Liquid chromatography tandem mass spectrometry method for quantification of topiramate in human plasma

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ABSTRACT

A simple and reproducible liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method for determination of topiramate in human plasma was developed and validated. The plasma samples were prepared by liquid-liquid extraction (LLE) method which composed of methyl-t-butyl ether and hexane. Topiramate and amlodipine (internal standard; IS) were separated on Luna HST 2.5 μm C18 (50x3 mm.) column. The mobile phase consisted of acetonitrile and 10 mM ammonium acetate (90:10, v/v) at pH 4.0 was run under isocratic condition at a flow rate of 0.3 mL/min. The total run time was 2.5 min. Mass detection was performed in multiple reaction monitoring (MRM) mode under negative electrospray ionization (ESI). The mass transitions were monitored at m/z 337.93>77.78 for topiramate and m/z 406.92>295.10 for IS. The lower limit of quantification (LLOQ) was 10 ng/mL which demonstrated good sensitivity and specificity. The linearity range of the method was 10-3,000 ng/mL with the correlation coefficients (r) ≥ 0.995. The method was economical since it required only 200 μL of sample, 1 mL of solvent and 2 μL of injection volume.

Keywords: LC-MS/MS, Topiramate, Amlodipine, Liquid-liquid extraction, Human plasma

1. INTRODUCTION

Topiramate is an antiepileptic drug indicated for monotherapy or adjunctive treatment of epilepsy in both children and adults. It is also approved for prophylaxis of migraine headache. Furthermore, topiramate has been evaluated for its effect in various neurological and psychiatric disorders. The chemical formula of topiramate is C12H13NO4S. It is a sulfamate-substituted monosaccharide that has a molecular weight of 339. Although, topiramate’s mechanism of action has not been fully elucidated, multiple drug actions have been proposed such as blockade of voltage-dependent sodium channels, antagonism of kainate/AMPA subtype of the glutamate receptors and augmentation of GABA activity.

Although, several LC-MS/MS methods for determination of topiramate in human plasma have been reported, there are some analytical limitations such as the need for large volume of plasma samples, time-consuming and complicated sample preparation method, high injection volumes, low sensitivity and narrow linearity range (10-2000 ng/mL).

This work describes the development and validation of an LC-MS/MS method for determination of topiramate in human plasma that is simple, less time-consuming of sample preparation and higher recovery compared to aforementioned methods. This method was fully validated and confirmed within the acceptance criteria according to the guidance of the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Topiramate (99.6% purity) and amlodipine besylate (99.8% purity), an internal standard (IS), were obtained from The United States Pharmaceutical Convention, Inc., USA. Their chemical structures were presented...
in Figure 1. Acetonitrile (HPLC grade), Methyl t-butyl ether (AR grade) and Hexane (AR grade) were purchased from Scharlau (Barcelona, Spain). Methanol and Propan-2-ol (HPLC grade) were purchased from Fisher Scientific (Loughborough, United Kingdom). Ammonium acetate (≥98% purity) was purchased from Sigma Aldrich Chemie (GmbH, Germany). Formic acid (AR grade) was purchased from Merck (Darmstadt, Germany). Milli Q water was generated by Type I water purification system (Millipore Corporation, Massachusetts, USA). Blank human plasma with Lithium heparin was obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2.2. Instruments

The LC-MS/MS system was the Acquity UPLC system (Waters, Co., Ltd. USA) equipped to the Quattro micro mass spectrometry (Micromass Technologies, UK). The system and data processing were performed by MassLynx software, version 4.1 SCN627. The chromatographic separation of topiramate and amlodipine was carried out at 40°C on the Luna HST 2.5 µm C18 (50x3 mm.) column from Phenomenex Ltd., USA. The temperature of auto sampler was set at 10°C throughout the run. The mobile phase consisted of 10 mM ammonium acetate (adjusted with formic acid to pH 4.0) and acetonitrile (10:90, v/v) was delivered under the isocratic condition at a flow rate of 0.3 mL/min. The total run time was 2.5 min. The injection volume was 2 µL. Mass detection of topiramate and IS was performed using multiple reaction monitoring (MRM) mode under negative electrospray ionization mode. The capillary and cone voltage were set at 3.50 kV and 40 V, respectively. The temperature of ion source was maintained at 120°C. Desolvation gas flow rate was 550 L/h at 350°C. The optimum collision energy for topiramate and amlodipine were 23 eV and 17 eV, respectively.

