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# New simple liquid chromatographic method for the determination of trimethoprim, sulfadiazine and $N^4$-acetylsulfadiazine in plasma of broilers

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**Abstract**

A new method for simultaneous quantification of trimethoprim, sulfadiazine and $N^4$-acetylsulfadiazine in plasma of broilers at levels down to 13–16 ng/ml has been developed. Samples were deproteinized with acetonitrile, defatted with hexane, and extracted with dichloromethane. Chromatographic analysis was carried out on a $C_{18}$ column in the presence of tetrabutylammonium hydrogen sulfate, a competing base, while detection was performed at 240 nm for trimethoprim, and 270 nm for both sulfadiazine and $N^4$-acetylsulfadiazine. Accuracy and precision data showed recoveries and relative standard deviation values better than 87.3% and 3.1%, respectively, for all three analytes. The good analytical characteristics of the method could allow limits of detection in the low ng/ml range to be realised. The method was successfully applied to determine drug concentrations in plasma samples from broilers administered a combination of sulfadiazine and trimethoprim.


*Keywords:* Trimethoprim; Sulfadiazine; $N^4$-Acetylsulfadiazine

## 1. Introduction

The introduction of diaminobenzylpyrimidines such as trimethoprim in combination with sulfonamides has provided useful treatments for diseases caused by a variety of pathogenic bacteria in both human and veterinary medicine [1]. These combinations act by blocking two successive steps in the biosynthesis of tetrahydrofolate, thus inhibiting the synthesis of purines required for DNA synthesis and leading to enhanced antibacterial and antiprotozoal activity [2].

In veterinary medicine, a combination of trimethoprim (TMP) with sulfadiazine (SDZ) has been widely used for the control of poultry diseases [1]. Despite this use, limited data are available on the pharmacokinetics of these drugs that are administered to poultry via the drinking water for several days. This shortage is mainly due to the fact that reliable analytical methods for simultaneous determination of these compounds and their main metabolites in biological fluids are generally lacking [3].

A liquid chromatographic (LC) method for simultaneous determination of TMP, SDZ and $N^4$-

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acetyl SDZ in human serum has been reported [4], but the proposed normal-phase isocratic system is not suitable for resolving matrix interferences when analyzing plasma of broilers. Reversed-phase LC methods for isocratic analysis of these compounds in plasma of broilers has also been reported [5,6], however, the employed acidic mobile phases cannot adequately resolve the late eluted TMP from the $N^4$-acetylated metabolite of SDZ. Moreover, the use of acidic mobile phases frequently results in a broadened and severely tailing peak of TMP, and such a behavior could be the reason that some workers [6] have used end-capped $C_{18}$ columns and applied an elution gradient with a pH 4.5 phosphate buffer–acetonitrile mobile phase in order to analyze TMP and SDZ in plasma of broilers.

In a study conducted in our laboratory on the pharmacokinetics of TMP, SDZ and $N^4$-acetyl SDZ in healthy and diseased broilers, a new analytical LC method was developed. This simple, fast, inexpensive, and reliable method that allows control of the chromatographic selectivity in a single isocratic run, is reported in this paper.

## 2. Experimental

### 2.1. Reagents

Standard trimethoprim and sulfadiazine were purchased from Sigma (St. Louis, MO, USA), whereas the $N^4$-acetylsulfadiazine was kindly donated by Daiichi Pharmaceutical (Seiyaku, Japan).

Analytical-grade disodium hydrogenphosphate and potassium dihydrogenphosphate were from Merck (Darmstadt, Germany), phosphoric acid, *n*-hexane and dichloromethane from Merck–Schuchard (Munich, Germany), and tetrabutylammonium hydrogen sulfate (TBA) from Sigma.

LC-grade acetonitrile and methanol were from Merck–Schuchard. The employed LC-grade water was produced in the laboratory using a Milli-Q (Millipore, Bedford, MA, USA) purification system.

