Technological research article

Application of high-performance liquid chromatography with diode-array detection (HPLC-DAD) for the determination of sulfadiazine and sulfadoxine in serum

Charli Rose Parra[1], Juan Pablo Betancourt Arango[2]*, Jorge Enrique Gómez Marín[1] & Gonzalo Taborda Ocampo[2]

[1] Molecular Parasitology Research Group (GEPAMOL), Biomedical Research Center, Universidad del Quindío, Armenia, Colombia. Cra 15 Calle 12N, Armenia, Colombia. Tel/Fax: +57 67 359959.
[2] Department of Chemistry, Universidad de Caldas, Calle 65 N. 26-10, A.A. 265, Caldas, Colombia.

* Corresponding author E-mail: chemquantum@outlook.com

Received: March 31, 2025
Corrected: September 14, 2025
Accepted: September 18, 2025

https://doi.org/10.15446/rcciquifa.v55n1.119599

SUMMARY

Introduction. Monitoring serum drug levels in children with congenital toxoplasmosis is essential for determining therapeutic efficacy. This study aimed to standardize the quantification of two sulfonamides, sulfadiazine (SDZ) and sulfadoxine (SDX), using high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD). Key attributes such as linearity, precision, accuracy, selectivity, limit of detection (LOD), and limit of quantification (LOQ) were evaluated, alongside secondary attributes like drug stability and matrix effects. **Methodology**. The quantification of SDZ and SDX was performed using HPLC-DAD, evaluating: linearity: Confirmed a linear correlation between analytical signal and drug concentration, precision: Evaluated using relative standard deviation (%RSD), accuracy: Determined by recovery percentages, selectivity: Ensured no significant interference from the biological matrix, LOD and LOQ: Assessed for method sensitivity. **Results**. The method demonstrated a clear linear relationship between concentration and instrumental response. Precision was within acceptable ranges for bioanalytical studies, with %RSD indicating consistent results. The accuracy was satisfactory with recovery percentages slightly below 90%, which was acceptable considering the complexity of the biological matrix. LOD and LOQ were consistent with previously reported values, confirming high sensitivity. **Conclusions**. The HPLC-DAD method is reliable, robust, and sensitive for monitoring sulfadiazine and sulfadoxine levels in serum. While recovery percentages were slightly below 90%, the method's performance was satisfactory considering the biological matrix. This method is suitable for therapeutic monitoring and can aid in assessing treatment efficacy in congenital toxoplasmosis.

Keywords: RP-HPLC-DAD; sulfadoxine; sulfadiazine; toxoplasmosis.

RESUMEN

Aplicación de la cromatografía líquida de alta resolución con detección por matriz de diodos (HPLC-DAD) para la determinación de sulfadiazina y sulfadoxina en Suero

Introducción. La monitorización de los niveles séricos de fármacos en niños con toxoplasmosis congénita es esencial para determinar la eficacia terapéutica. El objetivo de este estudio fue estandarizar la

cuantificación de dos sulfonamidas, sulfadiazina (SDZ) y sulfadoxina (SDX), mediante cromatografía líquida de alta resolución acoplada a un detector de matriz de diodos (HPLC-DAD). Se evaluaron atributos clave como la linealidad, la precisión, la exactitud, la selectividad, el límite de detección (LOD) y el límite de cuantificación (LOQ), junto con atributos secundarios como la estabilidad del fármaco y los efectos de la matriz. **Metodología.** La cuantificación de SDZ y SDX se realizó mediante HPLC-DAD, evaluando: linealidad: Se confirmó una correlación lineal entre la señal analítica y la concentración del fármaco, precisión: Evaluada mediante la desviación estándar relativa (%RSD), exactitud: Determinada por los porcentajes de recuperación, selectividad: Se garantizó que no hubiera interferencias significativas de la matriz biológica, LOD y LOQ: Se evaluó la sensibilidad del método. **Resultados.** El método demostró una clara relación lineal entre la concentración y la respuesta instrumental. La precisión estuvo dentro de rangos aceptables para estudios bioanalíticos, con %RSD indicando resultados consistentes. La exactitud fue satisfactoria, con porcentajes de recuperación ligeramente inferiores al 90%, lo que resultó aceptable teniendo en cuenta la complejidad de la matriz biológica. El LOD y el LOQ fueron coherentes con los valores comunicados anteriormente, lo que confirma la alta sensibilidad. **Conclusiones.** El método HPLC-DAD es fiable, robusto y sensible para monitorizar los niveles de sulfadiazina y sulfadoxina en suero. Aunque los porcentajes de recuperación fueron ligeramente inferiores al 90%, el rendimiento del método fue satisfactorio teniendo en cuenta la matriz biológica. Este método es adecuado para la monitorización terapéutica y puede ayudar a evaluar la eficacia del tratamiento en la toxoplasmosis congénita.

Palabras clave: RP-HPLC-DAD; sulfadoxina; sulfadiazina; toxoplasmosis.

RESUMO

Aplicação da cromatografia líquida de alta resolução com detecção por matriz de díodos (HPLC-DAD) para a determinação de sulfadiazina e sulfadoxina no soro

Introdução. A monitorização dos níveis séricos dos medicamentos em crianças com toxoplasmose congénita é essencial para determinar a eficácia terapêutica. O objetivo deste estudo foi normalizar a quantificação de duas sulfonamidas, a sulfadiazina (SDZ) e a sulfadoxina (SDX), por cromatografia líquida de alta resolução acoplada a um detetor de díodos (HPLC-DAD). Foram avaliados atributos-chave como a linearidade, a precisão, a exatidão, a seletividade, o limite de deteção (LOD) e o limite de quantificação (LOQ), bem como atributos secundários como a estabilidade do fármaco e os efeitos da matriz. **Metodologia.** A quantificação de SDZ e SDX foi efectuada por HPLC-DAD, avaliando: linearidade: foi confirmada uma correlação linear entre o sinal analítico e a concentração do fármaco, precisão: avaliada pelo desvio-padrão relativo (%RSD), exatidão: determinada pelas taxas de recuperação, seletividade: foi assegurada a ausência de interferências significativas da matriz biológica, LOD e LOQ: foi avaliada a sensibilidade do método. **Resultados.** O método demonstrou uma relação linear clara entre a concentração e a resposta instrumental. A precisão situou-se dentro de intervalos aceitáveis para estudos bioanalíticos, com %RSD a indicar resultados consistentes. A exatidão foi satisfatória, com percentagens de recuperação ligeiramente inferiores a 90%, o que foi aceitável tendo em conta a complexidade da matriz biológica. O LOD e o LOQ foram consistentes com os valores previamente comunicados, confirmando a elevada sensibilidade. **Conclusões.** O método HPLC-DAD é fiável, robusto e sensível para monitorizar os níveis séricos de sulfadiazina e sulfadoxina. Embora as taxas de recuperação tenham sido ligeiramente inferiores a 90%, o desempenho do método foi satisfatório, tendo em conta a matriz biológica. Este método é adequado para a monitorização terapêutica e pode ajudar a avaliar a eficácia do tratamento na toxoplasmose congénita.**Palavras-chave:** RP-HPLC-DAD, sulfadoxina, sulfadiazina, toxoplasmose.

Palavras-chave: RP-HPLC-DAD; sulfadoxine; sulfadiazina; toxoplasmose.

1. INTRODUCTION

Congenital toxoplasmosis is an infection caused by the protozoan *Toxoplasma gondii*, which is transmitted from mother to child during pregnancy [1]. In Colombia, this disease represents a significant public health challenge due to its prevalence and the severe complications it causes in the pediatric population [2]. The estimated prevalence in the country is approximately 0.4 per 1,000 children under the age of five, although other sources report values ranging from 2 to 10 cases per 1,000 newborns. These discrepancies can be attributed to regional variations and methodological differences in data collection [3]. The standard treatment consists of a combination of pyrimethamine with a sulfonamide, such as sulfadiazine (SDZ) or sulfadoxine (SDX) [4]. This treatment must be administered for a full year to maintain therapeutic drug levels, preventing the onset of retinochoroiditis lesions and new neurological complications while avoiding adverse effects such as hematological toxicity at high doses [4, 5].

