# Simultaneous Determination of Methotrexate and Sulfasalazine in Plasma Using an Agilent 1290 Infinity LC System

## Application Note

Pharmaceuticals


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### Abstract

This Application Note describes an HPLC/DAD method for the simultaneous determination of methotrexate (MTX) and sulfasalazine (SSZ) in plasma using an Agilent 1290 Infinity LC System. Using two different detector wavelengths, 304 nm for MTX and 358 nm for SSZ, we were able to selectively quantitate both analytes during the same chromatographic run. The method employed off-line SPE sample extraction. The chromatography was performed using an Agilent Poroshell Extend-C18 column 3.0 mm × 150 mm, 2.7 µm, and gradient elution using methanol (containing 10 mM ammonium acetate with 0.1 % formic acid) and water (containing 10 mM ammonium acetate with 0.1 % formic acid). The method has a Lower Limit of Quantification (LLOQ) of 0.02 ng/µL for MTX and 0.1 ng/µL for SSZ. The 15 minute method is linear for MTX and SSZ for a concentration range of the LLOQ to 100 ng/µL with a $R^2$ coefficient > 0.998. The method was evaluated, and the results are presented, using quality control samples for critical analytical performance criteria of recovery, stability, reproducibility, selectivity, accuracy, and precision.


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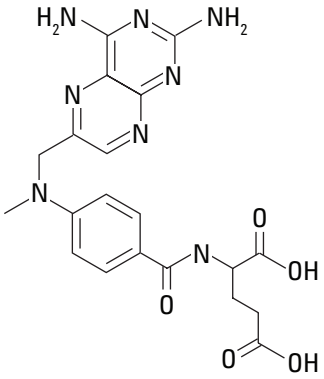
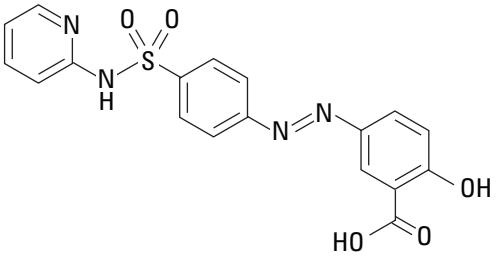
## Introduction

Approximately 99 % of rheumatologists[1,2,3] use MTX in combination with other nonbiological DMARDs such as sulfasalazine (SSZ, Figure 1). Additionally, both MTX and SSZ are highly protein bound (50 % for MTX, and 99 % for SSZ) and, thus, are likely to displace each other through interactions with plasma proteins[4,5]. Therefore, the simultaneous monitoring of plasma levels of MTX and SSZ used in combination may have relevance and aid further pharmacokinetic research into synergistic effects.

Given the sensitivities and dynamic range of detection typically needed from a bioanalytical method, it is without a doubt that an LC/MS is the instrument of choice for these analyses. However, a good chromatographic method up front of the mass spectrometer can have a big impact on the final analysis as well. This Application Note describes an HPLC/DAD method for the simultaneous determination of MTX and SSZ in plasma. We have also compared two sample preparation strategies of off-line solid phase extraction (SPE) as well as protein precipitation. Although the SPE approach allowed a more sensitive detection and a wider calibration range, protein precipitation is also discussed due to its simple and inexpensive nature. More details on this application are found elsewhere[6].



**MTX**
Formula $C_{20}H_{22}N_8O_5$
Mol. mass 454.44 g/mol



**SSZ**
Formula $C_{18}H_{14}N_4O_5S$
Mol. mass 398.394 g/mol

Figure 1. Structure of methotrexate (MTX) and sulfasalazine (SSZ).

## Experimental

### Chemicals and reagents

MTX, SSZ, methanol (Chromasolv grade), blank human plasma, ammonium hydroxide, formic acid, and ammonium acetate used in this study were obtained from Sigma-Aldrich India (Bangalore, Karnataka). Milli Q grade water was used throughout the work. All chemicals used were of analytical grade.

### Instrumentation

Chromatographic analysis was carried out using an HPLC-DAD system consisting of:

- Agilent 1290 Infinity Binary LC
- Agilent 1290 Infinity Binary Pump 4220A,
- Agilent 1290 Infinity Autosampler G4226A with an Agilent 1290 Infinity Thermostat G1330B
- Agilent 1290 Infinity Thermostatted Column Compartment G1316C
- Agilent 1290 Infinity Diode Array Detector G4212A with a 60-mm Max-Light flow cell (p/n G4212-60007)

### Software

Agilent ChemStation Openlab CDS Software (V. C.01.05).

