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Validation of Protein Precipitation and Solid Phase Extraction Clean-Up Procedure for Simultaneous Determination of Trimethoprim and Sulfamethoxazole in Human Plasma by a High Performance Liquid Chromatography

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Abstract

The combination of sulfamethoxazole (SMX) and trimethoprim (TMP) with a ratio of 5:1 is widely used in treating outpatient diseases against various gram-positive and negative bacteria as well as mycobacteria, parasites, and fungi. Monitoring these compounds in plasma is challenging due to the coexistence of complicated matrices. This study aimed to develop and validate the high performance liquid chromatography (HPLC-DAD) method combined with liquid-liquid extraction followed by an additional clean-up for the simultaneous determination of TMP and SMX in human plasma. The plasma sample was precipitated using the crashing solvent 1% acid formic in acetonitrile and then impurities were removed by a C18 sorbent (m = 100 mg). Two analytes were separated on a Hypersil Gold C8 column (100 mm × 2.1 mm inner diameter; 3 µm particle size) under isocratic elution with 0.3% formic acid in water and methanol (80/20, volume/volume). A washing column with 100% MeOH was employed for 5 minutes after each injection to eliminate any potential impurities retained in the analytical column. The flow rate and the column temperature were constantly set up at 0.4 mL.min^{-1} and 40^{o}C respectively. The maximum absorbance wavelengths were set at 241 nm for TMP and 279 nm for SMX to achieve the highest selectivity and sensitivity. The method shows high recovery at 80.4% and 82.6% for TMP and SMX, respectively. The limit of quantification (LOQ) in plasma was 11.8 µg/L for TMP and 28.0 µg/L for SMX and intra- and inter-day precisions were less than 15% for both analytes. This validated method could be applied to pharmacokinetic studies in treatments.

Keywords: Human plasma, HPLC-DAD, liquid-liquid extraction, protein precipitation, cleanup.

1. Introduction

Sulfamethoxazole (SMX), which belongs to the sulfonamide group, is usually combined with trimethoprim (TMP) in a 5:1 ratio to treat common outpatient diseases such as prostatitis, acute exacerbations of chronic bronchitis, urinary tract infections, and acute otitis media. It is also effective for treating serious infections that occur in hospitalized patients, such as acute pyelonephritis, *pneumocystis carinii* pneumonia, and certain types of gram-negative meningitis [1]. Sulfamethoxazole (SMX) is a structural analog of p-aminobenzoic acid, a basic component in the production of dihydrofolic acid by bacteria, which is the initial step in the reaction chain that produces folic acid. SMX inhibits bacterial synthesis of dihydropteroate by blocking the incorporation of p-aminobenzoic acid into dihydrofolic acid [2]. In addition, TMP inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid by competitively binding dihydrofolate reductase which is the metabolically active cofactor for the synthesis of purines, thymidine, and DNA [3]. Therefore, the presence of TMP will enhance the efficiency of SMX (Table 1). In the combination form, SMX-TMP is therapeutically used for treating chronic urinary tract infections, pneumocystis jirovecii pneumonia, shigellosis, and otitis media [4].

Hence, it is important to develop an accurate, precise, and sensitive analytical method for the simultaneous determination of SMX and TMP serving for therapeutic monitoring. Various analytical techniques have been proposed for the simultaneous determination of SMX and TMP in human plasma including high-performance liquid chromatography combined mass in tandem [5, 6] micellar electrokinetic capillary chromatography [7], and spectrofluorometric [8].

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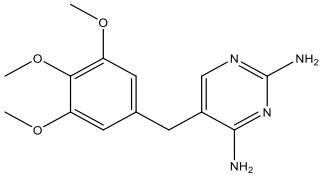
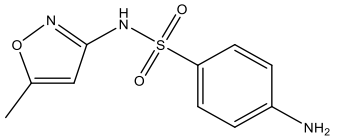
Table 1. Information on target pharmaceuticals

Analyte	Chemical Formula	pKa	log Kow	CAS number	Chemical Structure
Trimethoprim (TMP)	$C_{14}H_{18}N_4O_3$	7.2	0.79	738-70-5	
Sulfamethoxazole (SMX)	$C_{10}H_{11}N_3O_3S$	1.69, 5.57	3.10	723-46-6	

The high performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector is commonly used to ensure the sensitivity and specificity of the method [9, 10]. Liquid chromatography combined with a Fluorescence Detector (FLD) is not recommended to quantify TMP and SMX due to less sensitivity compared to variable wavelength UV- detectors [11].