Accurate quantification of SDZ and SDX in biological fluids is essential for therapeutic drug monitoring (TDM). In *T. gondii* infections, rigorous control of plasma antibiotic levels is necessary to ensure treatment efficacy [6]. However, the complexity of biological matrices, due to the presence of various metabolites and proteins, poses challenges for drug analysis. While methods for sulfonamide quantification in plasma have been reported, validated techniques for their determination in serum are scarce. Serum could be particularly useful for retrospective analyses in cases of therapeutic failure. Additionally, there is evidence that elevated concentrations of these drugs can lead to significant adverse effects [7]. Therefore, a validated methodology is crucial for providing reliable quantitative information on these analytes in biological samples.

Drug analysis in biological fluids presents challenges due to matrix complexity, as interfering metabolites and proteins can affect the quantification of target compounds [8]. Although several techniques for determining sulfonamides in plasma have been reported [9-11], no specific methodologies have been described for serum, which has potential applications in retrospective studies of therapeutic failures. Various analytical techniques have been employed for drug quantification in biological matrices, including gas chromatography (GC), high-performance liquid chromatography (HPLC), and spectrophotometry [8]. Among these, HPLC coupled with a diode array detector (HPLC-DAD) [12-15], stands out for its sensitivity, specificity, and ability to simultaneously analyze multiple compounds.

The validation of analytical methods is essential to ensure the reliability and reproducibility of results [14, 15]. This process involves evaluating parameters such as linearity, precision, accuracy, selectivity, limit of detection, and limit of quantification. In this context, the present study aims to standardize and validate an HPLC-DAD-based analytical method for the simultaneous quantification of SDX and SDZ in serum. Implementing this method will enable more precise monitoring of serum drug levels in pediatric patients with congenital toxoplasmosis, ultimately optimizing treatment and minimizing associated risks.

2. METHODOLOGY

2.1. Standards and Reagents

The solvents used in this study included HPLC-grade methanol (J.T. Baker, CAS: 67-56-1); analytical-grade formic acid (98%) (Merck, CAS: 64-18-6); and analytical-grade perchloric acid

(72%) (Merck, CAS: 7601-90-3) for the extraction process. HPLC-grade water was obtained from a Direct-Q system (Millipore).

2.2. Selection of Drugs

Certified standards of the antibiotics sulfadiazine (SDZ, CAS: 68-35-9) and sulfadoxine (SDX, CAS: 2447-57-6) were used, both purchased from Orbus Pharma Ltda. (Bogotá, D.C.), in solid form and with a purity close to 100%. Additionally, a fixed-dose combination of pyrimethamine-sulfadoxine tablets (500/25 mg, PYR-SDX) was analyzed, obtained from the pharmaceutical company BCN Medical (Bogotá). The initial solutions of each antibiotic were prepared from high-purity standards using HPLC-grade methanol as the primary solvent. The concentration was expressed in μg/mL and calculated according to the following equation:

$$Concentration\left(\frac{\mu g\ of antibiotic}{mL\ of solution}\right) = \frac{m \times \%Purity}{V}$$

Where, m is mass of the analyte (antibiotic) in μg, V is final volumetric capacity (L) and **%** *Purity* is purity percentage of the antibiotic.

Since SDZ and SDX exhibit low solubility in methanol and are practically insoluble in water, individual solutions were prepared at lower concentrations than those reported in the original method (100 mg/mL for SDZ). To enhance solubilization, the solutions were sonicated in a Branson-2510 device at a temperature of 36-37 °C for 5 minutes. The stock solutions prepared were: SDZ 624 μg/mL in methanol and SDX 644 μg/mL in methanol. From these, additional dilutions were prepared: SDZ 516 μg/mL, SDX 530 μg/mL, as well as a mixed solution containing both drugs at a concentration of 130 μg/mL.

2.3. Serum and Plasma Samples

Blood samples were collected from healthy volunteers with no diagnosis of toxoplasmosis and no history of treatment with sulfadiazine, sulfadoxine, or pyrimethamine. A total of 10 mL of venous blood was drawn from the forearm into two types of collection tubes: for serum, a tube without anticoagulant (dry tube) was used, while for plasma, a BD Vacutainer® tube containing lithium heparin as an anticoagulant was employed. The sample processing was performed as follows: Serum samples: Blood in the dry tube was left at room temperature (20-25 °C) for 30 minutes to allow complete coagulation. It was then centrifuged at 1,500 g for 10 minutes, and the supernatant (serum) was carefully recovered. Plasma samples: Blood collected in the lithium-heparin tube was centrifuged directly at 1,500 g for 10 minutes. The plasma (upper layer) was then carefully separated using a Pasteur pipette. Both serum and plasma samples were stored at -20 °C until analysis.

2.4. Chromatographic Conditions

The analyses were performed using an HPLC-DAD system (Shimadzu, Japan) equipped with a Prominence SPD-M20A photodiode array UV-VIS detector (operating at 270 nm), a Shimadzu CTO-10AS VP column oven, a Shimadzu SIL-10AF autosampler, and a Prominence DGU-20A5 degasser. A Waters C18 ODS2 column (250 × 4.6 mm, 5 μm) was used for the separation. Data acquisition and analysis were carried out using LC-Solution software (Shimadzu). A reverse-phase high-performance liquid chromatography (RP-HPLC) method was applied based on a previously described protocol (9), with modifications made to optimize the determination of SDX. Several comparative tests were conducted to assess the influence of the biological matrix (serum and plasma) on antibiotic quantification, as well as to compare cali-

bration curves prepared in serum and mobile phase (methanol). The mobile phase was prepared as follows: Mobile phase A: Methanol, water, and concentrated formic acid in a ratio of 50:950:1 (v/v/v). Mobile phase B: Methanol, water, and concentrated formic acid in a ratio of 500:500:1 (v/v/v). The injection volume was set at 20 μL. The injection port temperature was maintained between 25°C and 30°C, with a column flow rate of 0.5 mL/min and a working pressure range of 0.7–24.7 MPa. HPLC filters were from Millex (13 mm) and Advantec (0.22 μm). Standard solutions and analytes were injected in triplicate into the RP-HPLC-DAD system under the established conditions to determine the characteristic analytical signal and retention time for both individual solutions and the mixture. To evaluate the impact of the biological matrix on the analytical response, solutions were prepared in both serum and plasma, including blank matrices (without antibiotics) and spiked samples, ensuring that the detected signals corresponded exclusively to the analyzed drugs.

2.5. Calibration Curves

2.5.1. Linearity and System Variability Testing

To evaluate the linearity, seven calibration standard levels were prepared with concentrations of 120, 50, 30, 10, 5, 1, and 0.5 μg/mL. These standards were obtained by diluting the sulfadiazine (SDZ, 530 μg/mL) and sulfadoxine (SDX, 1070 μg/mL) stock solutions in methanol. The calibration curve was designed to cover a broad concentration range, from very low values to relatively high levels, within the expected range for therapeutic blood concentrations. To assess system variability (including the pump, detector, and column), the concentration range that satisfies Beer's Law was determined—specifically, the optimal range where the analytical signal is proportional to the concentration of the standards. Each standard of both drugs was injected once, and the resulting signal was averaged. To establish the relationship between the average peak area and the concentration of the standards, the following parameters were calculated using the least squares statistical method: slope of the calibration curve, intercept, correlation coefficient (r), and coefficient of determination (r^2).

2.5.2. Linearity and Variability Curve with Serum and Plasma Matrix

A calibration curve was constructed in a serum matrix, with seven concentration levels ranging from 0.2 μg/mL (the lowest concentration standard) to 50 μg/mL, for both sulfadoxine (SDX) and sulfadiazine (SDZ). These standards were analyzed under the same sample preparation conditions. For the serum calibration curve, two stock solutions were prepared: SDZ 624 μg/mL and SDX 644 μg/mL. From these solutions, two additional dilutions were made: 50 μg/mL and 20 μg/mL, respectively. The standard concentrations were diluted in serum from a volunteer to achieve the different calibration levels. Additionally, a calibration curve was prepared in plasma to assess potential variations in the analytical signal when using a different blood matrix.

