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Article

Article

A High-Performance Liquid Chromatographic Method for the Determination of Meropenem in Serum

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Abstract

In this study, a new, sensitive and selective high-performance liquid chromatographic method was developed for the determination of meropenem (MEM) in human serum. In the developed method, C18 column (3.9 × 150 mm, 5 µm) was selected as stationary phase at 30°C, and methanol: acetic acid solution mixture was used as mobile phase with gradient program. Chromatographic separation was carried out at a flow rate of 1 mL/min, and detection was performed at 300 nm with diode array detector. Doripenem was selected as an internal standard, and the analytes were extracted from serum using protein precipitation method with ortho-phosphoric acid: methanol. Detection wavelength was selected as 300 nm. The developed method was validated according to International Council for Harmonisation (ICH) guidelines. The calibration curve was linear over a concentration range of 4–240 µg/mL with correlation coefficient of 0.9985. The limit of detection and limit of quantification values were found as 0.057 and 0.192 µg/mL, respectively. The validated method was successfully applied for the determination of MEM in human serum samples collected from patient volunteers at different time intervals, and therapeutic drug monitoring of MEM has been investigated.

Key words: Carbapenems, Meropenem, High-Performance Liquid Chromatography, Human Serum, Validation, Therapeutic Drug Monitoring

Introduction

Meropenem (MEM) (4R,5S,6S)-3-[[(3S,5S)-5-[(Dimethylamino)carbonyl]-3 pyrrolidinyl]thio]-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (1) is a broad spectrum parenteral antibiotic of the carbapenem class (2). MEM is a white to off-white crystalline powder with acidic properties ($pK_{a1} = 2.9$ and $pK_{a2} = 7.4$; Figure 1). MEM is very slightly soluble in ethanol, not soluble in acetone and ether, but readily soluble in dimethylformamide, dimethylsulfoxide, 5% potassium dihydrogen phosphate and physiological saline solution. MEM was approved by the Food Drug Administration in 1996. It has come into clinical use (3). The clinical formulation includes anhydrous sodium carbonate (4).

MEM is extremely active with a broad spectrum of *in vitro* activity against Gram-positive and Gram-negative bacteria. It has indicated clinical and bactericidal activity in the treatment of a wide range of serious infections like upper respiratory tract, urinary tract, intra-abdominal and skin infections. It is similar with imipenem but there is a major difference between them. The difference is the addition of a methyl group to the 1-β position of MEM. This

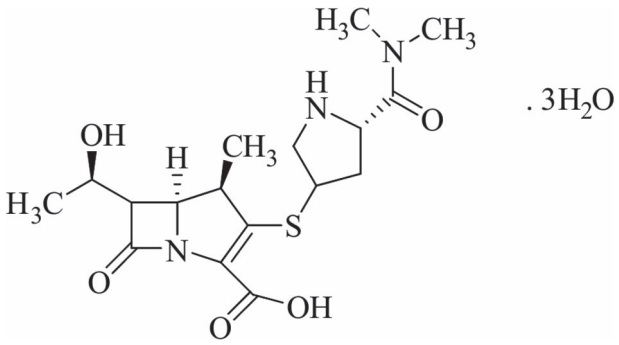

Figure 1. The chemical structure of meropenem.

modification has the advantage of being stable to hydrolysis by the proximal brush border enzyme DHP-I. Thus, it can be administered without an inhibitor of DHP-I, such as cilastatin (4,5).

MEM has been determined in biological fluids by several high-performance liquid chromatography (HPLC) techniques with ultraviolet spectroscopy (UV) detection (6,7). Few of them need several steps for the sample preparation such as solid phase extraction (8,9) but some of them required special equipment for example column switching (10,11), ultrafiltration (7,12) or HPLC-integrated system (13), which is not accessible in every laboratory. In recent years, HPLC methods with using mass spectrometry have been developed for determining MEM in different kinds of biological fluids. The liquid chromatography (LC)–MS methods (14–17) have lots of advantages over the traditional analytical techniques, such as short analysis time, high specificity and sensitivity. On the other hand, it requires expensive instrumentation and highly skilled personnel.

In this study, an easy and sensitive HPLC-UV method that has short analysis time and high recovery rate was developed for the determination of MEM in human serum. The developed method was successfully applied for the determination of MEM in human serum samples collected from patient volunteers who received 1000 mg of MEM intravenously.