2.3. Preparation of stock and working solutions

Topiramate stock solution used for generating a calibration curve was prepared in methanol to achieve a concentration of 2,835 µg/mL. The working solution was obtained by dilution of the stock solution with 50% methanol to various concentrations ranging from 100-30,000 ng/mL. For the quality control (QC) samples, topiramate stock solution was also prepared in methanol to acquire a concentration of 3,000 µg/mL. The working solution was prepared by further dilution of the stock solution with 50% methanol to acquire concentrations between 100-24,000 ng/mL. The stock solution of IS was dissolved in methanol to reach a concentration of 1,820 µg/mL and further diluted with 50% methanol to obtain the concentration of working solution at 30,000 ng/mL. All stock and working solutions were prepared under low light condition and stored in a -70±10°C freezer until analysis.

2.4. Preparation of calibration curve and QC samples

Calibration standard samples were prepared by spiking topiramate working solutions into pooled human blank plasma to obtain the concentration ranging from 10-3,000 ng/mL. The QC samples were prepared in a similar manner. The concentration of QC samples was 10 ng/mL at LLOQ, 30 ng/mL at LQC, 1,400 ng/mL at MQC and 2,400 ng/mL at HQC. Every sample was processed under light protection condition.

2.5. Sample preparation

Topiramate and IS were isolated from human plasma by liquid-liquid extraction technique under light protection condition. The IS working solution 20 µL was added to 200 µL of plasma sample. Then 1 mL of methyl-t-butyl ether and hexane mixture at 19:1 (v/v) was added, vortexed for 10 minutes and centrifuged at 10,000 rpm, 4°C for 10 minutes. The organic layer was then transferred to a 1.5 mL microcentrifuge tube and dried under nitrogen stream at 30°C. The residue was reconstituted by adding 200 µL mixture of acetonitrile

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**Figure 1.** Chemical structures of topiramate and amlodipine besylate.
and Milli Q water at 1:1 (v/v), then injected into the LC-MS/MS system.

2.6. Method validation

The developed method was validated according to the FDA and the EMA guidance. The selectivity was evaluated by comparing the response of interferences at the retention time of topiramate and IS in an extracted blank plasma sample against that of the QC sample at LLOQ level. Total 14 different sources of blank human plasma with lithium heparin as anticoagulant (10 normal, 2 hemolyzed and 2 lipemic sources) were screened. The minimum six sources of normal blank plasma that showed no interference at the retention time of topiramate and IS were pooled and used for full method validation.

2.6.1. Selectivity

Carry over effect was assessed by injecting a blank sample after the upper limit of quantification (ULOQ) sample. The interfering response in the blank sample following the high concentration standard should be less than 20% of the LLOQ of topiramate and less than 5% for the internal standard.

2.6.2. Carry over effect

Carry over effect was assessed by injecting a blank sample after the upper limit of quantification (ULOQ) sample. The interfering response in the blank sample following the high concentration standard should be less than 20% of the LLOQ of topiramate and less than 5% for the internal standard.

2.6.3. Linearity

Three independent calibration curves, each containing 8-point calibration standard, were used to establish linearity which ranged from 10-3,000 ng/mL. The LLOQ on a calibration curve required the signal to noise ratio (S/N) of an analyte to be greater than 5-time of the extracted human blank plasma.

2.6.4. Accuracy and Precision

The intra-assay accuracy and precision for topiramate were evaluated in six replicated analyses at four concentration levels of QC sample (LLOQ, LQC, MQC and HQC) in a single batch run. The inter-day accuracy and precision were calculated after repeated analyses in three different batches run on two different days.

2.6.5. Extraction recovery

The recovery of topiramate and IS were examined in six replicated analyses at LQC, MQC and HQC levels. The percentage recovery was assessed by comparing the peak area of plasma spiked standards before and after extraction.