2.5.3. Calibration Curve PYR-SDX in Serum

For the pyrimethamine-sulfadoxine (PYR-SDX) combination, a solution was prepared with a final concentration of 250 μg/mL, calculated based on pyrimethamine. The linearity of the analytical response was evaluated by preparing seven calibration levels in the region of highest graphical linearity, with the following concentrations in the serum matrix: 50, 30, 5, 2, 1, 0.5, and 0.2 μg/mL. Each standard was injected in duplicate into the HPLC system. Subsequently, the dependence relationship between the average peak area and the concentration of the

standards was determined, applying the same statistical parameters used for the evaluation of system linearity.

2.6. Statistical Analysis

The results were analyzed using the data processing programs Origin Pro 8 SR0 and GraphPad Prism. The relative standard deviation percentage (% RSD) was calculated using the following equation:

$$\%RSD = \left(\frac{Desviación\ estándar}{Área\ promedio}\right) x\ 100$$

To verify the concentration, range within which the linear model is valid, the following evaluations were conducted: a) Homoscedasticity Analysis: Using a residual plot versus concentration. b) Verification of the Linear Model Validity: Through the correlation coefficient. c) Analysis of Variance (ANOVA) of the regression, evaluating: Proportionality, through a t-Student test for the intercept. Slope and regression, comparing t_{exp} with t_{cal} at a 95% confidence level, where $t_{exp} > t_{cal}$ indicates a significant correlation between the chromatographic peak area and the analyte concentration. Snedecor's F-test to evaluate the equality of variances. Additionally, the limits of detection (LOD) and limits of quantification (LOQ) were calculated to ensure the accuracy of the analytical measurements: Limit of Detection (LOD): Determined by multiplying the average area of the blank signal noise by 4. Limit of Quantification (LOQ): Obtained by multiplying the average area of the blank signal noise by 16. From these values, the following definitions were established: LOD: The minimum detectable amount, which indicates the presence of the analyte, but whose quantitative determination is not valid. LOQ: The minimum quantifiable amount, necessary to perform a reliable prediction of the concentration in the sample.

3. RESULTS AND DISCUSSION

3.1. Qualitative analysis

According to the chromatographic results, it was found that the sulfonamides are easily separated by RP-HPLC-DAD under the established conditions for both mobile phases. However, it was not possible to differentiate the peak between sulfadoxine (SDX) and pyrimethamine (PYR) (Figure 1). For this reason, the measurement of PYR was not analyzed in this study.

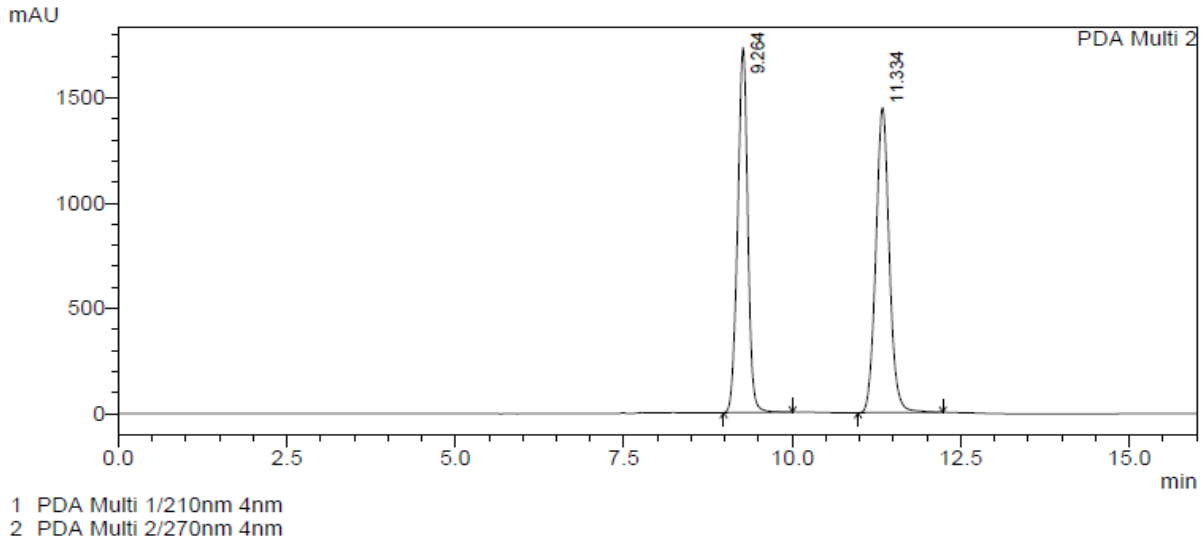


Figure 1. Chromatogram of a volunteer serum sample to which 300 µg/mL SDX, 200 µg/mL SDZ, and 200 µg/mL PYR were added. The peaks corresponding to SDZ and SDX are visible, but PYR is not detected. This likely indicates that the PYR peak elutes at a retention time very close to that of SDX, thus masking it under the current working conditions.

The retention times (RT) of the analytes after mixing were analyzed (**Table 1**). The RT of SDX and SDZ are not close to each other, so there is no overlap or interference between the signals of the two antibiotics. The RT of the fixed combination of PYR-SDX (although it is not possible to differentiate between the PYR and SDX peaks) shows a well-defined analytical signal with a RT similar to that of SDX in the standard solution, confirming the presence of SDX both in the solution and in the commercial tablets of this combination.

Table 1. Comparison of the retention times of the antibiotics in individual solutions and in the mixture.

Standard	Matrix	RT (min) individual	RT (min) in the mixture
Sulfadiazine (SDZ)	Methanol	9.341	9.451
	Serum		9.330
	Plasma		9.418
Sulfadoxine (SDX)	Methanol	11.599	11.658
	Serum		11.528
	Plasma		11.631
Fixed combination Pyrimethamine-Sulfadoxine (PYR-SDX)	Methanol	*11.503	
	Serum	*11.495	
	Plasma	*11.550	

3.2. Variability analysis in methanol matrix

Several calibration curves were prepared in duplicate, and the obtained peak areas were used to calculate the mean, standard deviation, and relative standard deviation percentage (%RSD), as recorded in **Table 2** and **Table 3** for the methanol matrix.

Table 2. Peak areas obtained for sulfadiazine standard in the system linearity assessment using a methanol matrix.

Sulfadiazine (SDZ) - System Linearity						
Level	**[ ] (µg/mL)**	**Area-1**	**Area-2**	**Media**	**SD**	**%RSD**
1	**0.5**	73364	52360	62862	14852.07	19.88
2	**1**	116523	123949	120236	5250.97	3.62
3	**5**	649031	316234	482632,5	235323.02	48.96
4	**10**	1467661	2520778	1994219,5	744666.17	38.84
5	**30**	4429106	4079225	4254165,5	247403.23	6.45
6	**50**	10114695	6872123	8493409	2292844.65	29.48
7	**120**	17909623	17261007	17585315	458640.77	2.78

Table 3. Peak areas obtained for sulfadoxine standard in the system linearity assessment using a methanol matrix.

Sulfadoxine (SDX) - System linearity						
Level	**[ ] (µg/mL)**	**Area-1**	**Area-2**	**Media**	**SD**	**%RSD**
1	**0.5**	86853	62540	74696,5	17191.89	23.02
2	**1**	129977	160046	145011,5	21261.99	14.67
3	**5**	579577	381794	480685,5	139853.70	29.09
4	**10**	1334516	2500208	1917362	824268.72	42.99
5	**30**	3819439	3855412	3837425,5	25436.75	0.66
6	**50**	8956182	6596526	7776354	25436.75	0.32
7	**120**	16772189	16266813	16519501	357354.79	2.16

3.3. Variability analysis in serum matrix

Several calibration curves were prepared in duplicate, and the obtained peak areas were used to calculate the mean, standard deviation, and relative standard deviation percentage (%RSD), as recorded in **Table 3** and **Table 4** for the calibration curve in the serum matrix.

