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Experimental determination of the pharmacokinetic properties of trimethoprim and sulfamethoxazole combination in the blood serum of broiler chickens

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Abstract: A rapid, simple and highly efficient analytical method for the targeted determination of trimethoprim and sulfamethoxazole in serum samples has been developed and used to measure the pharmacokinetic curve of these medicinal substances after administration to chicken broilers. The pharmacokinetics properties of trimethoprim and sulfamethoxazole were investigated in clinically healthy broiler chickens after the single oral administration of the commercial preparation Methoxasol (Eurovet Animal Health, B.V., The Netherlands) at a dose of 0.275 ml/kg b.w. After a single dose drug administration, the chickens were sacrificed by decapitation under general anaesthesia by Isoflurin 1 000 mg/g (Vetpharma AH, Spain) and the blood was collected at precisely defined intervals: 15, 30, 45, 60, 90, 120, 180, 360 and 720 min after the administration. The serum concentrations of amoxicillin were determined using Q Exactive tandem mass spectrometer (Thermo Fisher Scientific, USA) in conjunction with liquid chromatography. The detected pharmacokinetic parameters of trimethoprim after the oral administration were C_{max} = 2.1 ± 1.0 µg/ml; T_{max} = 1.5 h; $t_{½}$ = 0.88 h; k_{el} = 0.009 3 ± 0.001 1 1/h; AUC_t = 2.901 ± 1.4 µg.h/ml; AUC_∞ = 2.907 ± 1.5 µg.h/ml; V_d = 2.632 l/kg; Cl = 2.7 l/h. The pharmacokinetic parameters of sulfamethoxazole after the oral administration were C_{max} = 47.1 ± 15.3 µg/ml; T_{max} = 1 h; $t_{½}$ = 1.92 h; k_{el} = 0.004 6 ± 0.000 3 1/h; AUC_t = 89.676 ± 26.9 µg.h/ml; AUC_∞ = 94.612 ± 28.4 µg.h/ml; V_d = 0.584 l/kg; Cl = 0.21 l/h. To the best of our knowledge, this is the first pharmacokinetic study of the combination of sulfamethoxazole and trimethoprim in broiler chickens.

Keywords: antimicrobial treatment; drug concentrations; mass spectrometry; poultry; time dependence

Antimicrobials have an irreplaceable role in the treatment of bacterial infections in human and veterinary medicine, and there are currently no alternative drugs with the same efficiency against bacterial infections as antimicrobials. In recent years, there has been a significant decrease in the discovery and introduction of new antimicrobial molecules into clinical practice (Livermore 2012). For this reason, the key problem of modern medicine is to maintain the efficacy of the currently used

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antimicrobials, which is seriously threatened by the increasing prevalence of the resistance of microorganisms. Optimally defined antimicrobial dosages based on the knowledge of the pharmacokinetic and pharmacodynamic properties of substances that define the necessary exposure of the antimicrobials to the infection site to maximise their effect on bacteria are essential contributors to reducing the emergence and development of resistance (Jacobs 2001).

Scientific and clinical studies and data to determine the pharmacokinetic and pharmacodynamic properties of the antimicrobials needed to predict effective dosing schedules for all types of antimicrobials against all common infections are not often published. Every species has some pharmacokinetic peculiarity that determines its drug disposition patterns. Poultry are no exception. Knowledge of the origin of these characteristics is fundamental for the reasonable design of dosing schedules (Landoni and Albarellos 2015). In poultry, and in particular in broiler chickens, the pharmacokinetic and pharmacodynamic properties of antimicrobials were defined only for a limited spectrum of antimicrobials and the available data for combinations of trimethoprim and sulfamethoxazole were generated decades ago, measured by other, less accurate methods than required today by EU directives (Loscher et al. 1990; Dagorn et al. 1991; Baert et al. 2003). At that time, the breeds of domestic chickens had a different genetic basis in breeding, whereas broilers today, with Ross and Cobb as the breeds of choice, differently utilise food and have different daily increments, which is related to the amount of food and fluid intake and, thus, the possibility to make medicated feed or drinking water (Mebratie et al. 2019). Due to the lack of relevant data, marketing authorisations for newly authorised veterinary medicinal products for poultry are not possible. Nowadays, chickens are treated with medicines that do not have the desired efficacy, thus allowing the emergence and spread of resistant bacterial populations causing various diseases, or they are given antimicrobials with indication restrictions, which should only be administered in extreme cases when other treatment options fail and their effectiveness must be maintained primarily for human medicine (Sarkozy 2001; Landoni and Albarellos 2015). These antibiotics include fluoroquinolones, especially enrofloxacin, that are also an effective treatment choice used frequently in the field. However, fluoroquinolones are, nowadays, considered as last resort treatments, and are included in category B "Restrict" of the new, four-group categorisation, which corresponds to Category 2 in the first Antimicrobial Advise Ad Hoc Expert Group (AMEG) report (European Medicine Agency 2019). Sulfonamides are now classed in Category D "Prudence" for first line use, if suitable.

