Journal of Bioanalysis & Biomedicine (JBABM) under the open access category increases the range of requirement, makes it more flexible and increases the visibility for the upcoming as well as the domain researchers in the field of “physical and chemical biosciences”.

The objectives of JBABM are to establish an impressive and operational way of communication between researchers, practitioners and chemists related with the multiplex roles of various qualitative/quantitative bioanalytical methods and biomedical applications.
Keywords: HPLC; Sulfamethoxazole; Trimethoprim; Validation

Introduction

Cotrimoxazole, a combined drug consisting of 1 part of trimethoprim and 5 part of sulfamethoxazole, was introduced into clinical practice about 30 years ago and is still widely prescribed for various indications [14]. A combination of trimethoprim-sulfamethoxazole is effective treatment for a wide variety of infections including P. jiroveci pneumonia, shigellosis, systemic salmonella infections, urinary tract infections, prostatitis, and some nontuberculous mycobacterial infections. Trimethoprim in combination with a sulfonamide blocks sequential steps in folate synthesis, resulting in marked enhancement (synergism) of the activity of both drugs [11]. The combination often is bactericidal, compared with the bacteriostatic activity of a sulfonamide alone [14].

Because drug concentrations are an important element in determining individual or population pharmacokinetics, drug concentrations are measured in biologic samples, such as milk, saliva, plasma, and urine. Sensitive, accurate, and precise analytical methods are available for the direct measurement of drugs in biologic matrices. Such measurements are generally validated so that accurate information is generated for pharmacokinetic and clinical monitoring. In general, chromatographic methods are most frequently employed for drug concentration measurement, because chromatography separates the drug from other related materials that may cause assay interference [22].

Numerous HPLC methods for the quantification of cotrimoxazole in biological samples have been reported. These methods involved the use of protein precipitation, liquid-liquid extraction and solid-phase extraction coupled with ultraviolet detection or ion pair chromatography or high performance liquid chromatography tandem mass spectrometry method (HPLC/MS/MS) [15,16,19].

In bioequivalence studies, the proposed method should be simple and able to process hundreds of samples in a limited time. This paper describes a simple, rapid, precise, and accurate HPLC method for determining cotrimoxazole in tablet and human plasma in vitro.

Materials and Methods

Chemicals and reagents

Trimethoprim (100.1% on assay) and sulfamethoxazole (99.9% on assay) were obtained from F. Hoffman-La Roche. Sulfadimidine was obtained from Nanhai Beisha Pharmaceutical. Acetonitrile and methanol were HPLC-grade and were purchased from Merck. The other chemicals and reagents were analytical grade. Human plasma was obtained from Indonesian blood bank (Palang Merah Indonesia).

Chromatographic conditions

The HPLC system (Shimadzu, Japan) used consisted of a model LC-6A pump, a fixed manual injection loop of 20 μL, and a model SPD-6A UV detector; data acquisition was performed with the CBM-102 processor. The analytical column employed was a Kromasil C18 column (250 mm × 4.6 mm, i.d., 5 μm).

The mobile phase consisted of acetonitrile-water (20:80 v/v) with the addition of triethylamine (0.1% v/v). The mobile phases were adjusted to pH 5.9 ± 0.1 with 0.2 N NaOH or dilute acetic acid (1% v/v), filtered through a 0.45 μm cellulose membrane filter (Whatman).

Received: December 05, 2011; Accepted: March 24, 2012; Published: March 29, 2012

Citation: Harmita, Mansur U, Sartika J (2012) Optimization and Validation of Analytical Method of Cotrimoxazole in Tablet and Plasma In vitro by High Performance Liquid Chromatography. J Bioanal Biomed 4: 026-029. doi:10.4172/1948-593X.1000059

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and degassed before use (Elmasonic S60H). The detection wavelength was set at 240 nm. Chromatography separation was performed at room temperature and flow rate was maintained at 1 mL/min.

Standard solutions and quality control samples

As stated above, cotrimoxazole is fixed combination of 1 part of trimethoprim and 5 parts of sulfamethoxazole. Therefore, this study used that ratio on making the solutions. Primary stock solutions of cotrimoxazole consisted of trimethoprim (0.32 mg/mL) and sulfamethoxazole (1.60 mg/mL) were prepared in methanol [25]. Cotrimoxazole stock solution was further diluted with mobile phase to obtain different concentration. Sulfadimidine as the internal standard were made by the same steps as above to achieve a 10 gg/mL solution.

Human plasma calibration standards of cotrimoxazole were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. The concentration of trimethoprim (TMP) and sulfamethoxazole (SMX) in calibration curve was 150 ng/mL, 420 ng/mL, 670 ng/mL, 830 ng/mL, 1000 ng/mL, 1170 ng/mL, 1330 ng/mL for TMP and 750 ng/mL, 2080 ng/mL, 3330 ng/mL, 5000 ng/mL for SMX. Quality control (QC) samples were prepared at three concentrations that were low (416.7 ng/mL for TMP and 2083.3 ng/mL for SMX), medium (666.7 ng/mL for TMP and 3333.3 ng/mL for SMX), and high (1000 ng/mL for TMP and 5000 ng/mL for SMX) concentrations.

