

# Development of a Method for Identification and Quantification of Sulfadiazine and Pyrimethamine in Serum of Congenital Toxoplasmosis Patients

Marson Maria Elena<sup>1,2,4\*</sup>, Fleitas Ulises<sup>1,2,4</sup>, Pérez Montilla Carlos<sup>3</sup>, Prospitti Anabela<sup>1,2</sup>, Altcheh Jaime<sup>3,4</sup>, Moroni Samanta<sup>3</sup>, Moscatelli Guillermo<sup>3,4</sup>, García Bournissen Facundo<sup>5</sup> and Mastrantonio Guido<sup>1,2,4</sup>



<sup>1</sup>Toxicology Area, Department of Biological Sciences, Faculty of Exact Sciences, National University of La Plata, Argentina

<sup>2</sup>UPL Laboratory, National University of La Plata / Scientific Research Commission of the Province of Buenos Aires, Argentina

<sup>3</sup>Multidisciplinary Institute for Pediatric Pathology Research (IMIPP-CONICET), Parasitology and Chagas Service, Children's Hospital "Dr. Ricardo Gutiérrez". Buenos Aires, Argentina

<sup>4</sup>National Council for Scientific and Technical Research (CONICET), Argentina

<sup>5</sup>Division of Pediatric Clinical Pharmacology, Department of Paediatrics, Schulich School of Medicine and Dentistry, Western University, Canada

\*Corresponding author: Marson Maria Elena, Toxicology Area, Department of Biological Sciences. Faculty of Exact Sciences. National University of La Plata. La Plata, Province of Buenos Aires, Argentina

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## ABSTRACT

Infection with *Toxoplasma gondii*, is one of the most widespread zoonoses in the world. Congenital toxoplasmosis (CT) is particularly risky due to its fetal complications. Global risk of CT transmission is approximately 40%, reaching 90% in the last month of pregnancy. Children with CT frequently require treatment, usually in Argentina with sulfadiazine (SDZ) and pyrimethamine (PYR), to prevent morbidity. Therapy for pediatric patients is hampered by the absence of pediatric formulations. To address this problem, SDZ and PYR are prepared as extemporaneous formulations by hospital pharmacies in the form of syrups. At the moment, serological concentrations of these formulations have not been corroborated in patient serum samples. The objective of this study was to develop a bioanalytical method for identification and simultaneous quantification of SDZ and PYR by high performance liquid chromatography (HPLC) with UV detection. The validated method was tested with residual serum samples obtained from 6 pediatric patients undergoing treatment with SDZ 42.20 a 93.70 mg/kg/day and PYR 0.77 a 2.70 mg/kg/day. Calibration curves were made for SDZ and PYR by spiking both drugs on drug-free serum samples. Pretreatment consisted of a deproteinization step with trichloroacetic acid followed by centrifugation and then injection of supernatant. Limit of detection (LOD) and quantification (LOQ) were (0.17 ±0.02 and 0.13 ±0.02) µg/mL and (0.46 ±0.01 and 0.36 ±0.01) µg/mL for SDZ and PYR respectively. The validated method had a linear range of (< LOQ - 210.00 ±0.02) µg/mL for SDZ and (< LOQ - 15.05 ±0.02) µg/mL. Serum samples range concentrations found were (<LOD - 162.04 ±0.02) µg/mL for SDZ and (< LOD - 7.30 ±0.03) µg/mL for PYR.

We developed a rapid, accurate, precise HPLC method for quantification of SDZ and PYR simultaneously, using the most commonly employed C-18 column with UV detection with sufficient sensitivity to be applied to therapeutic monitoring in pediatrics. It is the first report of dosages of serum concentrations of SDZ and PYR in pediatric samples carried out in public institutions in Argentina.

## Introduction

Infection with *Toxoplasma gondii*, is one of the most widespread zoonoses in the world. It is estimated that one third of the world's population is infected. The infection is acquired mainly by food contaminated with parasite cysts and is usually asymptomatic. The congenital infection is particularly risky, with rates of mother to baby transmission approaching 90% in the last month of pregnancy. There are few studies on the incidence of congenital toxoplasmosis (CT) in Latin America, but the seroprevalence in women of childbearing age is high [1]. Mostly the infection is asymptomatic, and the diagnosis is made by serological screening. Treatment during pregnancy decreased fetal morbidity and sequelae in the child [2]. An early diagnosis followed by treatment of CT in infants provides a better resolution of clinical signs compared to those not treated [3,4]. Between 10 and 30% of prenatal infections result in abortion, death of the newborn or severe clinical signs at birth [5,6]. However, about 67% of congenital infections are clinically asymptomatic at birth and may develop symptoms later, predominantly ocular lesions [3,6]. The current therapy in pediatric patients is protocolized, but due to the absence of pediatric formulations of the drugs, these are prepared in the hospital pharmacy in the form of syrup and at the moment, pharmacological parameters of these drugs have not been locally corroborated in this population of patients, especially for the combination of SDZ and PYR.