Sulfonamides, in combination with diaminopyrimidines (usually with trimethoprim), also referred to as potentiated sulfonamides, are used for the treatment of chicken diseases caused by a wide range of pathogenic bacteria. They are particularly active against Gram-negative bacteria, e.g., *Escherichia coli* and *Pasteurella multocida*. However, sulfonamides have a narrow margin of safety, so their use is limited. Bone marrow suppression, thrombocytopenia and lymphoid and immune depression in poultry are some of the characteristic toxic effects observed in birds. Post-mortem changes include haemorrhagic infarcts in the liver and spleen, pale bone marrow, and petechial or ecchymotic haemorrhages in muscles (Frank 1947; Daft et al. 1989; Landoni and Albarellos 2015). Therefore, the safety of a potentiated sulfonamide treatment depends on the precise dosing.

In this study, we determined the pharmacokinetic properties of the trimethoprim and sulfamethoxazole combination in broiler chickens based on the measurement of time-dependent changes of their concentrations in the blood serum after a single oral administration of the therapeutic dose recommended by the manufacturer of the commercial veterinary drug containing trimethoprim and sulfamethoxazole.

MATERIAL AND METHODS

Animals

Forty-five healthy chickens of both sexes showing no signs of clinical disease – broilers of the Ross and Cobb lines, which are among the broiler lines most commonly used in commercial breeding, were included in the study. The chickens were fed with antibiotic-free feed for broilers and received drinking water *ad libitum* during the entire experiment. The health condition, individual weight gains and feed consumption in the groups were monitored daily.

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Animal experiment procedure

The animal experiment was carried out in the accredited animal facilities of the Veterinary Research Institute in Brno according to an animal experiment project approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permission MZe No. 2069). The animal care protocol for this experiment followed the Czech guidelines for animal experimentation.

Experiment

Forty-five chickens at 16 days old were divided into 7 groups of 5 chickens each. The commercial preparation Methoxasol (Eurovet Animal Health, B.V., Bladel, The Netherlands), containing trimethoprim and sulfamethoxazole in a ratio of 20 : 100 mg/ml, was individually administered by an oral route to all the chickens in a dose of 0.275 ml/kg b.w. (body weight).

After a single drug administration, the chickens were sacrificed by decapitation under general anaesthesia by Isoflurin 1 000 mg/g (Vetpharma AH, Barcelona, Spain) in a dose recommended by the manufacturer (8.9–10.2 mg/g b.w.) using a calibrated vaporiser in an anaesthetic unit in a closed system. The blood was collected at precisely defined intervals: 15, 30, 45, 60, 90, 120, 180, 360 and 720 min after the administration.

Chemicals and solvents

The analytical standards of trimethoprim (impurity B, European Pharmacopoeia reference standard), trimethoprim-d_9 (Vetranal™, analytical standard), sulfamethoxazole (impurity F, European Pharmacopoeia Reference Standard) and sulfamethoxazole-$^{13}C_6$ (Vetranal™, analytical standard) were purchased from Sigma-Aldrich. The acetonitrile hypergrade for LC-MS LiChrosolv® was purchased from Merck and the formic acid was purchased from Sigma-Aldrich. Deionised water was prepared with a water treatment system from Goldman water s.r.o.