Table 3. Peak areas obtained for the sulfadiazine standard in calibration curves using a serum matrix.

Sulfadiazine (SDZ)						
Level	**[ ] (µg/mL)**	**Area-1**	**Area-2**	**Media**	**SD**	**%RSD**
1	0.2	85930	55656	70793	21406.95	30.24
2	0.5	105626	97568	101597	5697.87	5.61
3	1	247518	248254	247886	520.43	0.21
4	2	464886	461915	463400.5	2100.81	0.45
5	5	1270344	1271269	1270806.5	654.07	0.05
6	30	7572169	7578400	7575284.5	4405.98	0.06
7	50	13182226	13115713	13148969.5	47031.79	0.36

Table 4. Peak areas obtained for the sulfadoxine standard in calibration curves using a serum matrix.

Sulfadoxine (SDX)						
Level	**[ ] (µg/mL)**	**Area-1**	**Area-2**	**Media**	**SD**	**%RSD**
1	0.2	182999	89186	136092.8	66335.81	48.74
2	0.5	131734	140722	136227.9	6355.48	4.67
3	1	236565	237114	236839.5	388.20	0.16
4	2	451158	423493	437325.4	19562.11	4.47
5	5	913446	914157	913801.6	502.75	0.06
6	30	6601460	6615340	6608400.1	9814.64	0.15
7	50	11562666	11528196	11545430.6	24373.97	0.21

3.4. Method linearity

The RP-HPLC-DAD methodology implemented for the detection and quantification of sulfadiazine (SDZ) and sulfadoxine (SDX) proved to be appropriate under the established chromatographic conditions. A strong linear response was observed between the analytical signal in methanol (**Figure 2A** and **Figure 2B**) and the drug concentration in the serum matrix (**Figure 2C** and **Figure 2D**).

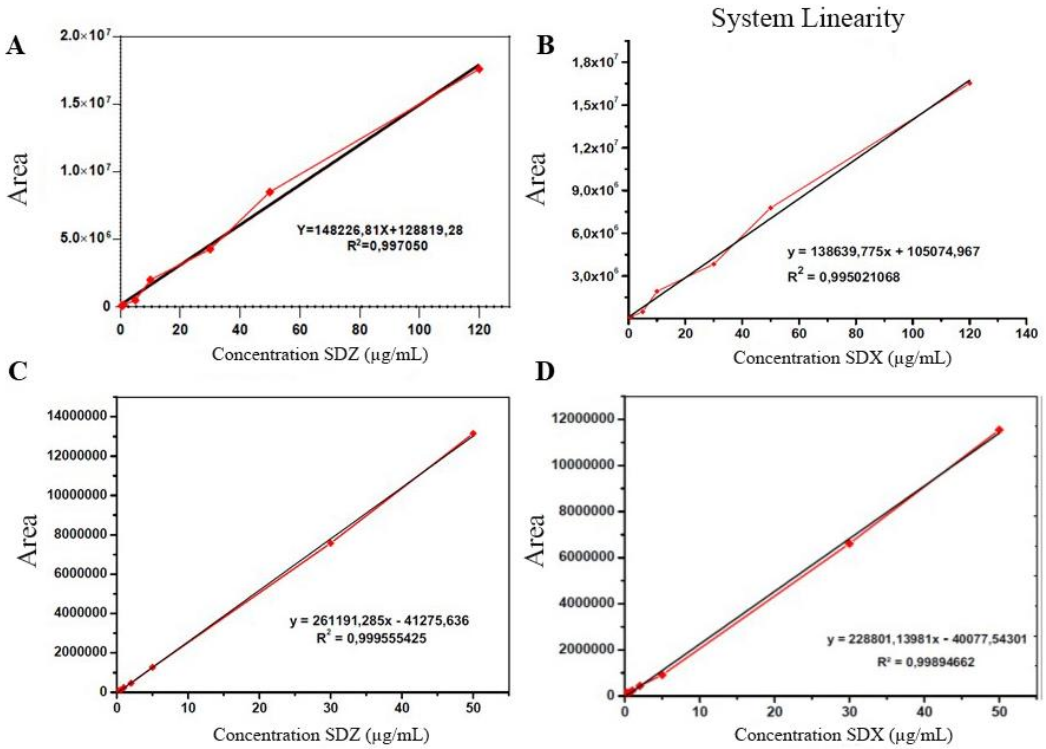


Figure 2. A. Calibration curve for sulfadiazine in methanol matrix. **B.** Calibration curve for sulfadoxine in methanol matrix. **C.** Calibration curve for sulfadiazine in serum matrix. **D.** Calibration curve for sulfadoxine in serum matrix.

For both matrices and both drugs (SDZ and SDX), correlation coefficients exceeded 0.99. The graphical analysis provided a visual evaluation of the lower limits established for the bioanalysis of the standard calibration curves, ensuring the method's linearity in the serum matrix.

Some data points in the system linearity curve (methanol matrix) presented slightly higher values. **Table 5** presents the calculated confidence limits for the correlation coefficients.

Table 5. Correlation coefficients, regression equations, and confidence limits for the studied drugs.

Drug	Correlation coefficient (r)	Confidence limits		Equation of the line $y = a + bx$
		Pending (b)	Ordered at the origin (a)	
Sulfadiazine (serum)	0.997	148226.81±3294.2	128819.28±117877.5	Y=128819.2+148226.8X
Sulfadiazine (methanol)	0.999	261191.29±6332.4	-41275.64±140180.2	Y = -41275.6+261191.2X
Sulfadoxine (serum)	0.997	138639.77±4385.8	105075.3±221947.5	Y = 105075.3+138639.7X
Sulfadoxine (methanol)	0.999	228801.14±8541.2	-40077.5±189076.6	Y = -40077.5+228801.1X

Additionally, **Table 6** summarizes the statistical significance test results performed on each calibration curve.

Table 6. Student's t-test results for the studied drugs and the method's linearity assessment.

Drug	r	r^2	t_{exp}	t_{tab}	H_o	Linear correlation
Sulfadiazine (serum)	0.997050	0.9941080	44.99626	2.57	≠ 0	Significant
Sulfadiazine (methanol)	0.999778	0.9995560	106.02686	2.57	≠ 0	Significant
Sulfadoxine (serum)	0.997507	0.9950211	48.97097	2.57	≠ 0	Significant
Sulfadoxine (methanol)	0.999473	0.9989462	68.859576	2.57	≠ 0	Significant

The statistical Student's t-test was performed for each drug using a 95% confidence level (α = 0.05) and considering seven concentration levels in both the method and system calibration curves. With n = 5 degrees of freedom, the tabulated t-values from the t-distribution table were 2.57 for all calibration curves. Based on the results presented in **Table 6**, a linear correlation was confirmed between the x and y variables for all analyzed calibration curves, both for the system and the method.

As a complementary analysis, homoscedasticity of the residuals was assessed to further verify the linear relationship between the variables in each calibration curve. The adjusted y-values were calculated from the selected regression equation, and residuals were determined as the difference between the adjusted y-value and the analytical signal (peak area). A Residuals vs. Concentration plot was then generated for the methanol calibration curves (**Table S1** and **Table S2**) and serum calibration curves (**Table S3** and **Table S4**). The homoscedasticity analysis confirmed that, for all studied drugs and both the system and method, the distribution of data points in the residual plots was random, without a discernible linear trend. Furthermore, the absence of a pattern in the sign of the residuals (±) supports the validity of the initially established linear model.

3.5. Precision

To estimate precision, an analysis of both instrumental repeatability and overall method reproducibility across the calibration range was conducted. Instrumental repeatability was assessed at three concentration levels by measuring two peak areas for both analytes (**Table 7**).

Table 7. Peak areas obtained at three concentration levels for sulfadiazine and sulfadoxine standards (instrumental repeatability).