### 2.2. Apparatus

Chromatography was carried out on a Shimadzu system consisting of a Model LC-10AD pump, a Model SIL-$10A_{XL}$ autoinjector, a Model CTO-10A column oven, and a Model SPD-10AV UV–Vis detector. Integration was achieved by using the software Class-LC10 (version 1.41, Shimadzu, Japan). Detection was performed at 240 nm for TMP and 270 nm for both SDZ and $N^4$-SDZ. The change in the wavelength setting was automatically controlled by the system controller CBM-10A (Shimadzu). Separation was carried out on a Spherisorb ODS-2, 250×4 mm I.D., 5 μm, reversed-phase column (MZ-Analysentechik, Mainz, Germany) preceded by a MZ-Analysentechik precolumn cartridge, 20×4 mm I.D., packed with Spherisorb ODS-2, 5 μm material.

A vortex mixer Model G-560E (Scientific Industries, NY, USA), an IEC centrifuge, Model Centra-MP4 (Needman Heights, MA, USA), and a thermoblock Model ReactiTherm heating/stirring module (Pierce, Rockford, IL, USA) were also used in sample preparation.

### 2.3. Standard solutions

Individual stock solutions of TMP, SDZ and $N^4$-acetyl SDZ were prepared in 20-ml volumetric flasks by dissolving ca. 5 mg of each compound and dissolving to volume with methanol. A mixed intermediate standard solution containing all three analytes was prepared by mixing appropriate aliquots (0.080–2 ml) from each of the stock solutions in a 10-ml volumetric flask and diluting to volume with methanol.

Successive dilution of the mixed intermediate standard solution with the mobile phase resulted in the preparation of working standard solutions in the range of 0.005–4.62 μg/ml for TMP, 0.005–60.48 μg/ml for SDZ, and 0.005–5.2 μg/ml for $N^4$-acetyl SDZ. All standards were protected from light with aluminum foil and kept at 4 °C in a refrigerator, when not in use.

### 2.4. Chromatographic conditions

The mobile phase was a mixture of acetonitrile–phosphate buffer (12:88, v/v) containing 20 m*M* potassium dihydrogenphosphate, 10 m*M* disodium hydrogenphosphate, and 2.5 m*M* tetrabutylammonium hydrogen sulfate, as a competing base, in

LC water. The pH of the phosphate buffer was adjusted to 3.5 by the addition of 1 *M* phosphoric acid prior to the addition of acetonitrile. Following mixing, the mobile phase was filtered through a 0.20-μm nylon 66 filter (Alltech, Deerfield, IL, USA) and degassed using helium.

The mobile phase was delivered to the column at a rate of 1.0 ml/min. The Spherisorb ODS-2 column was thoroughly equilibrated with mobile phase each time before use. Reproducible capacity factors ($k'$) could be realized after passage through the column of at least 150 ml of mobile phase. During runs, the column was kept thermostatted at 35 °C.

### 2.5. Sample extraction and cleanup

A 0.5-ml volume of plasma was pipetted into a 10-ml glass tube with the subsequent addition of 1 ml of acetonitrile. The mixture was vortexed for 30 s, centrifuged at 2000 *g* for 5 min, and the supernatant liquid was transferred to another tube in which 2 ml of hexane were also added. After vortex-mixing for 30 s and centrifugation at 1000 *g* for 3 min, the upper hexane layer was aspirated and discarded. A 0.2-ml volume of a pH 6.8 phosphate buffer and 6 ml of dichloromethane were then added to the tube, and the mixture was vortex-mixed for 60 s and centrifuged at 2000 *g* for 10 min. Following centrifugation, the top aqueous layer was discarded by aspiration, while a 5-ml aliquot of the bottom layer was transferred to another tube to be evaporated to dryness level under a stream of nitrogen at 50 °C. The residual was reconstituted in 0.5 ml of mobile phase, and 20 μl was injected into the LC system.

### 2.6. Determination

Calibration curves were obtained by running a series of working solutions and plotting the recorded peak areas (μV) versus the corresponding concentrations of the analytes injected (ng/20 μl). Slope, intercept and least-squared fit of standard curves were calculated. Quantification of each analyte in the unknown samples was carried out by reference to corresponding standard curves and multiplying by the appropriate dilution and recovery factors.