Drugs available for the treatment of toxoplasmosis only inhibit the growth of the parasite when it is in the active phase of its life cycle (tachyzoite), not being useful against the cystic or latent form of the parasite (bradyzoites). Most health centers do not hesitate to recommend treatment to infants with confirmed CT. However, to date, there is no controlled study in our country that determines its efficacy, the appropriate therapeutic dose and the optimal duration [6]. Indeed, there is a coincidence about the drugs to be used, but the duration of treatment has been more discussed. Prolonged treatments are associated with a lower rate of sequelae while short treatments have the advantage of reducing drug toxicity. The treatment scheme in Argentina is SDZ 50-100 mg / kg / d associated with PYR 1 mg/kg/d and folinic acid 5 mg / 48 hours. The duration is from diagnosis to one year of age with a minimum time of 6 months if the child is older. PYR (5-(4-Chlorophenyl)-6-ethyl-2,4-diaminopyrimidine) interferes with the synthesis of folic acid by inhibiting dihydropteroate synthase and dihydrofolate reductase and due to poorly studied pharmacological factors, treatment may not be successful. SDZ (N-amino-N-pyrimidin-2-yl-benzenesulfonamide) is the most active sulfamide against *T. gondii*. It has synergistic activity with PYR but being analogous to the PABA, necessary for the production of parasitic nucleic acids. It is excreted by the kidney, requiring dose adjustment in patients with renal impairment. It is not indicated in patients with glucose

deficiency 6-phosphate dehydrogenase (G6PDH) and replaced with clindamycin.

In other countries there are few studies in the pediatric population where PYR and SDZ on serum samples are quantified but they are framed in populations in which different drugs and combinations of drugs are used with different therapeutic and combination of drugs used [7-9]. A publication describing pharmacokinetic parameters for pediatric population treated several months for CT with PYR and sulfadoxine, proposes the existence of a wide interindividual variability and that at a dose adjusted to weight, plasma concentrations would be unpredictable. Therefore, it has not been possible to establish what plasma concentration in the combination of drugs is most effective in pediatrics. The relationship between therapeutic blood concentrations and toxicity is unknown and there are also no studies of interaction with new anticonvulsants or corticosteroids. On the other hand, the transfer information of these drugs through the placenta or breast milk is scarce. All these vacancies translate into important working hypotheses. Despite addressing drugs with a long time of use in therapeutics, there is no sufficient information in the literature in the pediatric field. To advance in any of the hypotheses, a simple, fast, precise and clinically adjusted method, such as the one presented in this work, is of great importance as a tool for systematization and improvement of the current pharmacological treatment protocols for this disease.

## Methods

A protocol designed to evaluate the response of a bioanalytical method for identification and simultaneous quantification of SDZ and PYR by high performance liquid chromatography (HPLC) with UV detection was followed. The aim is to validate the HPLC-UV method in order to transfer these capabilities to health institutions that perform therapeutic monitoring of these drugs. Instrumental techniques using HPLC-UV require equipment of medium complexity suitable for the monitoring of pharmacotherapy, available in hospitals and institutions of the public health system in Argentina.

### SDZ and PYR Stability Over Time

Accurate standard solutions of SDZ, PYR and a mix of both of them were evaluated at different concentration levels. Duplicate samples of these standard solutions were stored in batches in freezer and refrigerator respectively. After a defined period of time - between 24 hours and 30 days of storage - each solution was quantified. For every sample, its percentage coefficient of variation (CV %) intra-day (repeatability) and inter-day (reproducibility) was determined after its storage in refrigerator or freezer. Here, there was no pretreatment step needed for these samples, so as to