2.6.6. Matrix effect

Matrix effect was investigated in total 12 different sources including 10 normal, 1 hemolyzed and 1 lipemic blank plasma. Each source replicated four times at LQC and HQC levels. The matrix factors were calculated by comparing the peak area of post-extraction plasma spiked standard to the peak area of non-extraction plasma spiked standard. The IS normalized matrix factors were calculated by dividing the matrix factor of topiramate by that of the IS.

2.6.7. Dilution integrity

Dilution integrity was demonstrated by spiking topiramate standard into normal blank plasma to reach the concentration above the ULOQ (1.7 times of 85% the ULOQ), then frozen it for at least 24 h. Later, it was diluted by human blank plasma to a fraction of 1/2, 1/5 and 1/20. These fractions covered all the dilutions applied to the study samples and were evaluated by a fresh calibration curve in six replications.

2.6.8. Hemolysis and Lipemia effect

To investigate the impact of hemolyzed and lipemic human plasma on the accuracy and precision of the assay, six replications of LLOQ, LQC, MQC and HQC were prepared in hemolyzed and lipemic plasma and analyzed against calibration curve spiked in normal plasma.

2.6.9. Robustness

Robustness was evaluated on the same apparatus when changes occurred including different lot but same type of column, different organic solvent lots (reconstituted solvents and mobile phase). Intra-day robustness was assessed by analyzing six replications of quality control samples at LLOQ, LQC, MQC and HQC in a single batch run. Inter-day robustness was performed by analyzing 12 replications of quality control samples at each concentration through two robustness batches run on two consecutive days.

2.6.10. Reinjection reproducibility

Reinjection reproducibility was demonstrated by comparing the concentrations of LQC and HQC that were injected at time zero and at 25 hours after autosampler storage at 10±5°C. The variation within ±15% was acceptable.

2.6.11. Stability

The stability of stock solutions of topiramate
and IS were assessed after exposure at room temperature (25±2°C) for at least 6 hours and after storage at -70±10°C for 36 days by comparing the peak responses to that of the freshly prepared solution. The difference within ±7% was acceptable\(^{14}\).

The stability of topiramate in plasma were demonstrated by analysis four replications of LQC and HQC under various conditions against those of the freshly prepared QC samples. Freeze and thaw stability was evaluated after processing three cycles of freezing and thawing at -70±10°C and 25±2°C, respectively. For short term stability, the samples were exposed at room temperature (25±2°C) for 6 hours. Long term stability was demonstrated after samples storage at -70±10°C for 36 days. In addition, stability of the processed samples was evaluated by storage the reconstituted sample in an autosampler at 10±5°C for 48 hours and the dried sample at -70±10°C for 5 days before analysis. The accuracy and difference within ±15% were acceptable\(^{12-14}\).

3. RESULTS AND DISCUSSION

3.1. Method development

The concentrations of topiramate and internal standard, amlodipine, were analyzed by combined reversed phase liquid chromatography and tandem mass spectrometry (LC-MS/MS). The ion transition was examined on both positive and negative modes. As a result, negative ion mode was selected since it yielded more intense response in multiple reaction monitoring (MRM) transition. The mass spectra of parent and daughter ion were scan at m/z 337.93>77.78 for topiramate (Figure 2A) and m/z 406.92>295.10 for amlodipine, the internal standard (Figure 2B). Other parameters of mass spectrometry were also optimized. Although containing weakly acidic sulfamate group in the structure, topiramate is alkaline (pKa 8.7)\(^2\). In order to maximize topiramate peak shape and its ionization efficiency, a low viscosity compound, acetonitrile, and a volatile salt, ammonium acetate were chosen as the mobile phase and pH of the mobile phase was adjusted to below the pKa value of topiramate. The optimum chromatographic separation of topiramate and internal standard were investigated by varying pH of mobile phase from 4.0-8.0 and trialing different kinds of column including Luna HST 2.5 μm C18 (50x3 mm.) and Kinetex 1.7 μm C18 (50x2.1 mm.). The mobile phase containing acetonitrile and 10 mM ammonium acetate (90:10, v/v) at pH 4.0 run on the Luna HST 2.5 μm C18 (50x3 mm.) under isocratic elution at a flow rate of 0.3 ml/min was considered the most sufficient separation

Figure 2. Product ion MS/MS spectra of topiramate (A) and amlodipine (B).
because it resulted in symmetric peak shapes with short retention time. For liquid-liquid extraction, methyl-t-butyl ether, ethyl acetate and hexane were trialed either alone or in combination. A mixture of methyl-t-butyl ether and hexane at 19:1 (v/v) produced the highest recovery. The developed method was straightforward and utilized less solvent compared to other reports\textsuperscript{7,8,10,11}.