In addition, an extraction and clean-up step could help to remove proteins and other impurities. This step will help to reduce background signals and increase the sensitivity and selectivity of the method. The most common extraction techniques have been proposed to extract and eliminate the impurities from plasma such as liquid-liquid extraction [4, 12, 13], and solid-liquid extraction [5, 14]. However, the precipitation step could not eliminate protein, lipids, and impurities. Therefore, this paper aims to develop and validate an accurate, and sensitive HPLC method combined with liquid-liquid extraction and an additional clean-up for the simultaneous determination of TMP and SMX in human plasma. The developed method is a promising technique that could be applied to pharmacokinetic studies in treatment.

2. Materials and Methods

2.1. Reagents

SMX and TMP were purchased from Sigma-Aldrich (Singapore). Concentrated formic acid (FA, 98%, analytical grade), methanol, and acetonitrile (HPLC grade) were purchased from Fisher Scientific. Supel™ QuE PSA/C18 (55283-U) was purchased from Sigma (Singapore), DisquE^{TM} (1200mg $MgSO_4$/400mg PSA), Hydrophilic-lipophilic Balance (HLB) was purchased from Waters (Ireland), and Bond Elut C18, Captiva EMR-lipid cartridge (100 mg, 1 mL) were purchased from Agilent Technologies (Santa Clara, CA, USA),). The ultrapure water (18.20 MΩ.cm) was produced by the Barnstead GenPure Water Purification Systems (Thermo, England) and it was used throughout this study.

2.2. Preparation of Standard Solution and Calibration Curve

Single stock standard solutions of both analytes (1000 mg/L) were prepared by dissolving an exact amount of each compound in MeOH. The single stock solution was stored in a -20 ^oC freezer. The mixture of working solution at 10 mg/L of TMP and 50 mg/L of SMX was prepared monthly by a mixture of TMP and SMX stock solution in MeOH. The mixture standard solution was kept at -20 until use. A series of calibration curves from 20, 50, 100, 200, 500, 1000, 2000, and 5000 µg/L of TMP was prepared daily in the mobile phase from the working solution in the section above. The concentration of SMX in the standard solutions was 5 times higher than the concentration of TMP. The mobile phase was prepared by dissolving 0.3% formic acid in ultrapure water and methanol (80/20, volume/volume). The mobile phase was filtered and degassed in an ultrasonic bath before being used to remove dissolved gas.

2.3. Instrumentation and Chromatography Conditions

The Vanquish™ Core HPLC system (Thermo Scientific, USA) was used for analysis, which includes: a degassing unit for eliminating dissolved gas in the mobile phase, a solvent selection valve, an automatic quaternary pump, an autosampler for the liquid sample and a column compartment for controlling the column temperatures, a UV-Vis diode array detector, a software for instrument control, data acquisition, and processing (Chromeleon version 7.2, Thermo Scientific, USA). A Hypersil GoldC8 column (100 mm in length × 2.1 mm inner diameter; 3µm particle size) was used for the chromatographic separation. The flow rate was constantly kept at 0.4 $mL.min^{-1}$. The column temperature was set continuously at 40^oC and the injected volume was

20 µL with an analysis time of 6.0 minutes. A primary experiment using an UV visible spectrum scan (UV 1800i, Shimadzu) was performed to determine the maximum absorbance wavelength for both compounds. The maximum absorbance wavelengths of TMP and SMX were 241 nm and 279 nm, respectively (Fig. 1).