evaluate only the chromatographic system response and the stability of these standard solutions. An analogous treatment was performed for spiked serum samples at accurate and known concentrations of SDZ, PYR and a mix of both. Stability concentration after storage in the freezer between 24 hours and 30 days was also evaluated for these samples. Here, a pretreatment was needed to extract SDZ and PYR from the serum matrix. Percentage of recovery (R%) was calculated for all stored samples and for a fresh spiked serum sample with an accurate concentration as one of those stored, in every day of analysis [10]. Finally, another stability evaluation was performed in 4 serum samples after 3 cycles of defreeze / freeze at an accurate and known concentrations of SDZ, PYR and a mix of both, followed by extraction and quantification, according to protocols proposed by the National Administration of Medicines, Food and Medical Technology (ANMAT) for the stability of a bioanalytical method [11]. It was proposed to take as a stable criterion those samples that presented a CV %  $\leq$  15.0 % in all instances.

## Materials and Reagents

Trichloroacetic acid (TCA) pro analysis grade was purchased from Biopack (Buenos Aires, Argentina). Dimethyl sulfoxide (DMSO) pro analysis grade was obtained from Anedra (Buenos Aires, Argentina). Chromatographic grade demineralized water (<0.2  $\mu$ siemens) was obtained in our laboratory with ionic interchange resins. Acetonitrile (AcN) and Methanol (Me) (J.T. Baker, USA) HPLC-grade were used. Sulfadiazine (Stanton L\_1205050015269/0088) was obtained from the hospital pharmacy and pyrimethamine was obtained from sigma Aldrich. A Tabletop centrifuge (MRC, Scientific

## Standard Solutions

**Table 1:** Drug Standard Solutions evaluated in aqueous and serum matrix. SDZ St: Sulfadiazine Standard Solution; SDZ St dil: dilution 1/10 of Sulfadiazine Standard Solution; PYR St: Pyrimethamine Standard Solution; PYR St dil: dilution 1/10 of Pyrimethamine Standard Solution; HC SDZ: High Sulfadiazine Concentration on serum; HC SDZ + PYR: High Sulfadiazine Concentration with pyrimethamine on serum; LC SDZ: Low Sulfadiazine Concentration on serum; LC SDZ + PYR: Low Sulfadiazine Concentration with pyrimethamine on serum. HC PYR: High Pyrimethamine Concentration on serum; HC PYR + SDZ: High Pyrimethamine Concentration with sulfadiazine on serum; LC PYR: Low Pyrimethamine Concentration on serum; LC PYR + SDZ: Low Pyrimethamine Concentration with Sulfadiazine on serum.

Standard	SDZ ( $\mu$ g/mL)	PYR ( $\mu$ g/mL)	Standard	SDZ ( $\mu$ g/mL)	PYR ( $\mu$ g/mL)
SDZ St1	244.00	-	HC SDZ	154.57	-
SDZ St dil 1	24.40	-	HC SDZ + PYR	154.57	8.08
SDZ St2	240.00	-	LC SDZ	9.76	-
SDZ St dil 2	24.00	-	LC SDZ + PYR	9.76	8.08
SDZ St3	1189.0	-	HC PYR	80.80	-
SDZ St dil 3	118.90	-	HC PYR + SDZ	80.80	24.00
PYR St1	-	404.00	LC PYR	10.10	-
PYR St dil 1	-	40.40	LC PYR + SDZ	10.10	24.00

Standard solutions of SDZ and PYR were prepared separately. For SDZ standard solution, 0.0507g were dissolved in 10 mL of DMSO. To complete dissolution it was accurately diluted to 25.00 mL in a calibrated volumetric flask with a solvent mix of Me:water (50:50) to obtain a 2028.0  $\mu$ g/mL SDZ solution. For PYR standard solution, 0.0297 g were dissolved in 10 mL of AcN and then

Instruments, Argentina) and a rotary evaporator (Heidolph Laborota 4010) equipped with a ROTAVAP valve control equipment were used for the pretreatment procedures. A certified 0.1 mg analytical (Ohaus-Pionner, USA) was used in weighing operations. All micropipettes were calibrated before use. All HPLC solvents were degassed with a vacuum pump (Pascal, Buenos Aires, Argentina). An ultrasonic homogenizer (FAETA, Argentina) was also used on extraction procedures.