Sample preparation

One ml of the collected serum was spiked with internal standards (trimethoprim-d_9, sulfamethoxazole-$^{13}C_6$) to a concentration of 100 ng/ml. An amount of 200 µl of acetonitrile was added to 200 µl of the spiked serum and vortexed for 3 minutes.

After centrifugation (20 min, 14 100 *g*), 360 µl of the supernatant was transferred into a fresh tube and evaporated to dryness under a stream of nitrogen at a temperature of 30 °C. The pellet was dissolved in 150 µl of 30% acetonitrile in water, transferred to a vial and analysed by liquid chromatography-mass spectrometry/(high-resolution) mass spectrometry [LC-MS/(HR)MS].

Analytical methods

The chromatographic analysis was performed by an Accela high performance chromatographic system from Thermo Fisher Scientific (Waltham, MA, USA). The chromatographic separation was carried out on a column Luna Omega 1.6 µm Polar C18 100 × 2.1 mm from Phenomenex (Torrance, CA, USA). The composition of the mobile phases and gradient properties are summarised in Table 1.

The mass spectrometric detection was performed by a high-resolution Q Exactive Orbitrap mass spectrometer equipped with electrospray ion source from Thermo Fisher Scientific (Waltham, MA, USA). The mass spectrometer was operated in the positive ionisation mode. For confirmation, the m/z (mass/charge) of the precursor (sulfamethoxazole = 254; trimethoprim = 291) and three product ions (sulfamethoxazole = 108, 156, 171; trimethoprim = 123, 230, 261) was monitored. For quantification, the m/z of the most abundant product ions (sulfamethoxazole = 156; sulfamethoxazole-$^{13}C_6$ = 162; trimethoprim = 261; trimethoprim-d_9 = 264) was monitored (Table 1).

Table 1. HPLC gradient conditions: mobile phase A = 5% ACN/95% H_2O/0.1% formic acid; mobile phase B = 95% ACN/5% H_2O/0.1% formic acid

Time (min)	Flow rate (µl/min)	% solvent A	% solvent B
0	250	80	20
2	250	80	20
7	250	50	50
12	250	30	70
13	250	80	20
16	250	80	20

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Method validation

The analytical method was validated for the full range of the parameters according to the recommendations of the guideline documents European Medicine Agency (1998) and European Medicine Agency (2009). To evaluate the performance of the analytical method, the following validation parameters were calculated: specificity (identification), precision, linearity, range of linearity, limit of detection and limit of quantification.

The identification was performed by comparing the chromatography of the retention times (RT ± 10%) and mass accuracy (MA < 5 ppm) of the analytical standards sulfamethoxazole and trimethoprim (European Pharmacopoeia Reference Standard) against active substances in the applied drug alone dissolved in drinking water and also both substances in the analysed serum samples. The repeatability was determined by measuring the fortified serum samples at three concentrations/each repeated six times. The intermediate precision was determined by measuring the repeatability on three different days. The linearity was validated based on a matrix calibration curve determined for six concentration levels (including the blank) with six repeated measurements for each concentration. The calibration range was 0–50 µg/ml for sulfamethoxazole and 0–2 µg/ml for trimethoprim. The detection limit (LOD) and the quantification limit (LOQ) were calculated based on the standard deviation of the response and the slope from the matrix calibration curve.

Pharmacokinetic and data analysis

The time course of the change in the concentration levels of trimethoprim and sulfamethoxazole in the broiler organism after a single administration was described using a one-compartment open pharmacological model. Graphical methods for plotting the concentration (mean ± SD) versus time were used to estimate the pharmacokinetic curves of trimethoprim and sulfamethoxazole. The main pharmacokinetic parameters C_{max} – peak concentration, T_{max} – peak time, AUC_t – area under the curve to the last sampling time and AUC_∞ – area under the curve of the extrapolated area to time infinity were derived and calculated directly from the pharmacokinetic curves, in accordance with the EU directive (European Medicine Agency 1999). The parameters of the areas under the curve AUC_t and AUC_∞ were calculated using trapezoidal rules and with the addition of the extrapolated area from the last quantifiable trimethoprim and sulfamethoxazole concentration to time infinity for AUC_∞. The first-order kinetics were used to calculate the parameters $t_{½}$ – the half-life time and k_{el} – elimination rate constant. The parameter k_{el} was estimated by linearising the terminal phase of the pharmacokinetic curve and the parameter $t_{½}$ was derived from the corresponding relation $t_{½} = 0.693/k_{el}$ (Ritschel and Kearns 1984). Based on the pharmacological model approach, the parameters V_d – volume of distribution and Cl – clearance were further estimated. All the measured data were processed and evaluated using statistical software Statistica v13.0 (TIBCO, Palo Alto, USA).

