ± 3.13	1.5	−3.41	85.6

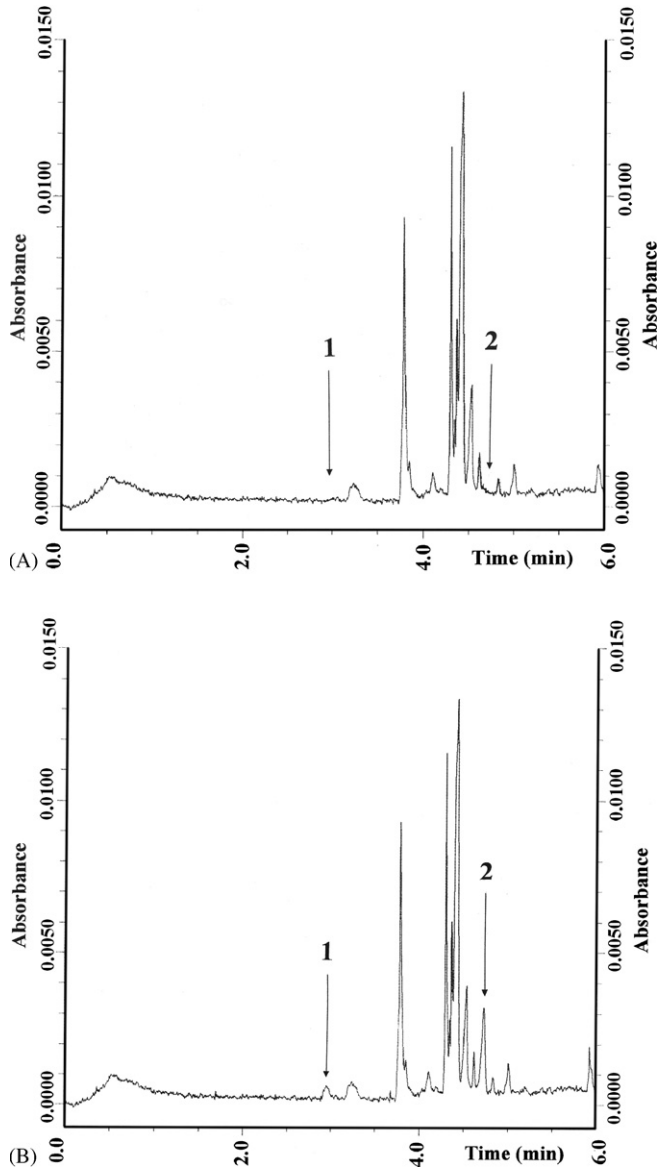

Fig. 1. (A) Electropherograms of drug-free turkeys serum. (B) Electropherogram of turkeys serum containing (1) ephedrine hydrochloride (I.S.) at 4 μg/ml and (2) thiamphenicol at 20 μg/ml. Conditions: UV detection at 200 nm, unmodified silica capillary (57 cm × 50 μm i.d.), temperature 25 °C, running buffer composed with 25 mM $Na_2B_4O_7$ and 20 mM NaH_2PO_4.

over a wide concentration range. TAP was stable when stored in the refrigerator and freezer. The freeze-thaw stability and long-term storage stability of TAP was determined by measuring the assay precision and accuracy for serum samples which underwent three freeze-thaw cycles during 2 months. The effect of freezing and thawing cycles was studied using six parallel samples at QC concentrations of drug. The stability data were used to support repeat analyses. The frozen plasma samples containing separated drug were thawed at room temperature for 3 h, refrozen for minimum of 2 weeks, thawed for 3 h, refrozen for next 6 weeks, thawed and then analyzed. The results showed that TAP was stable in turkey serum through three freeze-thaw cycles. The precision and accuracy for TAP form these extracted and stored serum samples (after 2 months) were 0.7–4.6% and

Table 2
The freeze-thaw stabilities of TAP in turkeys serum after stored at −20 °C for 2 weeks and 2 months