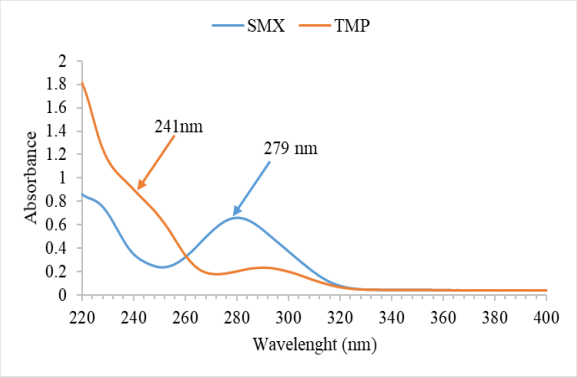


Fig. 1. The UV spectrum of SMX and TMP at 10 mg/L

2.4. Sample Preparation

A 200 µL plasma sample was transferred to a 2 mL microcentrifuge tube, then, 800 µL of crashing solvents was added and vortexed for 30s. The tube was sonicated for 5 minutes to ensure the complete precipitation of proteins, followed by centrifugation at 4200 rpm for 5 min. The upper layer was transferred to another tube containing cleanup sorbent, vortexed for 1 min, and centrifuged at 4200 rpm for 5 min. The extractant was then collected into a vial tube, evaporated under a gentle stream of nitrogen until dryness, and then reconstituted to 200 µL of H_2O/MeOH (80/20, v/v). Finally, the solution was filtered using a syringe filter (0.22 µm pore size, hydrophilic) and subjected to analysis by the HPLC-DAD method under optimized operating conditions.

2.5. Method Validation and Quality Control

To assess the method selectivity, blank plasma samples were processed following the protocol and compared with standard spiked plasma samples. The precision was assessed through repeatability (intra-day) and reproducibility (interday) based on the relative standard deviation (RSD) of the peak area. Five replicate quality control samples at low, medium, and high concentrations were investigated on the same day for intra-day precision, and on three separate days for inter-day precision. Blank plasma samples were spiked with standard solutions at three concentration levels and extracted as described above. The limit of determination (LOD) which is the lowest concentration of analyte in a sample is defined as the concentration of analyte that gives a signal-to-noise ratio of 3 (S/N = 3).

3. Result and Discussion

3.1. Chromatographic Conditions

A mixture of TMP and SMX standard solution was used for optimization of chromatographic separation. The separation of TMP and SMX was tested by using two different reversed-phase columns: C8-Hypersil Gold (100 mm in length x 2.1 mm inner diameter; 3 µm particle size) and C18-BDS Hypersil™ (100 mm in length x 2.1 mm inner diameter; 3 µm particle size), with a mobile phase composed of water and methanol (80/20, volume/volume). Both columns provided a good separation of TMP and SMX. However, the C8 column demonstrated a sharper peak for TMP when the mobile phase was acidified with formic acid. The addition of 0.3% FA converts both TMP and SMX to protonated forms which enhance the signal [15 - 17]. In this study, the C8 column was selected and the mobile phase in isocratic mode consisting of ultrapure water acidified by 0.3% FA and methanol was set up at isocratic mode at a ratio of 80/20 (volume/volume) to determine TMP and SMX. The flow rate and column temperature were constantly kept at 0.4 mL/min and 40 ºC, respectively. Fig. 2 shows that TMP and SMX were separated with retention times of 1.60 minutes for TMP and 2.82 minutes for SMX in such separation conditions.

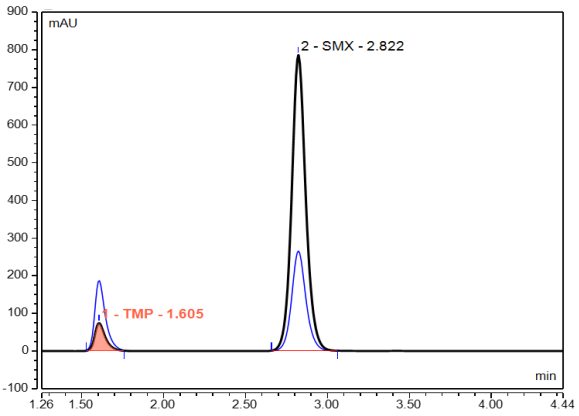


Fig. 2. The overlaid HPLC chromatogram of TMP and SMX at maximum absorbance wavelengths at 241 nm (light line) and 279 nm (bold line) with TMP and SMX concentrations of 1000 µg/L and 5000 µg/L respectively