### Calibration standards

Stock solutions of 2,000 ng/µL MTX and SSZ were prepared by dissolving each compound in methanol with ammonium hydroxide (100:0.1, v/v). A 200 ng/µL plasma stock solution of a combination of MTX and SSZ was prepared by spiking the appropriate volume of each stock solution into plasma. Serial dilutions from this plasma stock solution were made using blank plasma to achieve 11 concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 ng/µL. Aliquots of 200 µL of each of these plasma calibration standard solutions were extracted using protein precipitation or SPE, as described below, to prepare the calibration standards.

### Quality control samples

Three sets of quality control (QC) samples were prepared by spiking stock solutions of MTX and SSZ into plasma to yield 0.8, 8.0, and 80 ng/µL to be used as low, middle, and high QC samples respectively. QC samples were analyzed in five replicates each.

### Extraction from human plasma

Two different sample preparation techniques were evaluated for the extraction of each calibration standard from plasma, protein precipitation, and SPE.

For protein precipitation, a 0.4 M zinc sulphate in methanol (1:4) solution was used as a precipitating reagent in a 1:2 ratio. After adding the precipitating reagent and vortexing for 45 seconds, the mixture was centrifuged at 10,000 rpm for 5 minutes, and the resulting supernatant was used for LC analysis.

For SPE, 100 mg × 3 mL Agilent Bond Elut-C18 cartridges were used for the extraction of analytes from plasma. The SPE cartridges were preconditioned with 3 × 1 mL methanol containing 0.1 % ammonium hydroxide, followed by 3 mL of Milli Q water. Then, 200 µL of each plasma standard solution was loaded onto the SPE cartridge under gentle vacuum. The cartridges were then washed three times with 1 mL of mobile phase A under vacuum for 5 minutes to near dryness. The analytes were eluted from SPE cartridges with 2 × 0.5 mL of methanol containing 0.1 % ammonium hydroxide. Eluted fractions were concentrated to dryness using a concentrator (Eppendorf, Germany) and further reconstituted using 200 µL of 1:1 methanol and water containing 0.1 % ammonium hydroxide.

The details of the HPLC methodology are given in Table 1.

## Results and Discussion

### Optimization of plasma extraction

The analyte recovery for protein precipitation versus SPE was evaluated by comparing the area under the curve (AUC) of the analyte peaks from the plasma calibration standards with the AUC observed for standard solutions at a concentration of 12.5 ng/µL. As expected, analyte recovery proved significantly higher using SPE compared with protein precipitation. Additionally, a detailed inspection of the chromatographic data over baseline evidenced a better resolution of the analytes from matrix interferences using SPE (Figure 2). A closely eluting matrix peak (Rt = 3.57 minutes) just before MTX while using protein precipitation was found to be completely eliminated with SPE. Therefore, SPE allowed a higher recovery, better resolution, and thus, a more sensitive detection across a wider calibration range. However, due to its simple and inexpensive nature, protein precipitation may be used when the sensitivity requirement of the bioanalytical method is within the range observed here for protein precipitation.

Table 1. LC method parameters.

| Parameter | Value |
|---|---|
| Column | Agilent Poroshell 3.0 × 150 mm, 2.7 µm (p/n 693975-306) |
| Mobile phases | Solvent A) 10 mM $NH_4$ acetate + 0.1 % FA in water<br>Solvent B) 10 mM $NH_4$ acetate + 0.1 % FA in methanol |
| Gradient | 0 minutes, 10 % B<br>10 minutes, 95 % B<br>15 minutes, 95 % B<br>15.1 minutes, 10 % B<br>Post run time 5 minutes |
| Flow rate | 0.8 mL/min |
| Run time | 15 minutes |
| Column thermostat | 30 °C |
| Detection | DAD Signal 1) 304 nm (MTX)<br>DAD Signal 2) 358 nm (SSZ) |
| Injection volume | 5 µL |
| Needle wash | Methanol with 0.1 % $NH_4OH$ for 10 seconds |