RESULTS AND DISCUSSION

Method development for the analysis

A rapid, simple and highly efficient analytical method for the targeted determination of trimethoprim and sulfamethoxazole in the serum samples has been developed and used to measure the pharmacokinetic curve of these medicinal substances after administration to chicken broilers. The time required to prepare the sample was reduced by performing serum deprotection only using the addition of acetonitrile and centrifugation, followed by evaporation and reconstitution of the sample to the minimum volume available for the analysis. The chromatographic separation was performed on a high efficiency Luna Omega Polar analytical column with a fine grain size of 1.6 µm and a polar stationary phase C18, which is able to ensure unique selectivity within a wide elution window and increased retention for both polar and non-polar analytes. The Luna Omega Polar column, with these properties, was the optimal choice for the balanced retention of the trimethoprim and sulfamethoxazole analytes with well-developed, symmetric chromatographic peaks and a high signal-to-noise ratio, S/N = 100 : 1 and 150 : 1 for trimethoprim and sulfamethoxazole, respectively (see Figure 1).

The method was optimised and worked on the basis of the addition of isotopically labelled internal standards (trimethoprim-d_9, sulfamethoxazole-$^{13}C_6$)

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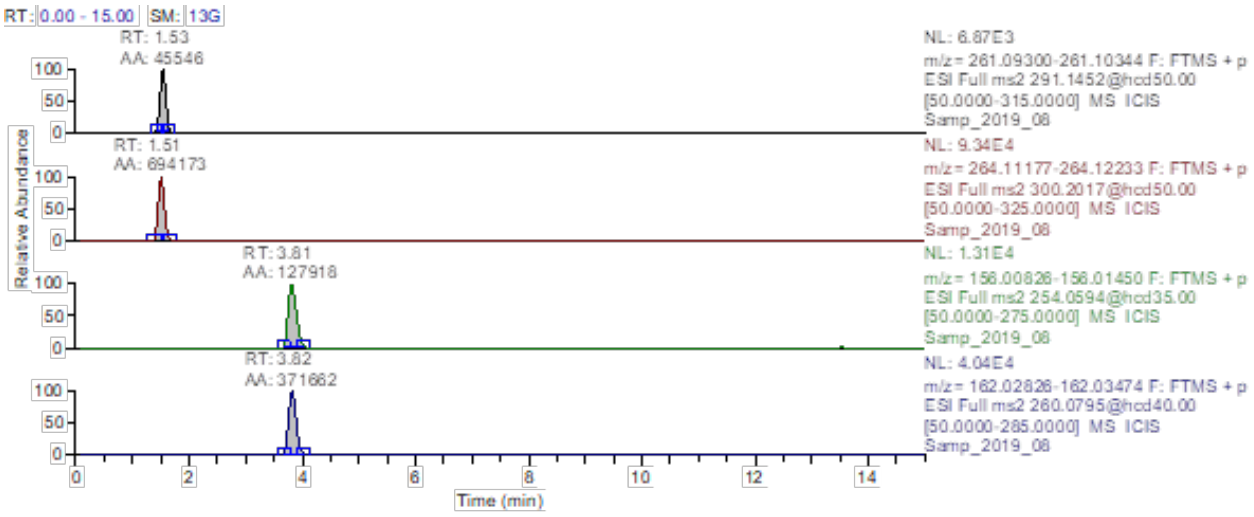

Figure 1. The chromatograms of the serum samples

so as to minimise the effects of the matrix and ion source suppression adversely affecting the overall measurement results. The parameters of the hybrid mass spectrometer were optimised using reference standards, and the HR-MS spectrometer was calibrated internally and externally using calibration standards for each mass accuracy with a positive ion calibration solution (Pierce Thermo Fisher Scientific) before each measurement.