Sulfadiazine (SDZ)						
Level	**[ ] μg/mL**	**Area 1**	**Area 2**	**Media**	**SD***	**%RSD****
2	0.5	105626	97568	101597	5697.87	5.61
4	2	464886	461915	463400.5	2100.81	0.45
7	50	13182226	13115713	13148969.5	47031.79	0.36
Sulfadiazine (SDX)						
Level	**[ ] μg/mL**	**Area 1**	**Area 2**	**Media**	**SD***	**%RSD****
2	0.5	131734	140722	136227.9	6355.48	4.67
4	2	451158	423493	437325.4	19562.11	4.47
7	50	11562666	11528196	11545430.6	24373.97	0.21

For SDZ and SDX, a greater dispersion of results was observed at the lowest concentration level. This is likely due to an increase in instrumental noise as the analyte concentration decreases, making it more challenging to fully resolve the chromatographic peaks. To evaluate method repeatability, recovery percentages (%R) were determined using a spiking approach with five concentration levels of standard solutions, analyzed on a single day under identical instrumental conditions in the serum matrix (**Table S5**).

For reproducibility assessment, Table S6 presents peak areas obtained over two different analysis days using real samples treated with Falcidar (PYR-SDX), focusing specifically on sulfadoxine. The samples were analyzed at unique concentration levels for each sample but on different days, covering a range from low to high along the calibration curve. The analyses were conducted 113 days apart, with a corrected injection volume of 20 μL. The acceptance criterion for the relative standard deviation (RSD) depends on the assay's purpose and the complexity of the biological matrix under study. For antibiotic analysis in serum, RSD values up to 15% are generally acceptable. However, for concentrations near the limit of quantification, RSD values of up to 20% may be considered acceptable.

3.6. Selectivity

A wavelength analysis was conducted to determine the optimal conditions for achieving the highest resolution and chromatographic peak distinction. It was found that at lower concentrations, better peak differentiation was obtained at 270 nm (**Figure 3**).

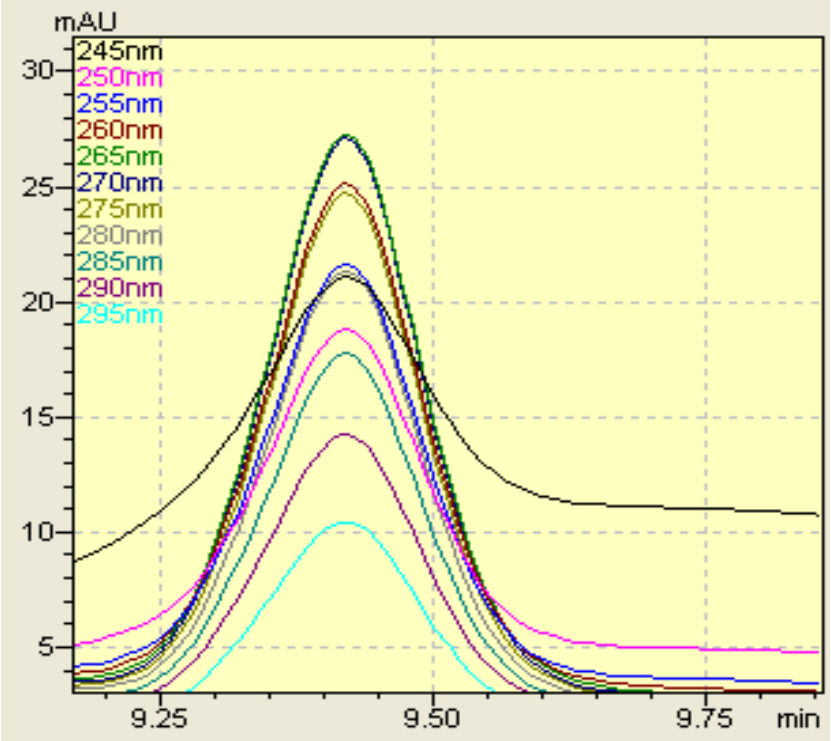


Figure 3. Chromatographic peaks obtained at different wavelengths (245–295 nm) for SDZ in a serum matrix using HPLC-DAD.

3.7. Identity confirmation: Selectivity/Specificity

To ensure that the chromatographic response was solely attributed to the analyzed compounds, all components involved in the sample processing and quantification were tested both together and separately. This included serum and plasma samples (negative controls) without the presence of the drugs (**Figure 4A** and **Figure 4B**). No chromatographic peaks were observed at the retention times of the target analytes, confirming the absence of interference in the method's measurements. In the chromatograms presented, the retention time (t_e) scale was maintained to allow direct comparisons between the observed drug signals.

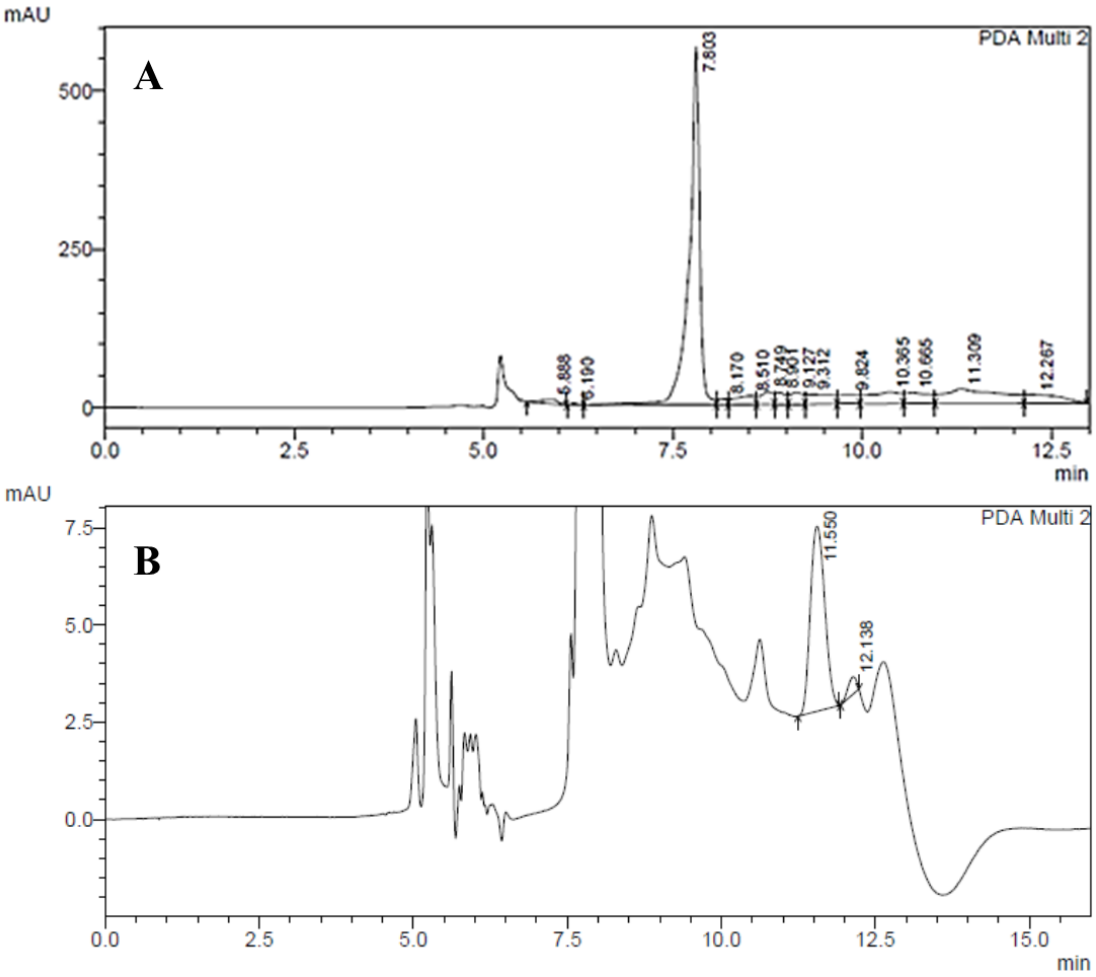


Figure 4. A. Chromatogram of undoped serum matrix. **B.** Chromatogram of undoped plasma matrix.