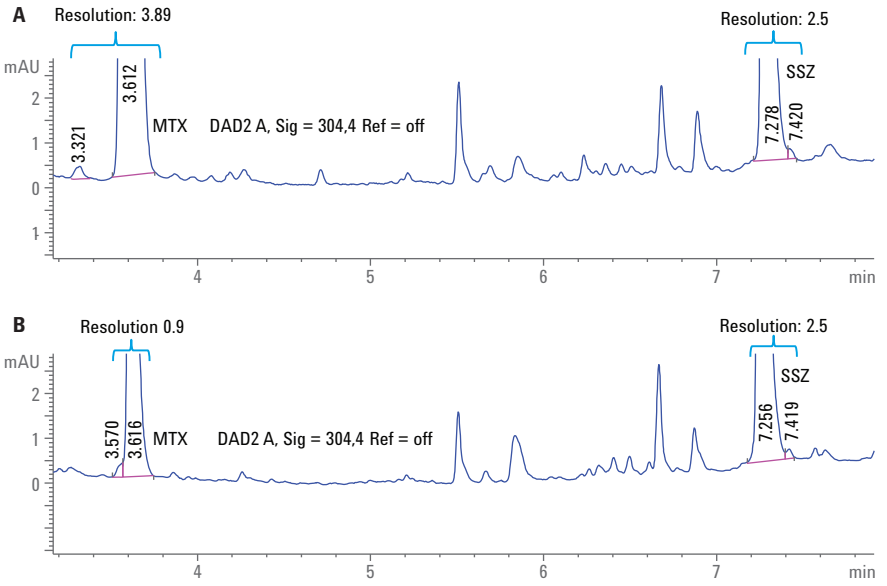



Figure 2. HPLC chromatogram of standard plasma solution, 12.5 ng/µL of MTX and SSZ using SPE (A) and protein precipitation (B) at 304 nm.

## Chromatographic method development

A C18 column was highly retentive for the analytes, and the best separation was achieved using a gradient of mobile phase B in mobile phase A, as described in the Materials and Methods section. The use of a low organic mobile phase concentration (10 %) at the beginning of the gradient resulted in good resolution of the analytes from the initial polar matrix background. Also, the late eluting analyte SSZ peak was well resolved from late eluting matrix interferences using a higher organic content (95 %) at the end of the gradient run. Baseline separation of the target analytes from each other, as well as from matrix peaks, was accomplished using these chromatographic conditions within a run time of 15 minutes (Figure 3).

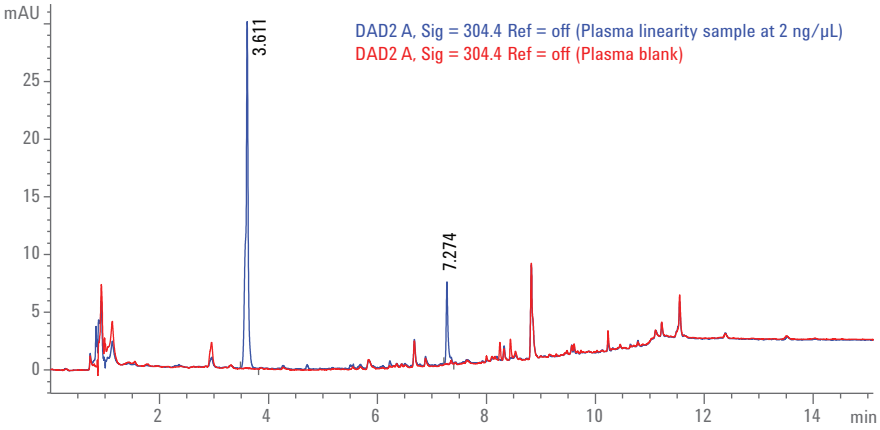



Figure 3. Elution profile of standard plasma solution of 2 ng/µL of MTX (Rt = 3.6 minutes) and SSZ (7.2 minutes) using SPE at 304 nm overlaid with blank plasma chromatogram.

## Method Evaluation

### Selectivity

In this study, the selectivity of the method for the two analytes, MTX and SSZ, during the chromatographic elution was examined across the elution window at LLOQ. The LLOQ was established as the lowest concentration on a calibration curve. At the LLOQ, the analyte response was at least five times higher compared to the blank response. Figures 4 and 5 show chromatograms of MTX and SSZ calibration standards at LLOQ respectively. Minor interferences were observed, but these did not impact peak integration and quantitation of analytes at LLOQ.