Method validation

The performance of the analytical method was verified by validation tests to the extent prescribed by the EU guidelines (VICH GL 2 and VICH GL 49). The analytical method used with a high-resolution Orbitrap mass spectrometer always brings the benefit of high specificity to the targeted analysis. The identification and confirmation of both active substances were performed on the basis of the RT and MA parameters for one precursor ion and two product ions, for each analyte. The detected and calculated RT and MA parameters with the resolving power of RP = 70 000 full width at half maximum (FWHM) used for each reference standard are shown in Table 2. An example of the exact identification of trimethoprim in an incurred serum sample after the oral drug administration is shown in Figure 2.

The intercept (A) and slope (B) linearity parameters for both substances were estimated using weighted linear regression according to the mathematical model Y = A + B × X calculated from the matrix calibration curve. The estimated intercept was A = 0.028 6 ± 0.011 4 and the slope was B = 0.000 64 ± 0.000 023 for trimethoprim, the intercept was A = –0.010 6 ± 0.082 6 and the slope B = 0.012 28 ± 0.000 164 for sulfamethoxazole. The correlation coefficients were r = 0.991 8 and 0.998 8

Table 2. Identification of trimethoprim and sulfamethoxazole by mass accuracy for MS data, RP = 70 000 (FWHM)

Analyte	Elemental composition	m/z of precursor ion (Da)	MA (ppm)	Elemental composition of product ion	m/z of product ion (Da)	MA (ppm)	RT (min)
Trimethoprim	$[C_{14}H_{19}N_4O_3]^+$	291.145 17	–1.03	$[C_{12}H_{13}N_4O_3]^+$	261.098 22	–1.91	1.53
				$[C_5H_7N_4]^+$	123.066 51	–0.08	1.53
Trimethoprim-d_9	$[C_{14}H_{10}D_9N_4O_3]^+$	300.201 66	–2.03	$[C_{12}H_{10}D_3N_4O_3]^+$	264.117 05	–2.95	1.51
Sulfamethoxazole	$[C_{10}H_{12}N_3O_3S]^+$	254.059 39	–0.08	$[C_6H_6NO_2S]^+$	156.011 38	0.19	3.81
				$[C_6H_6NO]^+$	108.044 62	2.13	3.81
Sulfamethoxazole-$^{13}C_6$	$[C_4{}^{13}C_6H_{12}N_3O_3S]^+$	260.079 52	0.04	$[{}^{13}C_6H_6NO_2S]^+$	162.031 50	–0.06	3.82

FWHM = full width at half maximum; MA = mass accuracy; MS = mass spectrometry; RP = resolving power; RT = retention time