## 3. Results and discussion

### 3.1. Extraction and cleanup

Preliminary experiments on the partitioning of TMP, SDZ and $N^4$-acetyl SDZ between phosphate buffers at various pH values and ethyl acetate or dichloromethane showed that the analytes could not be quantitatively extracted by each of these organic solvents when the pH value was higher than 8. Adjustment of the phosphate buffers at pH 6.8 substantially improved the extraction recoveries due to the amphoteric properties of the tested compounds. However, further experiments on plasma samples spiked with TMP, SDZ and $N^4$-acetyl SDZ showed that the use of ethyl acetate or dichloromethane resulted in concentrates which contained too many compounds that interfered with the chromatography of the analytes.

To reduce or eliminate interferences, a series of liquid–liquid partition cleanup procedures was evaluated. Results showed that deproteinization of the sample with acetonitrile followed by hexane washing of the aqueous acetonitrile extract could achieve significant purification. Subsequent extraction of the aqueous acetonitrile extract with ethyl acetate or dichloromethane showed a solvent-dependent recovery efficiency; ethyl acetate led to a recovery loss of more than 60% for the $N^4$-acetylated metabolite of SDZ, while dichloromethane increased the recoveries of all analytes to more than 85% eliminating, concurrently, interferences (Fig. 1).

### 3.2. Chromatography

Owing to their molecular structure (Fig. 2), TMP, SDZ and $N^4$-acetyl SDZ are not easily amenable to LC analysis in a single isocratic run. Using reversed-phase LC with mobile phases containing phosphate buffers in the pH range 3–6 and acetonitrile in the range of 10–30%, good separation of the analytes could generally be achieved. However, the late eluted TMP peak tailed badly under all examined conditions. Since this indicated that a strong adsorptive interaction between the protonated TMP and the negatively charged silanol groups on the silica-based stationary phase might exist, deactivation of these active sites by addition to the mobile phase of

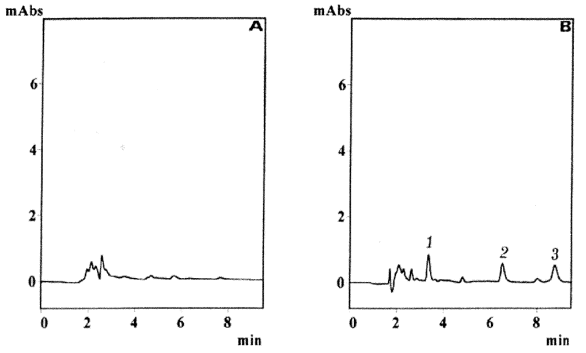
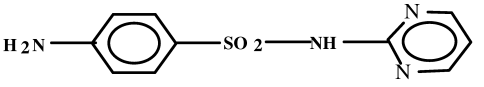
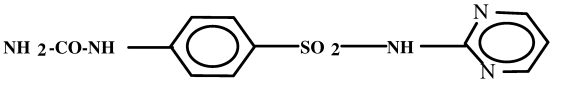


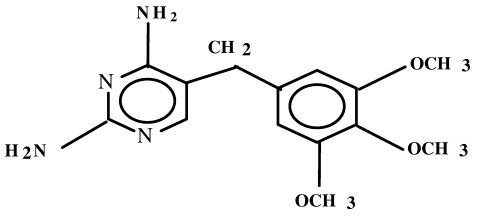

Fig. 1. Typical chromatograms of blank broiler plasma samples (A) and samples spiked with TMP (peak No. 1) 100 ng/ml, SDZ (peak No. 2) 90 ng/ml, and $N^4$-acetyl SDZ (peak No. 3) 105 ng/ml (B). Stationary phase: Spherisorb ODS-2; mobile phase: acetonitrile–phosphate buffer (12:88, v/v), containing 2.5 m*M* tetrabutylammonium hydrogen sulfate; column temperature, 35 °C; flow-rate, 1 ml/min; wavelength, 240 nm for TMP and 270 nm for SDZ and $N^4$-acetyl SDZ; injection volume, 20 μl.