Sample preparation

Cotrimoxazole tablet: The sample (cotrimoxazole tablet) was prepared by weighing 20 tablets and determining the average weight of the tablets. Then, we grind them and put some to weight accurately. We diluted the samples in methanol and sonication for 30 minutes. For the final step, we used milipore membrane to filter it.

In human plasma: To a 1.5-mL microtube were added 0.5 mL plasma, 25.0 µL of the cotrimoxazole standards solution, and 25.0 µL of the internal standard working solution (40 µg/mL). One part of acetonitril (190 µL) was added to precipitate protein in plasma, vortex-mixed for 20 sec, then add 4 part of mobile phase component into the tube so that the final volume was exactly 1.5 mL. The result was repeatedly vortex for 20 sec and centrifuged at 12,500 rpm for 15 minutes. A 20-µL aliquot of the supernatant was injected into the HPLC system.

Validation of this method

This method was successfully validated to measure cotrimoxazole in tablet [4,5]. The calibration curve was linear by r values 0.9994 and 0.9996, precision by coefficient of variation (CV) were 0.85% and 0.98% also accurate by % recovery for 3 concentrations were 98% - 102% for TMP and SMX, respectively. With the same method, the validation process was being conducted to measure cotrimoxazole in blank plasma.

Six randomly selected blank plasma samples [6] were processed by a similar extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to interference at the retention time of trimethoprim, sulfamethoxazole and sulfadimidine.

The calibration curves were constructed each day before the analysis of the samples by plotting the peak-area ratio (trimethoprim and sulfamethoxazole/sulfadimidine) versus the drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80-120%. The accuracy and precision of the method were assessed intra-day and inter-day during 5 days by determining QC plasma samples at three concentrations which were low (416.7 ng/mL for TMP and 2083.3 ng/mL for SMX), medium (666.7 ng/mL for TMP and 3333.3 ng/mL for SMX), and high (1000 ng/mL for TMP and 5000 ng/mL for SMX) concentrations, accompanying by a standard calibration curve on each analytical run.

The recovery of cotrimoxazole was evaluated by comparing measured concentration obtained from peak areas of pre-treated quality control plasma samples (n = 5) with mean measured concentration of those spiked-afterextraction samples at the same nominal concentrations. The recovery of I.S. from plasma was evaluated at a concentration of 670 ng/mL using the same process. Stability quality control plasma samples were conducted at low and high concentrations and were subjected to short-term (6 h and 24 h) incubation at room temperature, three freeze/thaw cycles, and storage for 30 days (-20°C). The stability of primary stock solutions were also being conducted for 40 days (5°C).

Results

Specificity

The current method showed excellent chromatographic specificity with no endogenous plasma interference at the retention times of trimethoprim, sulfamethoxazole and sulfadimidine as internal standard. Chromatograms obtained from human blank plasma and human blank plasma spiked with cotrimoxazole (150 ng/mL of trimethoprim and 750 ng/mL of sulfamethoxazole) and sulfadimidine (670 ng/mL) are shown in Figure 1A, 1B. Trimethoprim, sulfamethoxazole and sulfadimidine respectively were well resolved with respective retention times of 6.3 min, 14.2 min and 8.5 min.

Calibration curve and limit of quantification

The calibration curves were linear over the concentration range of 150 – 1300 ng/mL with a correlation coefficient of 0.9979 for trimethoprim and 750 - 6670 ng/mL with a correlation coefficient of 0.9976 for sulfamethoxazole. The correlation coefficient from

Figure 1: Representative chromatogram of human blank plasma (A), human blank plasma spiked with trimethoprim (1), sulfadimidine (2), and sulfamethoxazole (3) (B).
Precision and accuracy

The coefficient variation values of both inter- and intraday analysis for 5 days at three concentrations which each concentration is conducted at 5 replicates were less than 7.11% for trimethoprim and 6.51% for sulfamethoxazole whereas the % differentiation were less than 13.85% for trimethoprim and 13.63% for sulfamethoxazole. The inter- and intra-day precision and accuracy values of the assay method are presented in Table 1.

Recovery

The mean extraction recoveries of cotrimoxazole at three concentrations (low, mid, high) respectively were 85.62% - 102.91%, 95.17 - 101.27%, and 90.17% - 92.34% for trimethoprim whereas for sulfamethoxazole were 80.07% - 98.59%, 82.53% - 94.40%, and 87.78% - 89.14%.