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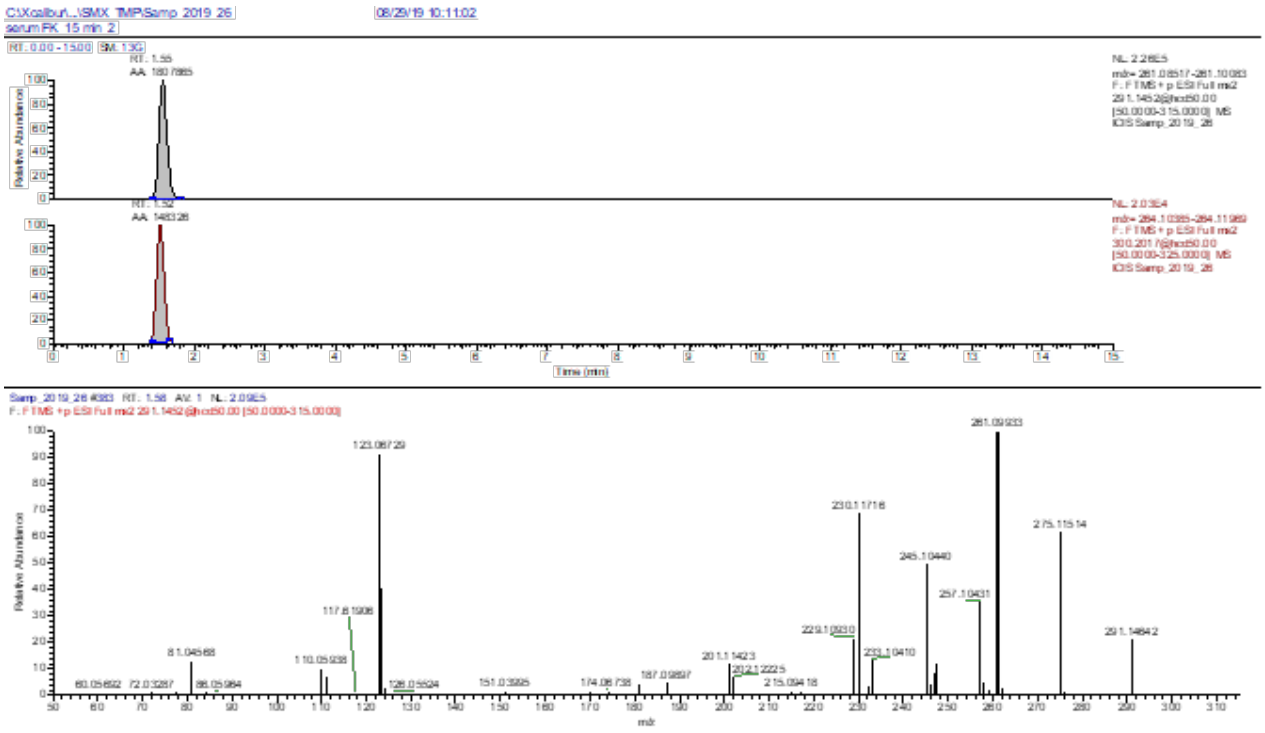

Figure 2. Chromatogram and mass spectrum of trimethoprim in a serum sample at time T_{max} after oral administration to the experimental broiler chickens

for trimethoprim and sulfamethoxazole, respectively. The sensitivity of the method was estimated based on the calibration curves which was LOD = 0.058 µg/ml, LOQ = 0.178 µg/ml for trimethoprim and LOD = 0.022 µg/ml, LOQ = 0.067 µg/ml for sulfamethoxazole. The repeatability was expressed by the relative standard deviation (RSD; %) and was < 6.2% (n = 18) for both analytes. The intermediate precision was < 7.8% of the measuring repeatability on 3 different days (n = 3 × 18). The validation parameters of the analytical method estimated for the sensitivity (LOD and LOQ) and precision (RDS) corresponded to the published values for standard triple-Q mass spectrometers. For example: LOQ = 0.5 µg/ml for sulfamethoxazole and LOQ = 0.05 µg/ml for trimethoprim in plasma samples (Bedor et al. 2008), LOD = 0.117 µg/g for sulfamethoxazole and LOD = 0.116 µg/g for trimethoprim in bovine muscle samples (Freitas et al. 2014) and LOD = 0.02 µg/g and LOQ = 0.04 µg/g for trimethoprim in feed samples (Yang et al. 2016).

Pharmacokinetics

All the broiler chickens that participated in our pharmacokinetic study were examined and found to be clinically healthy. The serum samples obtained in the study were analysed by ultra-high performance liquid chromatography (UHPLC) in combination with a high-resolution mass spectrometer. The used sulfamethoxazole and trimethoprim drug substances were detected in the serum samples by a targeted analysis based on an accurate identification (RT and MA criteria used) and quantified using a matrix calibration curve with the addition of an isotopically labelled internal standard. Based on the results thus obtained, the pharmacokinetic concentration-time profiles were compiled for both substances after a single oral administration and dosed in a 5 : 1 ratio of sulfamethoxazole to trimethoprim (Figures 3 and 4). The main pharmacokinetic parameters were calculated based on the time vs concentration data for the individual animals and subsequently statistically processed (Table 3).