Nominal concentration (μg/ml)	Concentration found (μg/ml ± S.D.)	S.D.	Precision R.S.D. (%)	Recovery (%)
After 2 weeks				
0.5	0.49	0.02	4.1	98.0
2	1.96	0.05	2.3	98.0
20	20.06	0.29	1.4	100.3
200	202.09	1.54	0.8	101.0
After 2 months				
0.5	0.49	0.02	4.6	98.0
2	2.02	0.05	2.3	101.0
20	20.42	0.48	2.4	102.1
200	203.33	1.38	0.7	101.7

98–102.1% (Table 2). The results demonstrated that serum samples could be thawed and refrozen without any decomposition. The mean absolute recovery values founded from turkeys serum in the conditions of the assay was 81.5 ± 2.3% for TAP and 78.8 ± 1.6% for I.S.

3.2. Application for pharmacokinetic study

The validated CE method was successfully applied to the pharmacokinetic studies and bioavailability of TAP in turkeys. Mean concentrations of TAP in serum after single i.v. and p.o. administration of drug at 30 mg/kg b.w. are presented graphically (Fig. 2). The basic pharmacokinetic parameters are listed in Table 3.

All animals in the study were normal following treatment and no adverse effects were observed in any of the birds after drug administration. The results of this study show that TAP is rapidly absorbed when administered by oral route into serum where it reached its peak at 1.42 ± 0.2 h after intragastric administration. Likewise, the time at which C_{max} was achieved was less than that reported for calves (4.5 h), lambs (3.5 h) [7] and pigs (3.42 h) [12], only in rabbits was observed shorter t_{max} (1.17 h) [14]. In the scientific literature, specific reports on intragastric adminis-

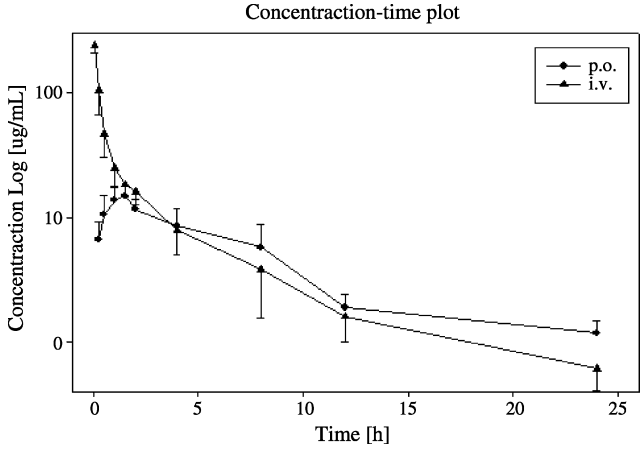

Fig. 2. Semilogarithmic plot of mean TAP serum concentrations vs. time after p.o. and i.v. administration of 30 mg/kg body weight in turkeys.

Table 3
Mean pharmacokinetic parameters of thiamphenicol after i.v. and p.o. administration at the therapeutic dose of 30 mg/kg b.w. for turkeys

Parameter	Thiamphenicol i.v.	Thiamphenicol p.o.
AUC_{0-t} (μg g/ml)	172.7 ± 38.4	104.8 ± 30.6
$AUC_{0-\infty}$ (μg g/ml)	178.9 ± 40.7	116.9 ± 30.6
T_{max} (h)	–	1.42 ± 0.20
C_{max} (μg/ml)	–	15.23 ± 3.24
C_{12} (μg/ml)	1.60 ± 0.60	1.88 ± 0.52
C_{24} (μg/ml)	0.50 ± 0.20	1.18 ± 0.30
K_{el} (1/h)	0.19 ± 0.08	0.10 ± 0.03
$T_{0.5}$ (h)	4.19 ± 1.58	7.40 ± 2.09
V_z (L)	8.51 ± 3.41	26.14 ± 13.23
MRT (h)	2.88 ± 1.18	6.54 ± 0.51
F (%)	–	65.36

(Values are mean ± S.D.); $AUC_{0-\infty}$, area under the curve extrapolated to infinity; AUC_{0-t}, area under the curve up to the last time (t) in which thiamphenicol was measured; T_{max}, the time to reach peak concentration; C_{max}, the maximum plasma concentration; C_{12}, serum drug concentration after 12 h; C_{24}, serum drug concentration after 24 h; K_{el}, the apparent elimination rate constant; $T_{0.5}$, the apparent elimination half-life; V_z, volume of distribution calculated by the area method; MRT, mean residence time; F, absolute bioavailability.

tration and disposition of TAP to turkeys were not found, thus comparisons could not be made for these species.