3.2. Optimization of Extraction

3.2.1. Effect of crashing solvent

Protein precipitation is a technique commonly used in biological sample preparation. Proteins in the plasma sample are precipitated by adding solvents like ACN or MeOH, and the precipitate is subsequently separated by centrifugation [14], [18]. When organic solvents are added, the hydration layer of proteins is destroyed and the repulsion between protein molecules is decreased, which lowers the solubility of the

proteins and causes them to precipitate. Numerous investigations demonstrate the practicality of protein precipitation utilizing the organic solvent mixture ACN/MeOH. A tiny quantity of basic or acid is added to the crashing solvent to lessen protein binding. Three different crashing solvents were used in the first experiment to optimize the extraction procedures at the 200 µg/L ultimate spiking concentration. In Fig. 3, when precipitated with crashing solvents like ACN/MeOH (95/5) and ACN/MeOH (95/5) added 1% NH_4OH), the recovery of the two target analytes was lower than that of ACN added 1%FA. Therefore, plasma samples were precipitated by 1% FA in ACN as the crashing solvent.

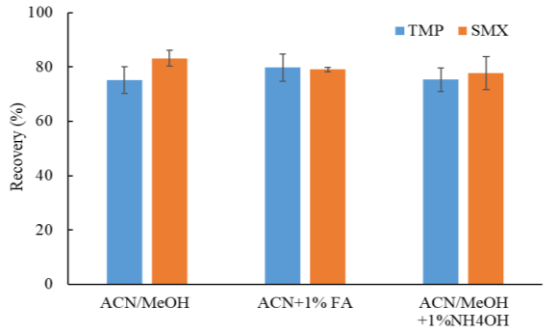


Fig. 3. Recoveries of analytes with different types of crashing solvent

3.2.2. Selection of sorbent for clean-up

Five types of sorbent at different weights were used to remove impurities after precipitating proteins step: 100 mg of EMR, 100 mg of C18, 100 mg of HLB, 100 mg of PSA, 100 mg of PSA/C18, 200 mg of PSA, and 200 mg of PSA/C18 (Fig. 4). The clean-up process was performed as described in section 2.4. The effect of sorbent on the recoveries of SMX and TMP was illustrated in Fig. 3. TMP shows a good recovery with most types of sorbent (higher than 70%) except for HLB sorbent with only (25%). In contrast, SMX showed a low recovery when PSA sorbent was used or at a higher amount of C18 sorbent (at 200 mg). The ERM sorbent as a clean-up phase showed average recoveries for both compounds (approximately 75%). The highest recovery was (82.6 ± 4.6)% for SMX and (80.4 ± 2.4)% for TMP, achieved with 100 mg of C18 sorbent, and these results comply with the Association of Official Analytical Chemists (AOAC) standard [19]. The hydrophobic properties of C18 sorbent play a role in absorbing non-polar molecules such as lipids and esters while the PSA sorbent with amine groups is commonly used to remove matrix samples containing carbohydrates, fatty acids, organic acids, phenols, sugars, and some water-soluble pigments by ion exchange mechanism [20]. When the mass of the C18 and PSA/C18 sorbents are increased, recovery of SMX is significantly decreased, indicating that a portion of SMX may be trapped in the sorbents. Consequently, following precipitation, the plasma sample was cleaned up using the C18 sorbent (100mg).

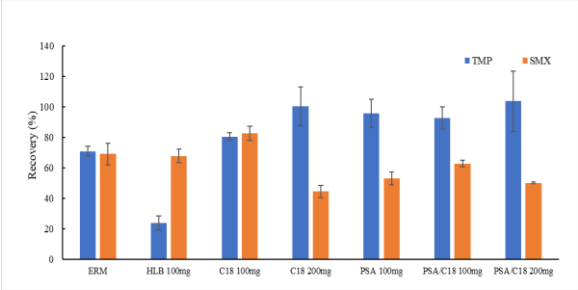


Fig. 4. Recoveries of TMP and SMX with different sorbent types used for sample clean-up