## HPLC Instrumentation and Calculation

The instrumental analytical procedure for samples measures were performed with an LC system consisting of an HPLC Merck-Hitachi LC-6200A and Merck-Hitachi UV/Vis L-4250 detector (Japan). Separations were carried out at room temperature using a C18 column 5  $\mu$ m, 100 mm  $\times$  4.6 mm I.D. Lichrospher-100 RP18 (Merck, USA). Samples were injected with a manual injector system with a 20  $\mu$ L sample loop. Peak areas were integrated automatically by Merck-Hitachi D-2500 Chromato-Integrator. All the calculations concerning the quantitative analysis were performed with an external standardization by the measurement of peak areas of a sample specimen series. Limits of detection (LOD) were established at 3.3 times of intercept coefficient standard error/slope coefficient ratio. Limits of quantization (LOQ) were established at nine times of intercept coefficient standard error/slope coefficient ratio. Accuracy and precision of the assays were calculated based on the analysis of three replicates for each level of the standard curve. Total uncertainty was calculated as the sum of accuracy and precision.

accurately diluted to 25.00 mL in a calibrated volumetric flask with the same solvent mix used for SDZ to obtain an 1189.0  $\mu$ g/mL PYR solution. These standard solutions and dilutions of them were used for stability studies (Table 1). Also, variable volumes of the described standard solutions of SDZ and PYR were added to drug-free serum to obtain matrix standards of 1,000  $\mu$ L volume

for calibration curves. These curves were made in triplicate for the lowest concentration point and duplicated for the rest of the points. Duplicated drug-free serum samples as a control specimen were included on calibration curves. Concentration points for both drugs analysed were (0.05; 0.56; 5.60; 70.50; 170.10; 240.66) µg/mL for SDZ and (0.03; 0.51; 0.91; 2.42; 7.07; 16.10) µg/mL for PYR (Table 2). In all cases the volume of the standard solution added to the serum matrix did not exceed 20% off its volume to minimize dilution effects. For control specimen a 20% serum sample volume of the solvent used for standard solutions was added.

**Table 2:** Concentration points used for calibration curves. CS: Control Specimen; St 1 to 6: Standard points at six concentration levels; a/b/c: indicates triplicate samples; a/b: indicates duplicate samples.

Mta	SDZ(µg/mL)	PYR(µg/mL)
CS a/b	-	-
St 1 a/b/c	0.05	0.03
St 2 a/b	0.56	0.51
St 3 a/b	5.60	0.91
St 4 a/b	70.50	2.42
St 5 a/b	170.10	7.07
St 6 a/b	240.66	16.10

### Serum Samples

Residual serum samples were obtained from six pediatric patients treated for TC from a clinical study with PYR (0.77 a 2.70 mg/kg/day), aged between 33 days and 3-year-old. Samples were stored at -20 °C until analysis. The clinical study and its informed consent for the use of the samples were approved by the institutional ethics committee of the Ricardo Gutiérrez Children's Hospital (HNRG).

### Sample Pretreatment and Chromatographic Conditions

All 1,000µL samples were deproteinized with 50 µL of TCA (30% p/v), vortexed for one minute, and sonicated for five minutes. The mixture was then centrifuged at 8000 g for another five 5 min. After this, 300µL of the supernatant were separated on an appendorf before injecting it into the HPLC system. The HPLC

analysis was performed by a gradient elution in a reverse phase (RP) mode. The mobile phase composition varied from 90 to 50% of water (1% v/v of formic acid) with methanol from 5 to 45% and 5% of acetonitrile that remained constant throughout the run. The flow ranged was 0.8 to 1.0 mL/min and the total running time was fourteen minutes. All solvents were filtered through a 0.45µm nylon membrane and degassed before use. The maximum UV absorption found for simultaneous identification of SDZ and PYR was at 273 nm, so this wavelength was chosen for the method. A value of 0.030 absorbance units (a.u.) threshold was used. Duplicate injections were made for all samples to test reproducibility of the detector response at each concentration level. Peak area was plotted against concentration to obtain calibration graphs. Linear regression and an analysis of variance (ANOVA) were applied to calculate calibration equation and statistical correlation coefficients.