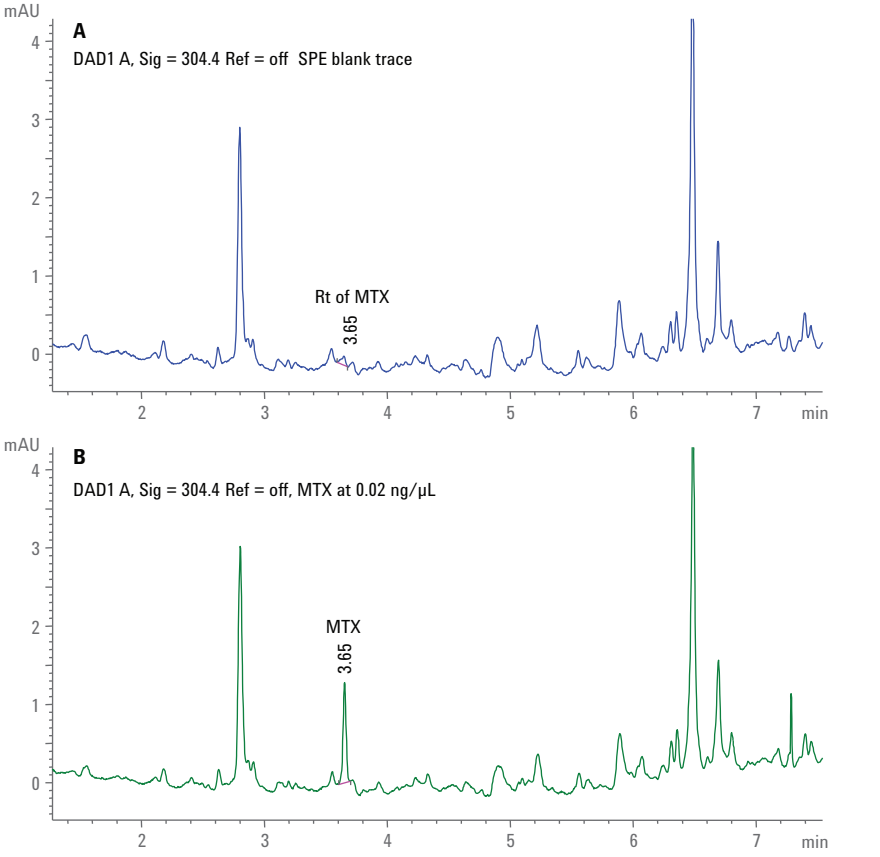



Figure 4. Elution profile of blank human plasma (A) and plasma spiked with MTX at LLOQ (B) extracted with SPE.

## Linearity study

A study of method linearity was performed by constructing a calibration curve consistent with the FDA draft guidance for bioanalytical method validation (2013) across several concentration levels including the LLOQ and Upper Limits of Quantification (ULOQ) in five replicates[7]. Calibration curves were constructed using peak area against concentration. It was observed that the area response was linearly and correctly regressed over a wide concentration range. The linear dynamic range for the current bioanalytical method is 0.02–100 ng/µL for MTX and 0.1–100 ng/µL for SSZ. The coefficient of correlation ($R^2$) was above 0.998 in each case. The calibration curves for both analytes are given in Figure 6. Observed accuracy values for each linearity level for MTX and SSZ are summarized in Figure 7. For MTX, the accuracy of the method at LLOQ was 85 %, while the accuracy of the method at LLOQ was 107.1 % for SSZ. Consistent with FDA guidelines, the observed precision standard deviation at LLOQ was below 20 %, and for all other concentration levels including ULOQ, the precision standard deviation observed was below 15 %.