The treatment with TAP at 30 mg/kg b.w. in this study gave maximum serum concentrations (15.23 ± 3.24 μg/ml) different to those reported in pigs (2.1) [12], calves (3.5), lambs (5,2) [7] or rabbits (69,8) [14]. Detectable quantities of TAP were found at 24 h after its i.v. (0.5 ± 0.2 μg/ml) and p.o. (1.18 ± 0.3 μg/ml) administration, and were above the LOQ of the analytical method. The elimination half-life in turkeys (7.4 h) determined in this study was longer than the value of 4.8 h in calves, 3.6 h in pre-ruminant lambs, reported by Mengozzi et al. [7], and also longer than the 3.79 h in pigs, obtained by Haritova et al. [12]. Likewise, the relatively long elimination half-life and MRT (6.5) values of TAP after its p.o. administration, determinated in present study are suggestive that the drug is eliminated relative slowly in turkeys, in contrast to other animal species. The p.o. elimination half-life was higher than that observed after i.v. administration (4.19 h). The absorption process probably continued for several hours, which would affect the drug elimination process. However, it should be noted that plasma half-life does not allow estimation of the tissue half-life as the drug could accumulate in the tissue. Moreover, in the present paper, TAP showed a high volume of distribution (particularly after p.o. administration), which suggests that penetration of biological membranes is very well. Thus TAP might easily reach well perfused tissues, possibly in levels high to achieve the value above of the MIC (minimum inhibitory concentration) (for TAP 0.5–2 μg/ml) of most pathogenic bacteria involved in the commonest infections in birds.

The bioavailability value for the p.o. route (65.36%) in turkeys (at a dosage of 30 mg/kg) was rather higher than those previously reported for in pre-ruminant lambs calves (60%) [7] and rabbits (64.2%) [14], indicated that the drug might have been partially absorbed from the gastrointestinal tract. Moreover, following p.o. administration of florfenicol (a structural analogue of TAP) to other bird species, bioavailability values are similar to values obtained in this study (55.3% in broiler chicken [19], 71% in infected chicken [20] and 73% in ducks [21]). In contrast, a previous assays concerning the pharmacokinetic disposition of TAP after oral treatment showed differences in bioavailability, from 30% in sheep [13] to 64.2% in rabbits [14]. Likewise, differences between kinetic parameters of TAP for male and female turkeys were evaluated, but there was no significant difference between sexes in any of the estimated pharmacokinetic parameters (data not showed).

The results obtained in this assay, suggest that TAP administered p.o. and i.v. at 30 mg/kg could be sufficient to achieve the MIC for most susceptible microorganisms. Therefore, TAP should be given twice a day at dosage of 30 mg/kg b.w. to maintain therapeutic concentrations, and that the interval between each administered dose could be extended up to 12 h.

4. Conclusion

The sensitive and reliable CE method for the measurement of thiamphenicol in turkeys serum has been developed and validated. In this study, validation experiments have shown that the assay has good precision and accuracy over a wide concentration range, and no interference caused by endogenous compounds was observed. The single step liquid–liquid extraction procedure was used to isolate the drug from serum samples. The cost and time of analysis decreased. In contrast, classical methods for the separation of TAP from biological matrixes require large volumes of extracting solvents, multiple extractions and evaporation steps. Moreover, the method is applicable for monitoring serum levels during clinical and pharmacokinetic trials with TAP to evaluate its most efficacious dosage regimen. The sensitivity of the assay method allows determination of drug concentrations in plasma samples collected 24 h after p.o. and i.v. route treatments. In conclusion, both p.o. and i.v. administration of TAP at dose 30 mg/kg in turkeys, may be effective for the treatment of susceptible infections and useful for its large scale therapeutic application.

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