As both active substances are well soluble in water, the primary choice for their medication in broilers was the administration through drinking water. Both trimethoprim and sulfamethoxazole were rapidly absorbed after the oral administration, T_{max} was 1 h for sulfamethoxazole and 1.5 h for trimethoprim. The maximum serum concentrations C_{max} after a single dose recommended by the manufacturer

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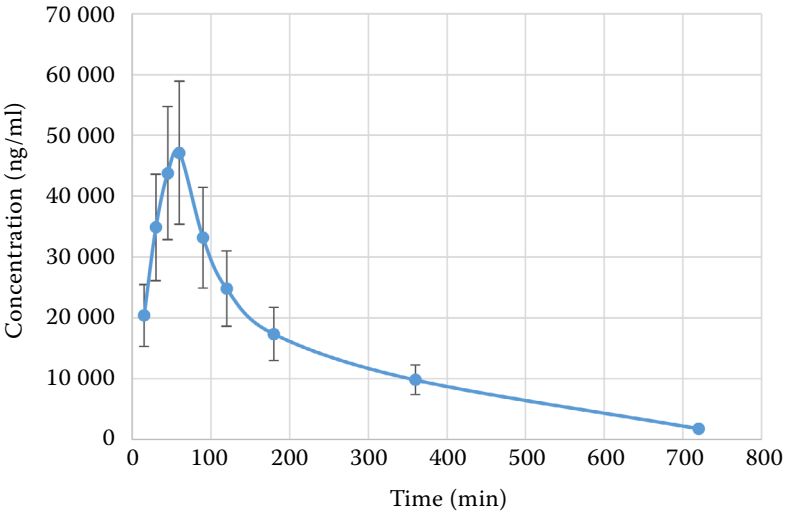

Figure 3. Serum concentrations in the broiler chickens – time profile of sulfamethoxazole after a single oral administration of 0.275 ml/kg b.w. Methoxasol = the points on the curve represent the mean ± SD concentrations of sulfamethoxazole in the individual chickens

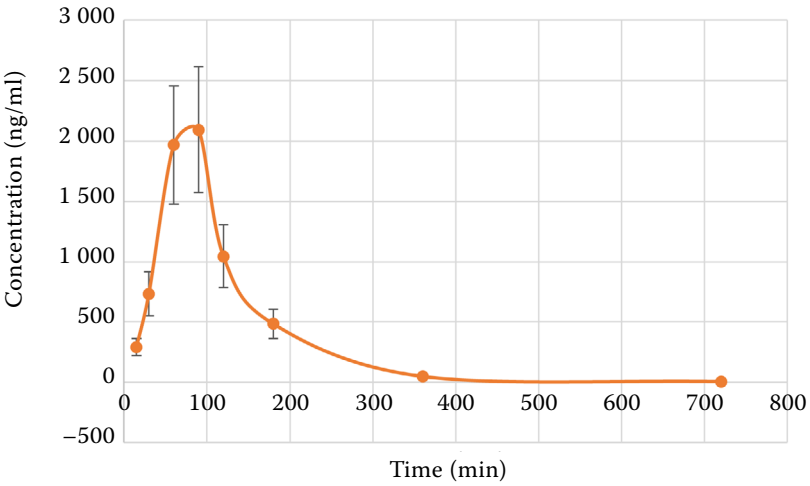

Figure 4. Serum concentrations in the broiler chickens – time profile of trimethoprim after a single oral administration of 0.275 ml/kg b.w. Methoxasol = the points on the curve represent the mean ± SD concentrations of trimethoprim in the individual chickens

Table 3. Pharmacokinetic parameters of sulfamethoxazole and trimethoprim after oral administration to broiler chickens at doses of 27.5 mg sulfamethoxazole and 5.5 mg trimethoprim per kg b.w. (5 : 1 ratio)

Pharmacokinetic parameters	Sulfamethoxazole	Trimethoprim
C_{max} (µg/ml)	47.1 ± 15.3	2.1 ± 1.0
T_{max} (h)	1.0	1.5
k_{el} (1/h)	0.004 6 ± 0.000 3	0.009 3 ± 0.001 1
$t_{½}$ (h)	1.92	0.88
AUC_t (µg.h/ml)	89.676 ± 26.9	2.901 ± 1.4
AUC_∞ (µg.h/ml)	94.612 ± 28.4	2.907 ± 1.5
V_d (l/kg)	0.584	2.632
Cl (l/h)	0.21	2.07