### Results

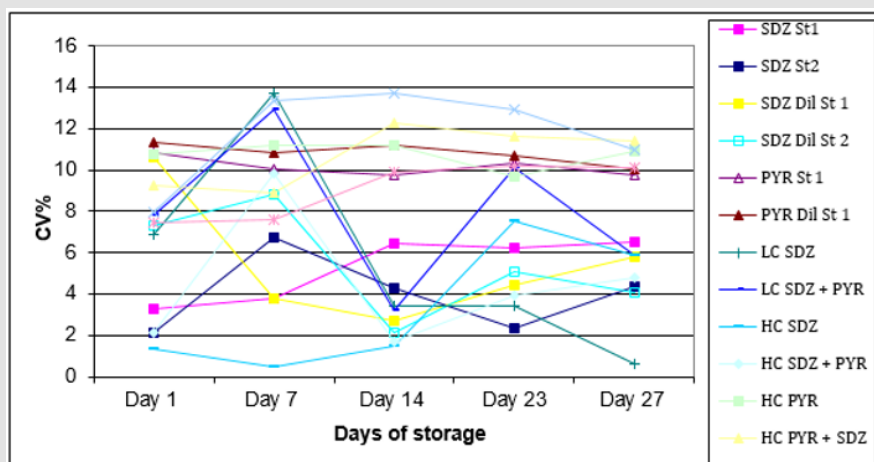
Table 3 and Figure 1 shows intraday CV % (repeatability), between duplicates of standard solutions in refrigerator and freezer after each storage time. The last two columns of the table present the CV% inter-day (reproducibility) for each standard in solvent or in serum for storage in refrigerator or freezer respectively. Higher concentrated standard solutions presented lower CV% than those diluted. Although the reproducibility in no case exceeds the maximum CV% proposed as acceptable, it was found that the standards stored in the refrigerator within the period evaluated, have lower CV% than those stored in the freezer. This variation may be due to factors such as the decrease in the solubility of drugs at low temperatures associated with some systematic error in the homogenization prior to injection into the chromatographic equipment. No evaluation on serum was made for sample storage in the refrigerator because it is well known that serum samples refrigerator storage is not recommended for periods longer than 24 hours. For this reason and not to add an extra instability factor, only its behavior stored in freezer was studied. For these serum samples, there were no significant differences in CV% or R% between high or low concentrations of both SDZ and PYR. Also, the presence of the two drugs together in the matrix did not cause analytical interference or significant increases in its CV% or %R.

**Table 3:** Sulfadiazine and Pyrimethamine Stability over time. Columns 2 to 5 shows intra-day (repeatability) percentage coefficient of variation (CV %) between duplicates in refrigerator and freezer after each storage time. The last two columns shows inter-day (reproducibility) CV% for storage in refrigerator or freezer respectively. Sample References were presented on Table 1.

Sample	Day 1 CV %	Day 7 CV %	Day 14 CV %	Day 23 CV %	Day 27 CV %	CV % (Fridge)	CV % (Freezer)
SDZ St1	3.31	3.80	6.44	6.20	6.50	3.08	10.9
SDZ St2	2.11	6.74	4.29	2.31	4.33	4.75	8.12
SDZ Dil St 1	10.60	3.80	2.67	4.42	5.78	9.02	7.90
SDZ Dil St 2	7.30	8.84	2.12	5.10	4.09	10.30	10.70
PYR St 1	10.82	10.01	9.73	10.32	9.78	10.73	10.69
PYR Dil St 1	11.32	10.80	11.16	10.68	10.04	10.90	11.25
LC SDZ	6.90	13.70	3.40	3.40	0.60	-	10.50



LC SDZ + PYR	7.80	12.90	3.20	10.1	5.90	-	9.70
HC SDZ	1.30	0.50	1.50	7.50	5.90	-	11.46
HC SDZ + PYR	2.10	9.80	1.70	3.90	4.80	-	10.68
HC PYR	10.75	11.20	11.20	9.70	10.90	-	11.40
HC PYR + SDZ	9.26	8.90	12.30	11.65	11.40	-	13.75
LC PYR	7.98	13.32	13.70	12.90	11.00	-	10.92
LC PYR + SDZ	7.45	7.56	9.89	10.20	10.10	-	11.62



**Figure 1:** Intra-day CV % (repeatability) between duplicates of standard solutions in refrigerator and freezer after each storage time.