3.2. Assay performance and validation

3.2.1. Selectivity

Selectivity was demonstrated in human blank plasma. The retention time of topiramate and the IS were 0.90 and 1.03 min, respectively. The area observed at the retention time was less than 20% of the LLOQ for topiramate and 5% for the IS. The chromatograms obtained from extracted blank plasma (Figure 3A) and plasma spiked with the IS (Figure 3B) indicated no interfering peaks from endogenous component at the retention time of both topiramate and IS. Figure 3C showed chromatogram of quality control sample at LLOQ concentration (10 ng/mL). The proposed method exhibited good specificity and efficient extraction for topiramate and the IS.

3.2.2. Carryover effect

The injection of blank samples following the ULOQ sample (3,000 ng/mL) showed no significant interference.

3.2.3. Linearity and sensitivity

The limit of detection (LOD) for topiramate was 2 ng/mL. Method sensitivity was evaluated at LLOQ concentration (10 ng/mL) with a signal to noise ratio of greater than 5. The percentage of accuracy and precision were 103.33% and 7.10%, respectively. The lower LLOQ concentration compared to previously reported methods\textsuperscript{9,15,17} indicated good sensitivity. The calibration curve was established from eight concentration points ranging between 10 to 3,000 ng/mL by plotting the peak area ratio of topiramate to the IS against the nominal concentration of topiramate. The simple linear equation was obtained by 1/x\textsuperscript{2} weighing factor. The correlation coefficient (r) was ≥0.995 which indicated good strength of linear relationship and was shown in Figure 4. The mean back-calculated concentration of three calibration curves was well within the acceptance criteria for accuracy and precision and was presented in Table 1.
Figure 4. Topiramate calibration curve.

Table 1. Accuracy and Precision of back calculated concentration data from three calibration standards curves.

| Nominal concentration (ng/mL) | CS1 | CS2 | CS3 | CS4 | CS5 | CS6 | CS7 | CS8 |
|-------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Linearity 1                  |     |     |     |     |     |     |     |     |
| 10               | 10.13| 24.71| 146.67| 770.79| 1504.06| 2053.73| 2427.24| 3022.07 |
| 25               | 10.15| 24.84| 137.18| 774.30| 1471.32| 2014.40| 2571.21| 3128.14 |
| 150              | 10.37| 23.31| 142.17| 764.36| 1498.70| 2089.10| 2543.39| 3050.98 |
| 750              | 10.22| 24.28| 142.00| 769.82| 1491.36| 2052.41| 2513.95| 3067.06 |
| 1500             |     |     |     |     |     |     |     |     |
| 2000             |     |     |     |     |     |     |     |     |
| 2500             |     |     |     |     |     |     |     |     |
| 3000             |     |     |     |     |     |     |     |     |
| Mean             |     |     |     |     |     |     |     |     |
| 10               | 10.14| 0.85| 4.75| 5.04| 17.56| 37.37| 76.37| 54.83 |
| 25               | 10.15| 0.89| 4.89| 5.52| 18.12| 38.20| 78.24| 56.81 |
| 150              | 10.37| 3.49| 3.34| 0.65| 1.18| 1.82| 3.04| 1.79 |
| 750              | 10.22| 3.49| 3.34| 0.65| 1.18| 1.82| 3.04| 1.79 |
| 1500             |     |     |     |     |     |     |     |     |
| 2000             |     |     |     |     |     |     |     |     |
| 2500             |     |     |     |     |     |     |     |     |
| 3000             |     |     |     |     |     |     |     |     |
| SD (±)           | 0.14| 0.85| 4.75| 5.04| 17.56| 37.37| 76.37| 54.83 |
| CV (%)           | 1.33| 3.49| 3.34| 0.65| 1.18| 1.82| 3.04| 1.79 |
| Accuracy (%)     | 101.57| 96.87| 94.58| 102.54| 99.33| 102.52| 100.46| 101.14 |