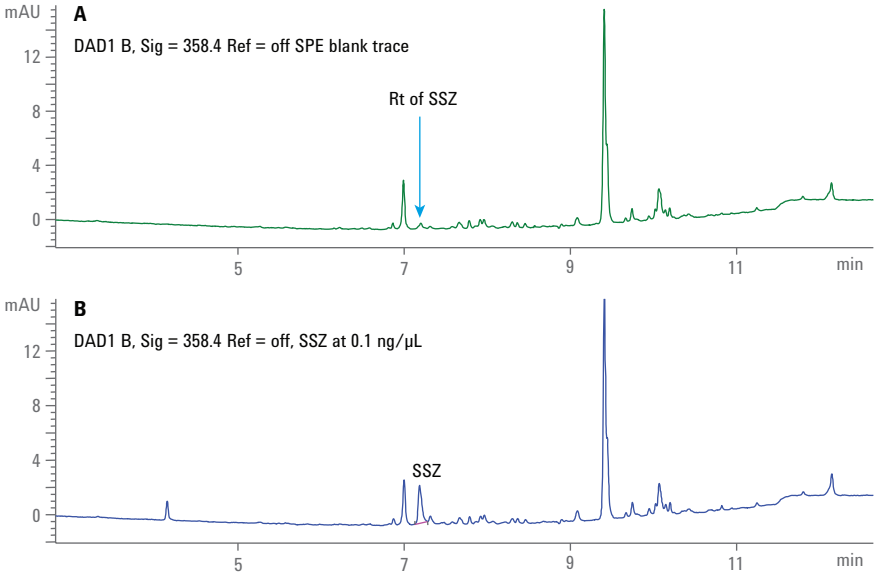



Figure 5. Elution profile of blank human plasma (A) and plasma spiked with SSZ at LLOQ (B) extracted with SPE.

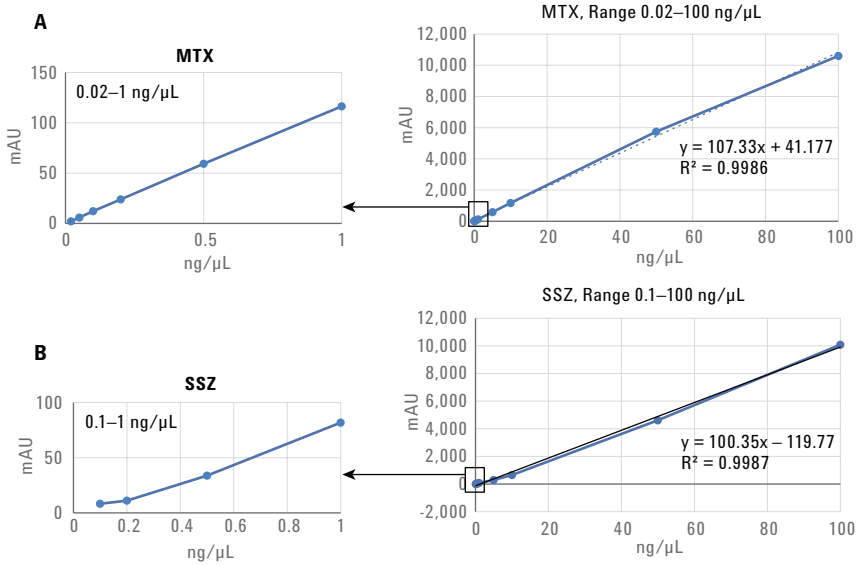



Figure 6. Linearity curves for MTX (A) and SSZ (B). Zoomed view for lower linearity levels are also shown.

## Limits of detection (LOD) and LLOQ

The LOD and LLOQ were determined at signal to noise (S/N) levels at or better than 3 and 10 respectively for both MTX as well as SSZ. Thus, the LOD and LLOQ for MTX were 0.01 and 0.02 ng/µL with S/N values of 6 and 10 respectively. While for SSZ, the LOD and LLOQ were 0.05 and 0.1 ng/µL with S/N values of 8 and 18. Figure 8 shows the typical chromatogram for MTX at LLOQ overlaid with blank traces in replicates. The chromatographic reproducibility at LLOQ was verified by replicate injections. The % CV of AUC and retention time at LLOQ were 0.02 % and 1.46 % for MTX and 0.01 % and 0.79 % for SSZ.

## Analyte recovery

Analyte recovery was measured consistent with FDA guidance. Thus, three sets of QCs for each analyte were analyzed in five replicates. The three sets of QCs were selected in such a way that, the concentration range covers the lower, mid and high region of the calibration curve. The analyte recoveries of MTX and SSZ were calculated using linearity equations of each analyte. These results were then compared with theoretical concentrations. The resultant mean recoveries and % CV are given in Table 2.