AUC_t = area under the curve to the last sampling time; AUC_∞ = area under the curve of the extrapolated area to time infinity; C_{max} = peak concentration; Cl = clearance; k_{el} = elimination rate constant; T_{max} = peak time; $t_{½}$ = the half-life time; V_d = volume of distribution

for chickens (Methoxasol: trimethoprim 5.5 mg, sulfamethoxazole 27.5 mg per 1 kg b.w.) were approximately 47.1 µg/ml for the free sulfamethoxazole and 2.1 µg/ml for the free trimethoprim. Queralt and Castells (1985), in their study, orally administered sulfamethoxazole and trimethoprim to hens at a dose of 80 mg/kg and 16 mg/kg b.w., respectively, and obtained C_{max} values of 54.5 µg/ml and 1.16 µg/ml, respectively. Trimethoprim was distributed to the body tissues to a greater extent than sulfamethoxazole; the detected distribution of the V_d for trimethoprim was approximately 5 times greater than the V_d for sulfamethoxazole. A similar pharmacokinetic study performed in broiler chickens, but with a different combination of sulfonamide (sulfadiazine) with trimethoprim, reported a volume of distribution (V_d) in the range of 1.63–4.02 l/kg for trimethoprim (Baert et al. 2003). The reported value of V_d corresponded to the value of V_d = 2.632 l/kg for the trimethoprim detected in our study. Both substances are excreted very rapidly in broilers,

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primarily in the urine due to their good solubility in the aqueous media. The elimination half-life of sulfamethoxazole and trimethoprim was about 1.92 and 0.88 hours, respectively. Estimates of the clearance Cl values for both substances correspond to the determined half-life values.

The sulfamethoxazole/trimethoprim substances were administered in a ratio of 5 : 1, but the detected ratio in the broiler serum in our study ranged from 20 to 35 : 1 for 0.5 h to 3 hours. A 20 : 1 ratio (sulfamethoxazole/trimethoprim) in animal plasma and in *in vitro* tests has been reported by many authors to be synergistically effective (Queralt and Castells 1985). In contrast, the synergism of this ratio has been critically discussed and questioned by some authors in recent years, primarily in clinical practice in human medicine (Wormser et al. 1982).

The trimethoprim/sulfamethoxazole combination exerts its antimicrobial effect by blocking the synthesis of tetrahydrofolic acid, the metabolically active form of folic acid. Sulfamethoxazole primarily inhibits the synthesis of dihydrofolic acid, whereas trimethoprim competitively inhibits dihydrofolate reductase, the final enzyme in the tetrahydrofolic acid synthesis pathway. The inhibition of the thymidine synthesis seems to be the primary net effect of this action (Wormser et al. 1982).

Trimethoprim/sulfamethoxazole is a 'broad spectrum' antimicrobial agent. *In vitro,* it is active against a wide range of organisms, including Gram-positive and -negative aerobic bacteria, chlamydia, Nocardia (actinomycetes), some mycobacteria and protozoa and many anaerobic bacteria. Organisms not susceptible to co-trimoxazole include *Mycobacterium tuberculosis, Treponema pallidum, Pseudomonas aeruginosa* and *Mycoplasma* species (Wormser et al. 1982). Moreover, trimethoprim/sulfamethoxazole is used against methicillin-resistant *Staphylococcus aureus* (MRSA) as an alternative to vancomycin (Eliakim-Raz et al. 2017). The European Medicines Agency listed the recommendation of veterinary medicinal products containing trimethoprim/sulfamethoxazole against poultry respiratory infections caused by *Escherichia coli*, *Pasteurella* spp. or *Salmonella* spp. and against infections caused by *Staphylococcus aureus* based on antimicrobial susceptibility testing (European Medicine Agency 2021).

There are a number of pharmacokinetic studies focused on the combination of different sulfonamides with trimethoprim in animals, but no comparable pharmacokinetic study on the combination of sulfamethoxazole and trimethoprim in broiler chickens has been published yet.

It is appropriate, in the future, to carry out further studies focusing, for example, on the pharmaceutical kinetic profile in animals affected by infections, which are animals that are, in fact, the target of the treatment.

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Conflict of interest

The authors declare no conflict of interest.

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