Table 2. Topiramate intra-day (within-run) and inter-day (between-run) accuracy and precision.

| Nominal concentration (ng/mL) | LLOQ | LQC | MQC | HQC |
|-------------------------------|------|-----|-----|-----|
| Within-run 1                  |     |     |     |     |
| Mean (n=6)                    | 9.71| 30.10| 1467.03| 2458.56 |
| SD (±)                        | 1.00| 1.09| 28.32| 63.67 |
| CV (%)                        | 10.34| 3.63| 1.93| 2.59 |
| Accuracy (%)                  | 96.23| 100.02| 104.65| 102.30 |
| Within-run 2                  |     |     |     |     |
| Mean (n=6)                    | 10.53| 30.65| 1422.87| 2398.67 |
| SD (±)                        | 0.89| 0.82| 34.90| 77.56 |
| CV (%)                        | 8.46| 2.69| 2.45| 3.23 |
| Accuracy (%)                  | 104.40| 101.87| 101.50| 99.81 |
| Within-run 3                  |     |     |     |     |
| Mean (n=6)                    | 10.43| 30.50| 1404.25| 2454.12 |
| SD (±)                        | 0.74| 0.99| 8.92| 28.98 |
| CV (%)                        | 7.10| 3.26| 0.64| 1.18 |
| Accuracy (%)                  | 103.33| 101.36| 100.17| 102.12 |
| Between run                   |     |     |     |     |
| Mean (n=18)                   | 10.22| 30.42| 1431.38| 2437.12 |
| SD (±)                        | 0.91| 0.95| 36.76| 63.20 |
| CV (%)                        | 8.93| 3.12| 2.57| 2.59 |
| Accuracy (%)                  | 101.32| 101.09| 102.10| 101.41 |
3.2.4. Accuracy and Precision

The intra-day and inter-day accuracy and precision were evaluated by analysis of six replicates of LLOQ, LQC, MQC and HQC samples. The data of accuracy and precision were summarized in Table 2. The percentage of accuracy and precision was well within ±20% at LLOQ level and within ±15% at other concentrations. The results revealed that this method was accurate, precise and reproducible for analysis.

3.2.5. Extraction recovery

The extraction recovery of topiramate at LQC, MQC and HQC levels were 83.28%, 83.07% and 82.92%, respectively whereas the overall average recovery of IS was 74.09%. The results indicated sufficient and reproducible liquid-liquid extraction technique for topiramate and IS. Although the solid phase extraction in other studies yielded better recovery (93.22-105.5%)\(^{10,11}\), the liquid-liquid extraction was continued because it produced acceptable recovery. Moreover, the liquid-liquid extraction for large number of samples was more affordable when compared to the solid phase extraction.

3.2.6. Matrix effect

The matrix factor (MF) of topiramate calculated at LQC and HQC level ranged from 0.91-1.05 with the CV of 4.12-4.17% and the IS normalized MF ranged from 0.93-1.06 with the CV of 3.00-3.03% (Table 3). The matrix factor of IS was 0.97 with the CV of 2.49%. The matrix factors of topiramate, IS and IS normalized MF within 0.85-1.15 indicated neither ion suppression nor ion enhancement from endogenous substances in plasma sample.

3.2.7. Dilution integrity

The dilution integrity covered the dilution to a fraction of 1/2 (2,550 ng/mL), 1/5 (1,020 ng/mL) and 1/20 (255 ng/mL). The accuracy of the dilution integrity was 99.34, 97.54 and 93.37% and the precision was 2.49, 1.69 and 1.73%, respectively. Accuracy and precision were well within ±15% of the acceptance criteria.

3.2.8. Hemolysis and Lipemia effect

The accuracy and precision of hemolysis and lipemia effects were summarized in Table 4. The accuracy varied within 20% of nominal concentration at LLOQ and within 15% at other concentrations. The precision (%CV) at LLOQ level was not exceed 20% and was within 15% at other concentrations. The results indicated no significant hemolysis and lipemia effects on accuracy and precision of this method.