It is worth noting that during plasma preparation, a metabolite with a distinct peak at approximately 7.8 min may have been removed, leading to the observed chromatogram. However, this peak was not significant due to its signal intensity being comparable to the instrument's baseline noise. In the analyses of undoped matrices, no quantifiable signals were detected except for the peak at 7.8 min, which exhibited high resolution and chromatographic intensity. Nevertheless, this peak did not interfere with the drug signals, which were observed at approximately 9.4 min and 11.6 min for SDZ and SDX, respectively. To further investigate potential matrix effects, SDZ and SDX were spiked into serum (**Figure 5A**) and plasma (**Figure 5B**).

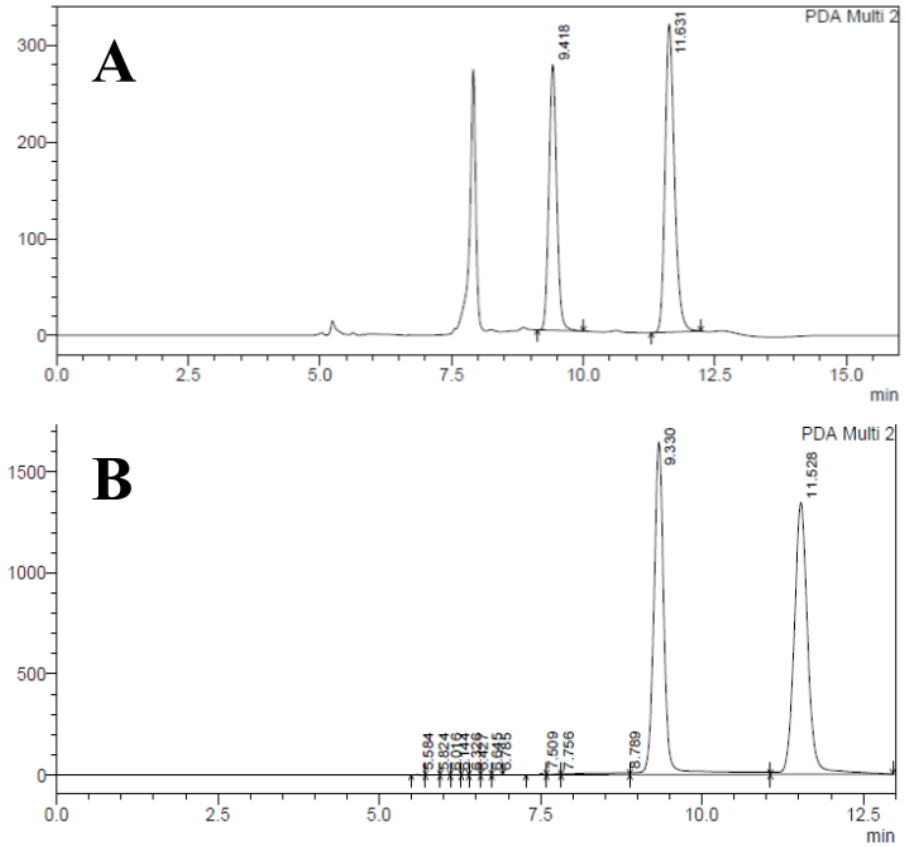


Figure 5. A. SDZ-SDX mixture in serum (130 μg/mL). **B.** SDZ-SDX mixture in plasma (50 μg/mL).

3.8. Accuracy

The statistical determination of accuracy was performed for five concentration levels within the calibration curve range for both antibiotics. A calibration curve was constructed to analyze the proportionality of recovered amounts at each level relative to the others, evaluate the recovery percentage (%R), determine the *t*-experimental value, and compare it to the *t*-tabulated value. A 95% confidence level ($p = 0.05$) was applied, considering five measurements without replicates, and the *F*-experimental value was calculated against the *F*-tabulated value (**Table S7** and **Table S8**).

This statistical procedure serves as an approximation for assessing method accuracy, as no replicates were performed for individual recovery percentage measurements. However, a proportional linear trend was observed in the amount of analyte recovered in each determination. This trend was confirmed by statistical estimators, including the *t*-Student and *F*-Snedecor tests. For both antibiotics, the *t*-Student test showed that a calculated *t* value greater than the tabulated *t* indicates method accuracy at a significance level of $\alpha = 0.05$. This confirms the presence of a statistically significant nonzero slope in the calibration curve.

3.9. Sensitivity

The analytical sensitivity was determined using the signal-to-noise ratio method, employing the instrumental signal provided by the blank sample. In this study, the blank corresponds to serum samples from individuals who have not received the treatment; therefore, no signal should be detected at the specific retention times for SDZ and SDX unless caused by an endogenous compound acting as an interfering signal. This possibility could be explained by the

broad range of the calibration curve. The estimated values for both parameters—Limit of Detection (LD) and Limit of Quantification (LQ)—are summarized in **Table 8**.

Table 8. Limits of detection and quantification for SDZ and SDX.

Drug	Average Area	Average Area	L.D (μg/mL)	L.Q (μg/mL)
Sulfadiazine	0.28409	1.13636	1.35883	4.52945
Sulfadoxine	0.32351	1.29420	2.09227	6.97424

4. DISCUSSION

Based on the obtained data and considering certain experimental limitations, the method was thoroughly evaluated in a biological matrix (blood serum), assessing key quality attributes such as linearity, precision, accuracy, selectivity, and sensitivity. The linearity assessment confirmed a direct correlation between the instrumental analytical signal and the concentration of the studied drugs within the established calibration range and under the applied chromatographic conditions. This clearly indicates that the results are directly proportional to the drug concentrations. Regarding precision, both instrumental and method precision were evaluated, yielding %RSD values within the acceptable ranges for bioanalytical studies. This suggests that the data are closely clustered around the mean value.

Although the recovery percentage was below 90%, considering the nature of the biological matrix, sample preparation and purification steps, dilutions, the actual analyte concentration in the original blood samples, and the analytical signal response, the method's performance is deemed satisfactory. Specifically, the average recovery was 77% for SDZ and 86% for SDX, with a %RSD of approximately 11.2%, which is below the 15% threshold established in the FDA guidelines for bioanalytical method validation in human studies. Based on this information, the analytical method can be considered reliable and sensitive for determining and monitoring SDZ and SDX levels within therapeutic concentrations.

In terms of reproducibility, the %RSD values were higher than those observed in repeatability studies but remained within the acceptance limit. Instrumental repeatability analysis showed %RSD values below 6%, indicating minimal dispersion in the peak areas obtained across different concentration levels in the calibration curves. This suggests a high level of system precision under the specified analytical conditions. The %RSD values for method repeatability were below 15%, although some were higher than those observed for instrumental repeatability. This discrepancy arises because method repeatability accounts for not only the instrument's precision but also all procedural steps involved in sample extraction and purification. The more steps involved, the greater the sample manipulation, leading to increased variability in the results due to potential analyte loss during the method's application. For reproducibility, variations in analysis time could increase %RSD due to potential fluctuations in the instrument's analytical response or signal stability from day to day, leading to data dispersion. However, all results remained below 15% RSD, demonstrating good reproducibility.

Precision is inherently associated with random errors in the determination process, which cause individual results to deviate from the mean value in an uncontrollable manner. Factors such as matrix complexity, analyte concentration, dilution steps, sample preparation, extraction procedures, instrument operating conditions, and analysis time can contribute to variability in the results. Nevertheless, all obtained values fall within the acceptance criteria for antibiotic analysis, indicating minimal variability among results and, therefore, good preci-

sion. Under the specified chromatographic conditions, the method exhibited adequate selectivity, as it allowed for the accurate and specific determination of the studied drugs without significant interference from the biological matrix components. Given the complexity of the serum matrix, interactions between the analytes and reagents used in sample preparation and purification likely played a role in the observed recovery rates. The presence of blood components such as lipids, salts, and hormones can compete with the target analytes for active sites in the stationary phase, reducing available binding sites and leading to analyte loss and lower instrumental responses.