**Sulfadiazine**




**N4-acetylsulfadiazine**




**Trimethoprim**

Fig. 2. Chemical structures of investigated compounds.

tetrabutylammonium hydrogen sulfate [7], a competing base, was examined.

Upon addition of 2.5 m*M* tetrabutylammonium cations to the mobile phase, the chromatographic performance of the LC system was substantially improved. Peak heights of all three analytes increased, while the distortion of the TMP peak was totally eliminated. Under these conditions, however, the late eluted TMP turned out to be the first eluted compound with a retention time of 3.2 min, whereas SDZ and its $N^4$-acetyl metabolite eluted from the column at 6.3 and 8.4 min, respectively (Fig. 1).

The dramatic decrease of the retention time of TMP in presence of tetrabutylammonium cations clearly indicates that the principle retention mechanism for this compound involves interaction with the surface silanols. With p$K_{a1}$ close to 1.3, p$K_{a2}$ equal to 6.6, and each p$K_a$ value referring to a deprotonation of the pyrimidine ring nitrogen, TMP carries a positive charge of around +1.03 in a mobile phase pH of 3.5 [8]. Hence, ion-exchange was possible at underivatized silanol sites in consistency with the reported [9] possible adsorption of diaminobenzylpyrimidines on the walls of untreated silica capillaries in capillary zone electrophoresis carried out at low pH. The drawn results lend support to previous investigators [8] who also reported that addition of tetrabutylammonium cations to the mobile phase reduced the retention time of TMP from 20 to 5.5 min without simultaneously accelerating the elution of several co-chromatographed sulfonamides.

Upon addition of tetrabutylammonium cations to the mobile phase, sulfadiazine and its $N^4$-acetylated metabolite also displayed some decrease in retention, a finding suggesting that these compounds were partly in the form of the positive charged conjugate acid. However, the loss of retention of these compounds was small compared with that of TMP. With a p$K_{a1}$ value close to 2.0 and p$K_{a2}$ equal to 6.5, SDZ carries a positive charge of around +0.14 in a mobile phase pH of 3.5, which is much smaller than that of TMP [8]. Therefore, it might be assumed that a correlation between the magnitude of the retention loss and the average charge of the analytes should exist, although the observed retention behavior might equally well be attributed not only to the efficient masking of the negatively charged silanols but also

to some electrostatic repulsion of the protonated analytes by the tetrabutylammonium cations adsorbed onto the octadecylsilica surface [10].

### 3.3. Linearity and limit of quantification

Regression analysis of the data obtained by running a series of working solutions showed linear response in the range examined (0.1–92.4 ng of TMP injected; 0.1–1209.6 ng of SDZ injected; 0.1–104 ng of $N^4$-acetyl SDZ injected). Calibration curves were described by the following equations: $y=52.1+3409.4x$, $r^2=0.99998$, for TMP, $y=-54.1+4184.1x$, $r^2=0.99999$, for SDZ, and $y=49.0+3569.3x$, $r^2=0.99999$, for $N^4$-acetyl SDZ where $y$ represents peak area in μV and $x$ the quantity in ng of the compound per 20 μl injected.

The efficiency of the ion pair chromatographic system coupled with the cleanliness of the extracts, and the excellent response of TMP at 240 nm, and SDZ and $N^4$-acetyl SDZ at 270 nm, allowed low limits of detection to be realized (5 ng/ml for a signal-to-noise ratio of 3:1) for all three analytes. The limits of quantification, as they were determined by the smallest validated concentration with spiked samples (Table 1), estimated at 13.8 ng/ml for TMP, 12.6 for SDZ, and 15.6 ng/ml for $N^4$-acetyl SDZ.