3.2.9. Robustness

Intra- and inter-day accuracy were 97.29-105.02% and 100.77-102.66%, respectively. Intra- and inter-day precision were 1.45-9.04% and 3.21-7.67%, respectively. These results ensured the robustness of this method.

3.2.10. Reinjection reproducibility

Reinjection reproducibility was performed to ensure a sample re-analysis when an unexpected event had interrupted the analysis. The percentage variation of the reinjected samples was 5.67% and 2.55% at LQC and HQC concentration, respectively after autosampler storage for 25 hours. The results suggested that the samples were consistent within 25 hours.

3.2.11. Stability

The stock solution of topiramate and IS were
stable at room temperature (25±2°C) for 11 hours and at -70±10°C for 36 days.

Stability studies were performed under various conditions and summarized in Table 5. Topiramate in human plasma was stable at 25±2°C for 6 hours, at -70±10°C for 36 days and after three cycles of freeze and thaw. In addition, the reconstituted topiramate samples were unaffected when kept in an autosampler at 10±5°C for 48 hours. Similarly, the dry extracted of topiramate samples were not altered after freezing at -70±10°C for 5 days. These results indicated the stability of topiramate in human plasma under storage or operating conditions.

Table 4. Effect of hemolysis and lipemia on accuracy and precision.

| Nominal concentration (ng/mL) | LLOQ 10 | LQC 30 | MQC 1400 | HQC 2400 |
|-------------------------------|---------|--------|----------|----------|
| Mean (n=6)                    | 10.61   | 30.51  | 1519.85  | 2484.29  |
| SD (±)                        | 0.58    | 1.28   | 21.90    | 59.67    |
| CV (%)                        | 5.46    | 4.19   | 1.44     | 2.40     |
| Accuracy (%)                  | 105.19  | 101.41 | 108.41   | 103.37   |

Table 5. Topiramate stability data in human plasma under various conditions.

| Stability test                   | QC samples       | Mean±SD (ng/mL) | CV (%) | Accuracy (%) | Difference (%) |
|----------------------------------|------------------|-----------------|--------|--------------|----------------|
| Freeze and thaw stability at -70±10°C for 3 cycles | LQC (30 ng/mL) | 27.10±0.51     | 1.87   | 90.08        | 9.27           |
|                                  | HQC (2400 ng/mL) | 2229.90±28.97  | 1.30   | 92.79        | 8.60           |
| Short term stability at 25 ± 2°C for 6 h | LQC (30 ng/mL) | 30.72±1.78     | 5.80   | 102.11       | 2.85           |
|                                  | HQC (2400 ng/mL) | 2535.42±59.62  | 2.35   | 105.50       | 3.93           |
| Long term stability at -70±10°C for 36 days | LQC (30 ng/mL) | 28.15±1.49     | 5.29   | 93.57        | 5.42           |
|                                  | HQC (2400 ng/mL) | 2519.59±33.59  | 1.33   | 104.84       | 3.51           |
| Post-preparative stability at 10 ± 5°C for 48 h | LQC (30 ng/mL) | 30.00±1.16     | 3.88   | 99.71        | 0.44           |
|                                  | HQC (2400 ng/mL) | 2612.24±112.01 | 4.29   | 108.69       | 7.08           |
| Post-preparative stability at -70±10°C for 5 days | LQC (30 ng/mL) | 28.82±2.09     | 7.24   | 95.79        | 3.51           |
|                                  | HQC (2400 ng/mL) | 2400.54±42.31  | 1.76   | 99.89        | 1.60           |

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Conflict of interest
All authors declare no conflicts of interest in preparing this article.

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4. CONCLUSIONS

In this study, the LC-MS/MS method for quantification of topiramate in human plasma has been successfully developed and validated according to the USFDA and EMA guidelines. This method employed uncomplicated LLE technique that demonstrated acceptable sensitivity and specificity at reasonable cost. Furthermore, the method allowed small quantity of sample (200 µL), small volume of solvent (1 mL) and only needed 2-µL injection volume. A run time of 2.5 minutes per sample permitted an analysis of over 500 samples per day.

Ethical approval
The Institutional Review Board of Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand approved the study protocol. (COA no. Si167/2017)

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