Stability

Treated plasma samples were found to be stable at least 24 h when the samples were kept at room temperature (%diff<15%). The concentrations of cotrimoxazole in plasma which underwent three freeze-thaw cycles or storage at -20°C for 30 days were found to be stable with % differentiation less than 15%. The stability data of cotrimoxazole stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2. The primary stock solutions were also found to be stable for 40 days when were kept at 5°C.

The lower limit of quantification with a coefficient of variation of less than 20% was 150 ng/mL for trimethoprim and 750 ng/mL for sulfamethoxazole.

| Concentration | Mean ± SD (μg/mL) | CV (%) | % diff |
|---------------|------------------|--------|--------|
|               | TMP              | SMX    | TMP    | SMX | TMP | SMX |
| Low           | 0.4240 ± 0.0219  | 2.1572 ± 0.1002  | 5.23 | 4.71 | Min = 0.92% | Min = 0.02% |
|               | Max = 12.09%     | Max = 13.62%     |
| Mid           | 0.6444 ± 0.0269  | 3.3959 ± 0.1645  | 4.15 | 4.87 | Min = 0.10% | Min = 0.02% |
|               | Max = -11.74%    | Max = 13.22%     |
| High          | 0.9884 ± 0.0358  | 5.1658 ± 0.1981  | 3.65 | 3.90 | Min = 0.50% | Min = -0.02% |
|               | Max = -14.60%    | Max = 13.63%     |

Table 1: Accuracy and precision from the determination of cotrimoxazole in human plasma (n = 25/concentration).

| Concentration | CV (%) | % diff |
|---------------|--------|--------|
|               | TMP    | SMX    | TMP | SMX |
| Short term stability for 24 h in plasma at room temperature |       |        |     |
| Low           | 6.32   | 1.45   | 2.06 - 14.83% | 0.78 - 3.36% |
| High          | 1.22   | 1.29   | -3.57 - (-)1.46 % | -0.16 - 2.19% |
| Long term storage at -20°C for 30 days |       |        |     |
| Low           | 3.95   | 9.18   | -14.22 - (-)7.18% | -7.17 - 10.57% |
| High          | 3.20   | 2.70   | 2.04 - 8.77 % | -0.07 - 4.82% |
| Three freeze/thaw cycles |       |        |     |
| Low           | 0.64   | 3.31   | -11.74 - (-)10.60% | -10.80 - (-)5.51% |
| High          | 0.06   | 0.04   | -13.92 - (-)3.75 % | -11.56 - (-)4.08% |

Table 2: Stability data of cotrimoxazole in human plasma.
data of cotrimoxazole stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2.

Discussion

Preparation of plasma samples

Protein precipitation has the advantages of simplicity and universality, so it was used to prepare the plasma samples [3]. The reason of choosing acetonitrile as the precipitation agent was caused by its ability to precipitate protein, especially when given not less than the volume of blood. It is also usually become the component of mobile phase with the result that the system will be able to accept. Results indicated that direct protein precipitation with acetonitrile was simple and rapid and good separation of the drug and I.S. was achieved using the precipitation method. The centrifugator was used optimally on 12500 rpm for 15 minutes. The aim was to obtain the pure supernatant which was ready to be injected.

Optimization of mobile phase

The chromatographic conditions were optimized by injecting analytes with mobile phase containing varying percentages of organic phase, flow rates and pH of mobile phase to achieve good resolution and symmetric peak shapes for trimethoprim, sulfamethoxazole and sulfadimidine, as well as a short retention time. As expected, the retention times increased with decreasing acetonitrile percentage and system flow rates. The chosen mobile phase pH was 5.9 ± 0.1 by giving the most symmetric peak shapes for trimethoprim, sulfamethoxazole, and sulfadimidine.

Triethylamine was added to improve peak shapes. Thus, optimal conditions were a mobile phase consisting of acetonitrile, water, and triethylamine (20:80:0.1, v/v/v) pH 5.9 ± 0.1 arranged by 0.2 N NaOH or 1% acetic acid. Under optimum conditions, the chromatographic run time for each sample was completed within 18 min.

Advantages of the method

In comparison to previously published HPLC methods for separation and quantitation of cotrimoxazole, the major modifications incorporated into the current method include: simple sample preparation procedures, common and cheap HPLC equipment and mobile phase additives, and a relative short analysis time as well.

Thus the assay is suitable for routine analysis when determining assay on tablet and biological samples to perform bioequivalence studies. A simple, rapid, precise, and accurate HPLC method for determining cotrimoxazole in human plasma has been presented. Although lower sensitivity was obtained in comparison to previously published LC methods with mass spectrometry detection, the resulting LLOQ (150 ng/mL for trimethoprim and 750 ng/mL for sulfamethoxazole) was sufficient for human pharmacokinetic studies.

Conclusion

An analytical method developed for cotrimoxazole quantification in tablet and plasma samples showed good specificity, sensitivity, linearity, precision, and accuracy over the entire range of clinically significant and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials.

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