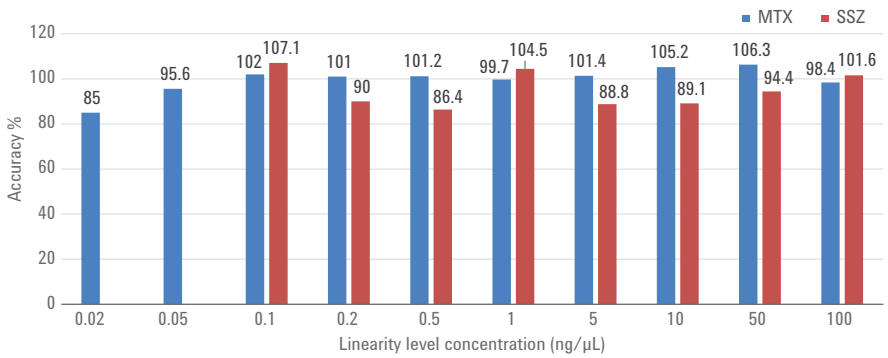



Figure 7. Accuracy values for each linearity level of MTX and SSZ.

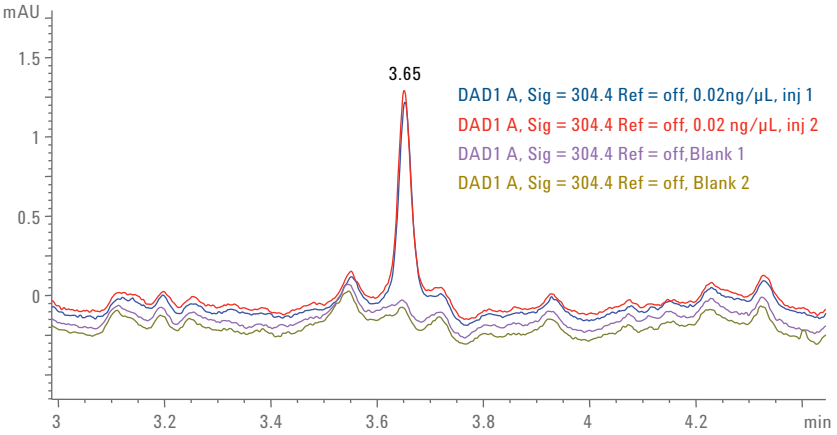



Figure 8. Chromatogram for MTX at LLOQ overlaid with blank traces in duplicates.

Table 2. QC sample results summarizing mean recoveries, accuracies, % CV of accuracies, retention time (RT) RSD, and AUC RSD.

| | | MTX (n = 5) | | | | | SSZ (n = 5) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| QC | Target (ng/µL) | Mean recovery (ng/µL) | Accuracy (%) | CV (%) | RT RSD (%) | AUC RSD (%) | Mean recovery (ng/µL) | Accuracy (%) | CV (%) | RT RSD (%) | AUC RSD (%) |
| Low QC | 0.8 | 0.70 | 87.7 | 1.02 | 0.03 | 0.26 | 0.82 | 102.2 | 0.86 | 0.09 | 0.34 |
| Middle QC | 8.0 | 8.1 | 101.1 | 0.26 | 0.03 | 0.25 | 7.5 | 94.2 | 0.28 | 0.02 | 0.41 |
| High QC | 80.0 | 75.5 | 94.3 | 0.29 | 0.03 | 0.29 | 78.1 | 97.6 | 0.29 | 0.02 | 0.29 |

### Method reproducibility

Reproducibility of the method was determined in accordance with the FDA draft guidance for bioanalytical method validation[11] by measuring the accuracy and precision of the method across three QC samples in the calibration range, using five replicates at each concentration. The results for intraday precision and accuracy in plasma quality control samples for MTX and SSZ are also summarized in Table 2. Calculated relative standard deviation for retention time and area was found to be within 0.1 and 0.4 % respectively. These results promise high reproducibility of the method.

## Conclusions

The simultaneous monitoring of plasma levels of MTX and SSZ may aid further pharmacokinetic research into any synergistic effects. This Application Note describes a simple, sensitive, and robust HPLC/DAD method for the simultaneous determination of MTX and SSZ in plasma using an Agilent 1290 Infinity LC System. The method is linear within the required concentration ranges of 0.02 to 100 ng/µL (0.04 to 220 µM) for MTX and 0.1 to100 ng/µL (0.25 to 251 µM) for SSZ with an $R^2$ coefficient > 0.998 for each. The method has an LLOQ of 0.02 ng/µL for MTX and an LLOQ of 0.1 ng/µL for SSZ. In addition, this method is more sensitive, allows a wider calibration range, and can be performed using a much smaller (200 µL) volume of plasma sample than previously reported methods for the bioanalysis of MTX. The method was evaluated using quality control samples for critical analytical performance criteria of recovery, stability, reproducibility, selectivity, accuracy, and precision.

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