### 3.4. Accuracy and precision

The accuracy of the method was studied by spiking drug-free plasma samples with TMP, SDZ and $N^4$-acetyl SDZ at four fortification levels (13.8–1104.0 ng/ml for TMP; 12.6–1677.0 ng/ml for SDZ; 15.6–1252.0 ng/ml for $N^4$-acetyl SDZ) and analyzing six replicates. Least-squares and regression analysis of the data as presented in Table 1, showed that the relationship between ''added'' and ''found'' was adequately described by linear regression for each of the analytes: $y=-0.0002+0.971$ ($\pm 0.017$)$x$ for TMP, $y=0.0027+0.929$ ($\pm 0.016$)$x$ for SDZ, and $y=0.0045+0.872$ ($\pm 0.013$)$x$ for $N^4$-acetyl SDZ. Therefore, the slope of each of these lines could be used as an estimate of the overall recovery of the method, which for TMP was 97.1±0.02%, for SDZ 92.9±1.6%, and for $N^4$-acetyl SDZ 87.2±1.3%.

The precision of the method was also studied by spiking drug-free plasma samples with all analytes at three levels (138–1104 ng/ml for TMP; 126–1677 ng/ml for SDZ; 156.5–1252.0 ng/ml for $N^4$-acetyl SDZ), and assaying four replicates on each of three different days. The results of the assays presented in Table 2 suggest that the precision of the method, expressed as percent relative standard deviation (RSD%), ranged from 2.3 to 3.1% for both TMP and $N^4$-acetyl SDZ, and 1.3 to 2.5% for SDZ.

Table 1
Accuracy data in the analysis of broiler plasma spiked with trimethoprim, sulfadiazine, and $N^4$-acetylsulfadiazine

| | Analyte added (ng/ml) | Mean concentration found* (ng/ml±SD) | RSD (%) | Mean recovery (%±SD) |
|---|---|---|---|---|
| Trimethoprim | 13.8 | 13.4±0.2 | 1.3 | 97.1±1.8 |
| | 138.0 | 135.9±3.2 | 2.4 | 98.3±2.5 |
| | 552.0 | 532.9±9.7 | 1.8 | 96.6±1.7 |
| | 1104.0 | 1073.1±17.6 | 1.6 | 97.2±1.6 |
| Sulfadiazine | 12.6 | 12.3±0.1 | 1.1 | 97.9±1.1 |
| | 126.0 | 118.9±0.3 | 0.3 | 94.4±0.2 |
| | 838.5 | 792.9±16.6 | 2.1 | 94.6±1.9 |
| | 1677.0 | 1556.9±30.6 | 2.0 | 92.8±1.8 |
| $N^4$-Acetylsulfadiazine | 15.6 | 13.7±0.3 | 2.5 | 87.8±2.2 |
| | 156.6 | 142.3±3.7 | 2.6 | 90.9±2.4 |
| | 626.0 | 556.4±10.3 | 1.9 | 88.9±1.6 |
| | 1252.0 | 1092.8±16.7 | 1.5 | 87.3±1.3 |

*Six replicates.

Table 2
Precision data on the analysis of broiler plasma samples spiked with trimethoprim, sulfadiazine and $N^4$-acetylsulfadiazine on 3 different days

| | Analyte added (ng/ml) | Overall mean* (ng/ml±SD) | RSD (%) |
|---|---|---|---|
| Trimethoprim | 138.0 | 134.5±4.1 | 3.1 |
| | 552.0 | 542.4±13.8 | 2.5 |
| | 1104.0 | 1069.2±24.6 | 2.3 |
| Sulfadiazine | 126.0 | 117.6±1.5 | 1.3 |
| | 838.5 | 797.9±19.6 | 2.4 |
| | 1677.0 | 1538.1±37.3 | 2.4 |
| $N^4$-Acetylsulfadiazine | 156.6 | 140.8±4.0 | 2.9 |
| | 626.0 | 560.7±17.3 | 3.1 |
| | 1252.0 | 1089.4±24.8 | 2.3 |

*Four replicates.