From a clinical perspective, therapeutic drug levels could be considered an acceptance criterion, particularly at concentrations where a positive treatment response is expected. This is supported by Trenque *et al.*, who reported plasma SDX concentrations of 46.1 μg/mL within a broad range, although under different chromatographic conditions [11] (11). Another study reported a median of 42.39 μg/mL for malaria treatment. Given that malaria is also caused by a parasite (*Plasmodium falciparum*), a comparable therapeutic response might be inferred for toxoplasmosis, suggesting that the determined LD and LQ values are appropriate for the study's objectives. In conclusion, the method was successfully standardized and validated for the determination of sulfadiazine and sulfadoxine in serum, demonstrating its suitability for monitoring antibiotic levels in clinical samples.

5. CONCLUSIONS

Based on the obtained data, and considering some experimental limitations, as well as the particular characteristics of the biological matrix (blood serum), the relevant quality attributes of the methodology were thoroughly assessed, including linearity, precision, accuracy, selectivity, and sensitivity. The linearity assessment confirmed a correlation between the instrumental analytical signal and the concentration of the studied drugs within the established calibration range, under the applied chromatographic conditions. This clearly indicates that the results are directly proportional to the concentration of the drugs used.

Regarding precision, both instrumental and method precision were evaluated, with %RSD values within the acceptance range for bioanalytical studies, indicating that the data are closely clustered around the mean value. In the reproducibility study, the %RSD showed values higher than those observed in repeatability but remained below the acceptance limit. Although the recovery percentage was below 90%, considering the biological matrix type, sample preparation and purification processes, dilutions, the actual analyte concentration in the original blood samples, and the analytical signal generated by the analytes under the established chromatographic conditions, it can be concluded that the method developed in this research demonstrates satisfactory performance. The method shows reliability and sensitivity for determining and studying the levels of SDX and SDZ in therapeutic concentrations for treatment.

ACKNOWLEDMENTS

The authors would like to express their gratitude to the Master's program in Chemistry and Biomedicine at the University of Quindío. Additionally, they would like to thank the Chromatography and Related Techniques Research Group (GICTA) at the University of Caldas.

AUTHORS CONTRIBUTIONS

CRP: Development of the experimental part, manuscript draft preparation. **JPBA:** Proofreading, document translation, and validation. **JEGM:** Data evaluation, statistical application, and information confirmation. **GTO:** Project supervisor and manuscript evaluation.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

SUPPLEMENTARY INFORMATION

Table S1. Residual analysis (homoscedasticity) for sulfadoxine (methanol).

X	Y	Y original	Y adjusted
[ ] (µg/mL)	Residue	Area	Area
0.5	59830.68	74697	10962.62
1	12277.09	145012	89320.01
5	27970.25	480686	219915.65
10	-17706.43	1917362	481106.93
30	6125.61	3837426	1264680.79
50	-219178.51	7776354	7794462.91
120	130681.30	16519501	13018288.60

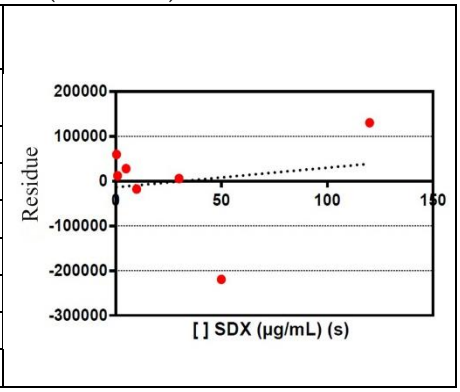


Table S2. Residual analysis (homoscedasticity) for sulfadoxine (serum).

X	Y	Y original	Y adjusted
[ ] (µg/mL)	Residue	Area	Area
0.2	130410.12	136092.8	5682.7
0.5	61904.87	136227.9	74323.0
1	48115.90	236839.5	188723.6
2	19800.66	437325.4	417524.7
5	-190126.6	913801.6	1103928.2
30	-215556.7	6608400.1	6823956.7
50	145451.6	11545431	11399979.4

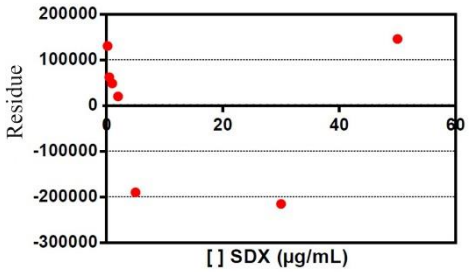


Table S3. Residual analysis (homoscedasticity) for sulfadiazine (methanol).

X	Y	Y original	Y adjusted
[ ] (µg/mL)	Residue	Area	Area
0.2	59830.68	70793.3	10962.62
0.5	12277.09	101597.1	89320.01
1	27970.25	247885.9	219915.65
2	-17706.43	463400.5	481106.93
5	6125.61	1270806.4	1264680.79
30	-219178.51	7575284.4	7794462.91
50	130681.30	13148969.9	13018288.60

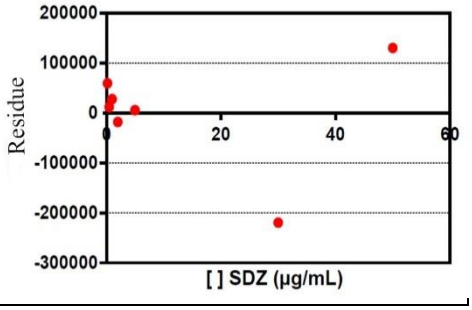


Table S4. Residual analysis (homoscedasticity) for sulfadiazine (serum).

X	Y	Y original	Y adjusted
[ ] (µg/mL)	Residue	Area	Area
0.5	-140070.69	62862	202932.69
1	-156810.10	120236	277046.10
5	-387320.84	482632.5	869953.34
10	383132.11	1994219.5	1611087.39
30	-321458.10	4254165.5	4575623.60
50	953249.18	8493409	7540159.82
120	-330721.56	17585315	17916036.56

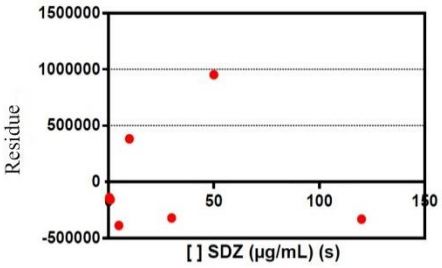


Table S5. Method repeatability after doping in matrix.

Drug	Serum		
	Media	SD*	%RSD**
Sulfadiazine	77.126	8.633309	11.19
Sulfadoxine	86.706	7.303717	8.423

*SD: Standard deviation

** %RSD: percentage relative standard deviation

Table S6. Areas obtained for the drug sulfadoxine, on two different days to assess reproducibility.

[ ] µg/L Calculated	Drug	Day 1	Day 2	%RSD	RT
		Area 1	Area 2		
12.253	M1-SDX	2750704	2763386	0.325	11.4540
18.965	M2-SDX	4263388.4	4299246	0.597	11.4235
30.655	M3-SDX	8195176.8	6973772	11.397	11.4325
33.925	M4-SDX	7460823.2	7721949	2.432	11.4340
39.862	M5-SDX	8902518.8	9080447	1.399	11.4520
42.777	M6-SDX	10667234.8	9747345	6.372	11.4295

Table S7. Recovery percentages obtained for sulfadoxine in the study for accuracy evaluation.