The CV % obtained for serum samples after 3 cycles of defrosting / frizzling are presented in Table 4. There, it can be observed that the CV% are slightly higher for PYR than for SDZ (average CV% of 12.88 for the SDZ and 14.03 for the PYR) but they do not exceed the values proposed as acceptable in any case. Calibration curves for simultaneous detection and quantification of SDZ and PYR were made on three different days with six concentration levels. Retention time range for gradient mode used on chromatographic analysis was (5.01-5.36) minutes for SDZ and (12.16-12.60) minutes for PYR. Also, SDZ and PYR retention time ratio had a range of 0.41- 0.43 and a total run time was 14 minutes per sample. The LOD and LOQ were (0.17 ±0.02 and 0.13 ±0.02) µg/mL and (0.46 ±0.01 and 0.36 ±0.01) µg/mL for SDZ and PYR respectively. The

validated method had a linear range of (< LOQ - 210.00 ±0.02) µg/mL for SDZ and (< LOQ - 15.05±0.02) µg/mL for PYR. A summary of calibration curve statistical parameters is presented on Table 5. Chromatographic parameters such as resolution, selectivity, and peak asymmetry were satisfactory for SDZ and PYR determination with this method. In addition, analytes showed no decomposition products detectable in chromatograms profiles. Figure 2 presents a characteristic chromatogram profile for the proposed RP gradient chromatographic system for the detection and quantification of SDZ and PYR in

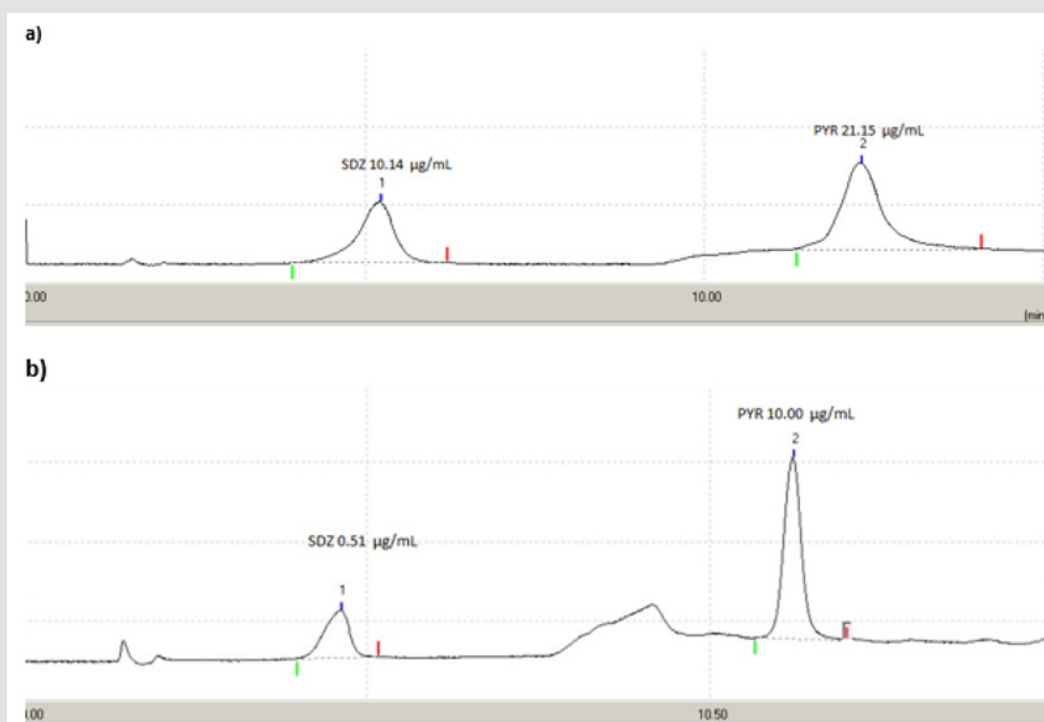
- a) standard solutions mix.
- b) serum extracts for SDZ and PYR.

**Table 4:** The CV% obtained for serum samples at different concentrations after 3 cycles of defrosting / frizzling are presented here.

Sample	CV % for SDZ	CV % for PYR	SDZ (µg/mL)	PYR (µg/mL)
Serum A	14.0	13.78	118.9	24.0
Serum B	13.30	12.84	10.1	24.0
Serum C	11.98	14.59	118.9	24.0
Serum D	12.24	14.90	10.1	24.0

**Table 5:** Statistical parameters found for simultaneous calibration curve for SDZ and PYR: LOD: Limit of detection; LOQ: Limit of quantification; R (%): Percentage of recovery; CV % interday: inter-day (reproducibility) percentage coefficient of variation; R2: R-squared value.