3.2.3. Matrix effect

The matrix effects are a common phenomenon because complicated matrix with high plasma levels can affect the sensitivity, selectivity, and recovery. To evaluate the impact of the matrix effect, a total of 15 pooled samples were split into three sets. The first set of pooled samples was extracted as described above (section 2.3) without spiking standards. The second set was spiked with standards before precipating with crashing solvent and the third set was performed as the first set but the standards were spiked after extraction and the clean-up step. These samples were spiked with the concentration of TMP and SMX in the final concentration *via* analysis at 1000 µg/L and 5000 µg/L, respectively. After processing, the samples were analyzed using the HPLC-DAD system under the same conditions in section 2.2. The matrix effect, extraction efficiency, and total recovery rates for both compounds were ranged of 86.2-90.3%, 83.3-86.4%, and 89.8-93.1%, respectively, as depicted in Fig. 5. Notably, the observed matrix effect is deemed acceptable, as it falls within the range of 80% to 120%, as reported in previous studies [21]. This underscores the negligible impact of the sample matrix on the detection signal of the target analytes after clean-up processes.

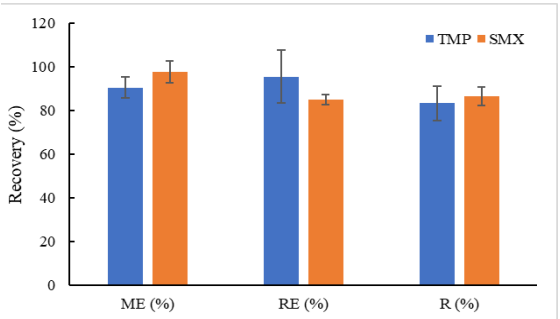


Fig. 5. Overall recovery (R), extraction efficiency (RE), and matrix effect (ME) of TMP and SMX

3.3. Validation of the Analytical Method

3.3.1. Selectivity

Fig. 6 shows the chromatogram of TMP and SMX in both blank plasma and spiked plasma samples. It is clear that neither of the two compounds was detected in the blank plasma sample (light line) and two peaks at 1.64 minutes for TMP and 2.81 minutes for SMX after extraction and clean-up with C18 sorbent. In addition, Fig. 6 shows the difference in the *S/N* before and after the addition of C18 sorbent. Without adding C18 sorbent (bold line), the background noise increased and reduced the recoveries of both compounds. By contrast, the background noise was significantly decreased when the samples were cleaned up with 100 mg of sorbent (light line). This shows that the C18 sorbent helped to remove well potential impurities retained in the sample after the precipitation process and enhance the method sensitivity.

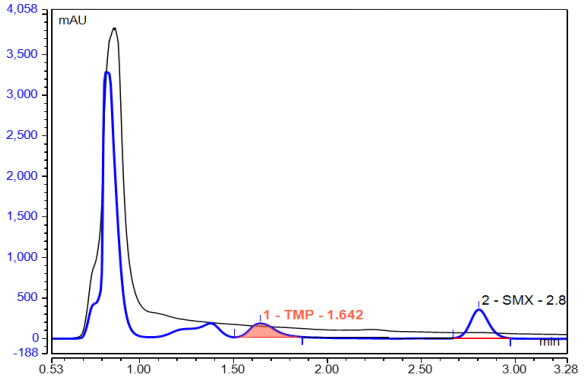


Fig. 6. The overlay HPLC chromatogram of a blank plasma (light line), and a spiked plasma (bold line) cleaned up by C18 sorbent at a concentration of 1000 µg/L for TMP and 5000 µg/L for SMX

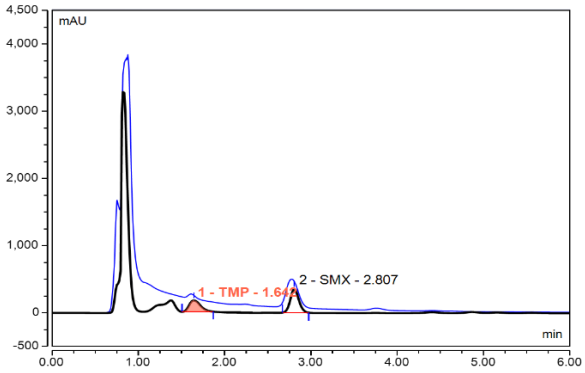


Fig. 7. The ovelay HPLC chromatogram of a spiked plasma without clean-up (light line), and a spiked plasma cleaned up by C18 sorbent (black line) at a concentration of 1000 µg/L for TMP and 5000 µg/L for SMX

3.3.2. Linearity

The calibration curve for TMP was established with eight independent solutions with concentrations from 20 to 5000 µg/L, whereas that of SMX was five times higher. The HPLC-DAD system was utilized to analyze standard/samples in triplicate. Other chromatographic separations are listed in section 2.2. The peak area of both analytes was integrated and used for quantification. The regression equation and correlation coefficient are listed in Table 2. As clearly shown in Table 2, an excellent correlation between peak area and concentration was achieved ($R^2 > 0.999$) for both analytes.