Sulfadoxine (SDX)		
[ ] µg/mL added	**[ ] µg/mL retrieved**	**(%R)**
2	1.940	97
5	3.817	76.36
10	8.620	86.23
20	17.404	87.02
50	44.695	86.92
	$\%\overline{R}$	86.71
	SD	7.303717
	%RSD	8.423
	t exp.	94.64213
	t tab.	3.18245
	F. exp.	8957.1329
	F. tab.	10.13

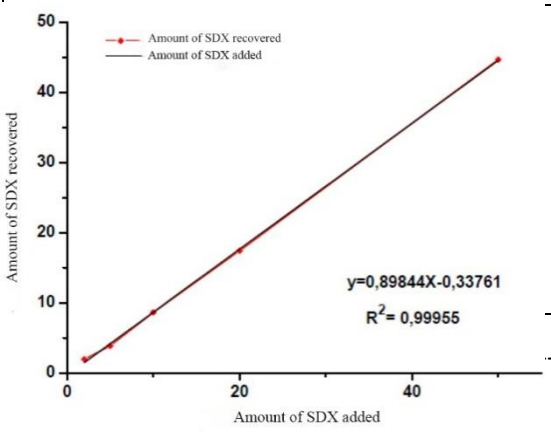
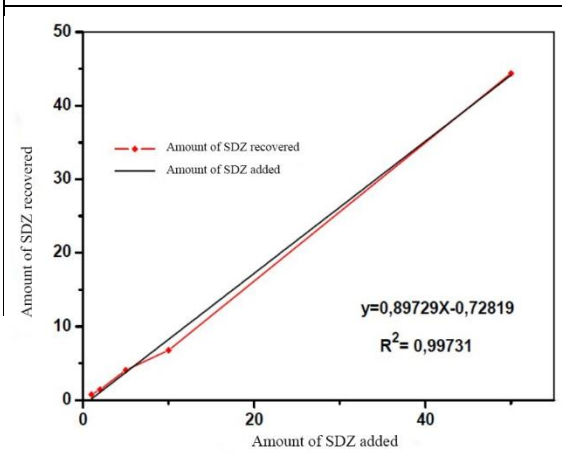


Table S8. Recovery percentages obtained for sulfadiazine in the study for accuracy evaluation.

Sulfadiazine (SDZ)		
[ ] µg/mL added	**[ ] µg/mL retrieved**	**(%R)**
1	0.759	75.9
2	1.411	70.57
5	4.055	81.09
10	6.775	67.75
50	44.375	88.75
	$\%\overline{R}$	77.126
	SD	8.628264
	%RSD	11.19
	t exp.	38.54768684
	t tab.	3.182
	F. exp.	1485.924
	F. tab.	10.13

REFERENCES

1. J. Espinoza-Rojas, E. López-Mora, J. Dabanch-Peña & R. Cruz-Choappa. Recomendaciones para el diagnóstico y tratamiento de la infección por *Toxoplasma gondii*. *Revista Chilena de Infectología*, **39**(2), 132–137 (2022). https://doi.org/10.4067/s0716-10182022000200132
2. J.E. Gómez-Marin, A. de-la-Torre, E. Angel-Muller, J. Rubio, J. Arenas, E. Osorio, *et al.* First Colombian multicentric newborn screening for congenital toxoplasmosis. *PLoS Neglected Tropical Diseases*, **5**(5), e1195 (2011). https://doi.org/10.1371/journal.pntd.0001195
3. G. Pedraza-López, P.A. Otálora-Vargas & L.A. Vargas-Torres. Toxoplasmosis congénita en primera infancia y toxoplasmosis en mujeres en edad reproductiva en Colombia. *Salud y Sociedad UPTC*, **7**(2), 21–35 (2021). https://doi.org/10.19053/uptc.2744953x.15515
4. R. McLeod, K. Boyer, T. Karrison, K. Kasza, C. Swisher, N. Roizen, *et al.* Outcome of treatment for congenital toxoplasmosis, 1981-2004: the national collaborative Chicago-based, congenital toxoplasmosis study. *Clinical Infection Diseases*, **42**(10), 1383–1394 (2006). https://doi.org/10.1086/501360
5. R. McLeod, F. Kieffer, M. Sautter, T. Hosten & H. Pelloux. Why prevent, diagnose and treat congenital toxoplasmosis? *Memórias do Instituto Oswaldo Cruz* (Rio de Janeiro), **104**(2), 320–344 (2009). https://doi.org/10.1590/s0074-02762009000200029
6. J.J. Berzas-Nevado, G. Castañeda-Peñalvo & F.J. Guzmán-Bernardo FJ. Simultaneous determination of sulfaquinoxaline, sulfamethazine and pyrimethamine by liquid chromatography. *Journal of Chromatography A*, **870**(1–2), 169–177 (2000). https://doi.org/10.1016/s0021-9673(99)00880-8
7. D.R. Schmidt, B. Hogh, O. Andersen, S.H. Hansen, K. Dalhoff & E. Petersen. Treatment of infants with congenital toxoplasmosis: tolerability and plasma concentrations of sulfadiazine and pyrimethamine. *European Journal of Pediatrics*, **165**(1), 19–25 (2006). https://doi.org/10.1007/s00431-005-1665-4

8. FDA. *Bioanalytical Method Validation: Guidance for Industry*. Office of Communications, Division of Drug Information Center for Drug Evaluation and Research, Food and Drug Administration. Silver Spring, (MD), 2018; 44 p. URL: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf
9. J.K. Johannessen, I. Christiansen, D.R. Schmidt, E. Petersen & S.H. Hansen. Simultaneous determination of pyrimethamine, sulfadiazine and *N*-acetyl-sulfadiazine in plasma for monitoring infants in treatment of congenital toxoplasmosis. *Journal of Pharmaceutical and Biomedical Analysis*, **36**(5), 1093–1098 (2005). https://doi.org/10.1016/j.jpba.2004.09.001
10. S. Corvaisier, B. Charpiat, C. Mounier, M. Wallon, G. Leboucher, M. Al-Kurdi, J.-F. Chaulet & F. Peyron. Population pharmacokinetics of pyrimethamine and sulfadoxine in children treated for congenital toxoplasmosis. *Antimicrobial Agents and Chemotherapy*, **48**(10), 3794–3800 (2004). https://doi.org/10.1128/aac.48.10.3794-3800.2004
11. T. Trenque, N. Simon, I. Villena, C. Chemla, C. Quereux, B. Leroux, *et al.* Population pharmacokinetics of pyrimethamine and sulfadoxine in children with congenital toxoplasmosis. *British Journal of Clinical Pharmacology*, **57**(6), 735–741 (2004). https://doi.org/10.1111/j.1365-2125.2004.02077.x
12. M.M. Ghanem & S.A. Abu-Lafi. Development and validation of a stability-indicating HPLC method for the simultaneous determination of sulfadiazine sodium and Trimethoprim in injectable solution formulation. *Scientia Pharmaceutica*, **81**(1), 167–182 (2013). https://doi.org/10.3797/scipharm.1210-12
13. J.Q. Sun, D.S. Sun & H. Chen. Simultaneous determination of sulfadiazine and sulfamethoxazole in synthetic samples and pharmaceutical tablets using HPLC-DAD combined with second-order calibration method. *Advanced Materials Research*, **554–556**, 1781–1784 (2012). https://doi.org/10.4028/www.scientific.net/amr.554-556.1781
14. I. García, M.C. Ortiz, L. Sarabia & J.M. Aldama. Validation of an analytical method to determine sulfamides in kidney by HPLC-DAD and PARAFAC2 with first-order derivative chromatograms. *Analytica Chimica Acta*, **587**(2), 222–234 (2007). https://doi.org/10.1016/j.aca.2007.01.054
15. K. Madej, Ł. Paprotny, D. Wianowska, J. Kasprzyk, M. Herman & W. Piekoszewski. A fully validated HPLC–UV method for determination of sulthiame in human serum/plasma samples. *Biomedical Chromatography*, **35**(3), e5002 (2021). https://doi.org/10.1002/bmc.5002

HOW TO CITE THIS ARTICLE

C. Rose-Parra, J.P. Betancourt-Arango, J.E. Gómez-Marín & G. Taborda-Ocampo. Application of high-performance liquid chromatography with diode-array detection (HPLC-DAD) for the determination of sulfadiazine and sulfadoxine in serum. *Rev. Colomb. Cienc. Quim. Farm.*, **55**(1), 68–87 (2026). https://doi.org/10.15446/rcciquifa.v55n1.119599