Table 3
Stability of trimethoprim, sulfadiazine and $N^4$-acetylsulfadiazine in spiked plasma samples, at 25 °C in the autosampler and at −30 °C following 6 months storage

| | Analyte added (ng/ml) | Mean concentration found* (ng/ml±SD) | |
|---|---|---|---|
| Storage at 25 °C | | 0 h | 24 h |
| Trimethoprim | 217.4 | 216.1±0.4 | 215.4±1.7 |
| Sulfadiazine | 218.2 | 216.2±0.6 | 217.0±0.6 |
| $N^4$-Acetylsulfadiazine | 201.0 | 197.7±2.9 | 197.4±2.5 |
| Storage at −30 °C | | 0 months | 6 months |
| Trimethoprim | 434.8 | 430.3±3.2 | 431.6±3.7 |
| Sulfadiazine | 872.8 | 868.7±0.6 | 867.7±2.4 |
| $N^4$-Acetylsulfadiazine | 402.0 | 397.3±2.5 | 394.2±2.0 |

*Six replicates.

### 3.5. Storage stability

To determine the stability of TMP, SDZ and $N^4$-acetyl SDZ on sample storage, plasma samples spiked with all three target compounds were analyzed after either a 24-h waiting time in the autosampler of the LC at 25 °C, or 6 months storage at −30 °C. The analytical results presented in Table 3 showed that none of the tested procedures could result in any significant changes (Student $t$-test, $P<0.05$) in the concentrations of the analytes.

### 3.6. Applicability

To test the method with real samples, a trial was undertaken to quantitate all three analytes in plasma from four broilers intravenously administered a SDZ–TMP combination (5:1) at a single dose of 30 mg/kg body mass. The results presented in Table 4 demonstrate the efficiency of the method in pharmacokinetic studies application, and/or therapeutic drug monitoring. Typical chromatograms of plasma samples from broilers intravenously or orally administered a single dose of SDZ and TMP, alone or in combination (5:1), are also illustrated in Fig. 3.

## 4. Conclusion

The new method exhibits quite satisfactory analytical characteristics with respect to recovery, precision, sensitivity, and selectivity. It is rapid since a daily throughput of 50–70 plasma samples could be easily achieved by manual sample preparation and

Table 4
Mean levels of sulfadiazine, trimethoprim and $N^4$-acetylsulfadiazine in plasma of four broilers after a single i.v. administration of SDZ–TMP combination (5:1) at a dose of 30 mg/kg body mass

| Hours after administration | Concentration in plasma (ng/ml±SD) | | |
|---|---|---|---|
| | Sulfadiazine | Trimethoprim | $N^4$-Acetylsulfadiazine |
| 1 | 41 687±5054 | 1665±151 | 1667±109 |
| 3 | 28 832±5240 | 924±169 | 1278±201 |
| 6 | 15 449±4850 | 459±119 | 809±196 |
| 12 | 5622±1650 | 112±45 | 287±84 |
| 24 | 198±60 | <LQ | <LQ |
| 36 | 37±18 | <LQ | <LQ |
| 48 | <LQ* | <LQ | <LQ |

*Limit of quantification.

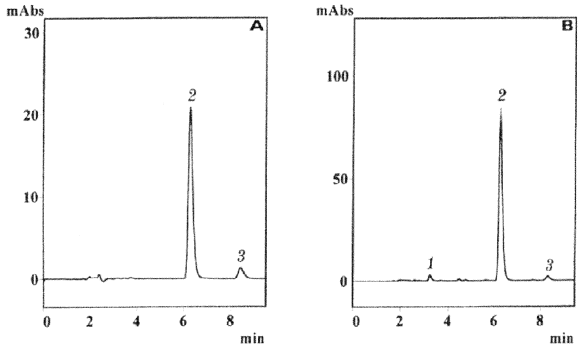



Fig. 3. Typical chromatograms of plasma samples taken from broilers, which received a single oral dose of 25 mg SDZ/kg body mass (A, sample at 0.5 h after the administration, 22 368 ng/ml SDZ and 610 ng/ml $N^4$-SDZ) or a single intravenous dose of 30 mg SDZ–TMP/kg body mass (5:1), (B, sample at 0.5 h after the administration, 2062 ng/ml TMP, 52 289 ng/ml SDZ and 1245 ng/ml $N^4$-SDZ). Chromatographic conditions and peak labels as in Fig. 1.

automated LC analysis. Therefore, the developed method might be useful as a simple, inexpensive and reliable means for quantitative determination of TMP, SDZ and $N^4$-acetyl SDZ in plasma samples.

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