3.3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were estimated by the signal-to-noise ratio (*S/N*), in which *S* is peak height and *N* is baseline noise. The *S/N* was evaluated by injecting ten times the lowest concentration. LOD and LOQ were found to be 3.54 µg/L and 11.82 µg/L for TMP, 8.41 µg/L, and 28.03 µg/L for SMX, respectively (Table 2). Due to their low levels, the LOD and LOQ were intended for the quantification of pharmaceuticals in plasma samples. Quantitation limits were 10 µg/L for TMP and 50 µg/L for SMX respectively, and were estimated as proposed by E. Sayar [22].

Table 2: Analytical characteristic of the developed HPLC-DAD method for analysis of TMP and SMX

Analyte	**TMP**	**SMX**
Regression equation	$y = 0.0137x + 0.1266$	$y = 0.839x - 0.1977$
Correlation coefficient (R^2)	0.999	0.999
Linear range (µg/L)	20-5000	100-25000
LOD (µg/L)	3.54	8.41
LOQ (µg/L)	11.82	28.03

Table 3: The intra- and inter-day precision for the analysis of TMP and SMX by the developed HPLC-DAD method

Analyte	Concentration (µg/L)	Inter-day		Intra-day	
		Mean ±SD (µg/L)	RSD (%)	Mean ±SD (µg/L)	RSD (%)
TMP	20	19.72±1.14	5.8	19.70±1.09	5.6
	100	95.69±1.29	1.3	95.08±1.93	2.0
	500	496.98±3.07	0.6	499.87±5.29	1.0
SMX	100	91.40±2.73	3.0	92.27±2.54	2.5
	500	489.36±9.62	2.0	480.55±10.35	2.2
	2500	2467.42±5.78	0.2	2456.40±13.97	0.6

3.3.4. Precision

The intra-day precision was conducted by injecting 5 times standard solution with 3 concentration levels: low, medium, and high on the same day. The inter-day precision was evaluated by injecting 5 times standard solutions of the same concentration for three consecutive days. The mean value of the concentration and relative standard deviation (% RSD) are summarized in Table 3. The calculated RSD for the precision of the system repeatability was lower than 7% for both TMP and SMX, suitable according to the AOAC standard [19].

3.4. Recovery

Table 4 illustrates the recoveries of these two compounds extracted by the optimized procedure. The protein precipitation method combined with solid phase extraction (SPE) has increased the recovery of the plasma sample. The recoveries of TMP from plasma samples spiked at 20 µg/L and 100 µg/L, whereas that of SMX was five times higher. The recovery of TMP averaged 80% with RSD less than 6%. The recovery of SMX ranged from 82-101% with RSD less than 12%. The recovery in this study is similar to some other studies conducted by the HPLC-DAD method [5, 13].

4. Conclusion

In this study, a simple, fast, and reliable HPLC method was developed for the simultaneous determination of SMX and TMP in human plasma-based protein precipitation and solid-phase extraction. The method achieved a good linearity from 20 – 5000 µg/L for TMP and five times higher for SMX with regression coefficients (R^2>0.999). The method was validated by assessing its sensitivity, precision, accuracy, and selectivity. Extraction recoveries were achieved higher 80% after a liquid-liquid extraction with acetonitrile in 0.1% formic acid followed by a clean-up step with C18 sorbent. The matrix effects were also evaluated and there was no matrix effect observed. Finally, this analytical method is promising to apply in therapeutic monitoring.

Table 4: Recoveries of TMP and SMX in two different concentrations

Analyte	Spiked concentration (µg/L)	Recovery (%)
TMP	20	79.9 ± 5.1
	100	80.4 ± 2.4
SMX	100	101.1 ± 11.8
	500	82.6 ± 4.6

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