	SDZ	PYR	R <sup>2</sup>	Linear Range
LOD µg/mL	(0.13 ± 0.02)	(0.17 ± 0.02)	0.97797	(0.36 – 15.05)
LOQ µg/mL	(0.36 ± 0.01)	(0.46 ± 0.01)	0.97566	(0.46 – 210.00)
R%	89 ± 2	86 ± 4	-	-
CV % interday	3.1	4.4	-	-
accuracy %	97 ± 2	94 ± 2	-	-



**Figure 2:** Characteristic chromatogram for the validated method for SDZ and PYR quantification in  
a) standard solutions  
b) serum extracts for SDZ and PYR.

### SDZ And PYR on Pediatric Samples

With the validated method, SDZ and PYR were measured in a set of 30 samples from 6 pediatric patients who participated in a clinical study to evaluate the pharmacokinetics of both drugs on serum samples. The concentrations range found were (<LOD - 162.04 ± 0.02) µg/mL for SDZ and (< LOD - 7.30 ± 0.03) µg/mL for PYR. It should be noted that there is no average value informed for each drug measure. The reason is that samples were taken at different times post dose for use in pharmacokinetics so report an average value has no clinical meaning or bioanalytical interest here. In this sense, these measures have the purpose of validating the technique and its application in a real clinical context. The drug stability determinations, plus the parameters evaluated in the validation of the method as a whole, configure measures of the robustness of the method for use in clinical studies and therapeutic monitoring.

### Discussion

The two drugs analytically evaluated on this study SDZ and PYR, are currently available for the treatment of TC on public health Institutions in Argentina. As the fact that an appropriate pediatric formulation is not commercially available, administration of SDZ and PYR requires to be prepared in the hospital pharmacy in the form of syrup. Actually, serological concentrations of these formulations have not been corroborated in patient serum samples before. Until now, there were no validated methods by HPLC/UV developed for the simultaneous detection of both drugs. In this sense, it is important to remark that with this method it was possible to corroborate stability of these drugs on different matrices and its dependence on sample storage period between fridge and freezer. This proves that the robustness of the method is suitable for therapeutic monitoring, pharmacokinetics and toxicokinetics.

In literature, there is a developed method for determination of PYR, sulfadoxine, mefloquine, and ibuprofen by HPLC/UV for determination of these drugs in raw materials and dosage of pharmaceutical formulations but SDZ was not included [12]. Also, there are methods developed for SDZ and its hydroxy metabolite and its quantification by reverse phase HPLC [13], and there are others for PYR by HPLC and fluorescence detection but applied to the malaria pharmacotherapy [14] or for TC but with sulfadoxine instead of SDZ also by HPLC/UV.

There is an interest in the determination of clinically significant serum range of SDZ and PYR concentrations. To our knowledge, there are two different scenarios: adult serum concentration and pediatric serum concentrations. The LOQ of the method described here for both SDZ and PYR, seems to be appropriate in pediatric contexts. Most of the pediatric samples obtained were of 1.00 mL or less volume of serum. In this sense, minor sample volume may imply a decrease in the sensibility of the method, so there is a compromise between these two variables, also attending that blood samples volumes in pediatrics are normally smaller than in adults. It is important to note that no extra samples were taken for the evaluation of the method developed from the pediatric patients because residual volumes of serum were taken from an ongoing clinical study. The development of HPLC methods for determination of drugs has received considerable attention in recent years because of their importance in the quality control of drugs and drug products. The goal of this study was to develop a rapid, accurate, precise, and less time-consuming HPLC method for analysis of SDZ and PYR simultaneously, using the most commonly employed C-18 column with UV detection.

Instrumental techniques using HPLC-UV require equipment of medium complexity suitable for the monitoring of pharmacotherapy, available in hospitals and institutions of the public health system, giving its advantages over others reported HPLC methods for determination of some of these two drugs but through more expensive and sophisticated detection systems [15].

## Conclusion

A rapid, precise, accurate, low-cost, RP-HPLC-UV method for simultaneous identification and quantification of SDZ and PYR was developed, validated and tested its applicability on real samples. The results are accurate and precise, confirmed by the statistical parameters. The proposed method allows simultaneous determination of both drugs with sufficient sensitivity to be applied to therapeutic monitoring in pediatrics. It is the first report of dosages of serum concentrations of SDZ and PYR in pediatric samples carried out in public institutions in Argentina.

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## Compliance with Ethical Standards

The clinical study protocol and its informed consent for the use of human samples were approved by the institutional ethics committee of the Ricardo Gutiérrez Children's Hospital.

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Marson Maria Elena. Biomed J Sci & Tech Res



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