Experimental

Chemicals and reagents

MEM trihydrate and doripenem (DOR) monohydrate (internal standard, IS) were obtained from Sigma Aldrich (Taufkirchen, Germany). Sodium chloride, HPLC Grade Methanol, analytical grade % 85 o-phosphoric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from Pure Lab Elga apparatus.

Equipment and chromatographic conditions

The chromatographic separations were performed on a Shimadzu LC 20A chromatography system (Kyoto, Japan), which consisted of an LC-20AT pump, a DGU-20A5 vacuum degasser, a SIL-20 AC autosampler, a CTO-20A column oven and an SPD-M20A diode array detector (DAD). The samples were separated on an Inertsil C18 (150 × 3.9 mm, 5 µm) column with a Phenomenex C18 (4.0 × 3.0 mm, 5 µm) guard column at 30°C. The mobile phase was a mixture of acetic acid solution (1.75 mM) and methanol with gradient program (Table I) at a flow rate of 1 mL/min. The detector was set at 300 nm, and the injection volume was 20 µL. Chromatographic data were collected and computed by LC Solution Version 1.24 system software.

Table I. The Mobile Phase with Gradient Program

Time (min)	% A (Methanol)	% B (Acetic acid solution)
0	10	90
1.65	10	90
1.66	15	85
10	15	85

Preparation of stock and working solutions

The stock solution of MEM was prepared daily (50 mg/mL) in physiological saline solution. The working solutions were prepared by dissolving the stock solution at final concentrations of 0.5, 1, 5 and 10 mg/mL. The calibration standards were diluted from the working solutions at final concentrations of 4, 20, 40, 120, 200 and 240 µg/mL. Standard calibration samples were prepared daily by spiking 500 µL of drug-free human serum.

Preparation of IS solution

Stock solution of IS was prepared at 5 mg/mL concentration of DOR in physiological saline solution. Standard solution was prepared daily by diluting the stock solution at final concentration of 600 µg/mL.

Sample collection

Blood samples were obtained from five patient volunteers with different infections after taking an intravenous administration of Maxipen (MEM 1000 mg three times a day). Samples were collected into the serum tubes and centrifuged at 6000 rpm for 10 min at 4°C, then the supernatant was stored at −20°C.

Sample preparation

Protein precipitation was used for extraction of MEM from samples. A total of 0.5 mL serum was diluted to 1 mL with physiological saline solution. The 50 µL MEM (at six different concentrations from 4 to 240 µg/mL) and 50 µL IS (600 µg/mL concentration) solutions were added to 0.5 mL diluted serum samples. Then 400 µL ortho-phosphoric acid solution in methanol (75 µL/10 mL) was added and final solution was mixed by a vortex mixture for 2 min. The samples were centrifuged for 10 min at 6000 rpm and 4°C. After centrifugation the supernatant was filtered through 0.2 µL nylon filter before 20 µL injection into the HPLC system.

Method validation

Selectivity. The selectivity of the method was evaluated by injecting six different blank human serum samples. The data obtained from the blank serum samples were examined for interference at the retention times of analyte including IS by comparing with those data obtained from spiked serum samples. In addition, both MEM and IS peak purities were investigated by DAD. The peak purity index values of the MEM and IS in spiked serum samples were found as 0.999977 and 0.999981, respectively.

Linearity. The linearity of the method was determined by preparing a series of solutions of working standards at concentration range from 4 to 240 µg/mL in human serum. Six replicates of the calibration standards were prepared, and the calibration curve was made by plotting peak area ratios of MEM to IS versus the drug concentrations with least-squares linear regression analysis. The sensitivity was evaluated by the limit of quantitation (LOQ), the lowest concentration of the serum spiked with MEM in the calibration curve.

Precision and accuracy. Intra-day and inter-day precision and accuracy were determined in serum samples by determining Quality control samples at three concentration levels (4, 120 and 200 µg/mL). For intra-day assay precision and accuracy, six replicates of samples at each concentration were assayed all at once within a day. The inter-day assay precision and accuracy were determined by samples on three different days. Six replicates at each concentration were assayed per day.

Recovery. Absolute recoveries of MEM at three concentration levels (4, 120 and 200 µg/mL) ($n = 5$) were measured by comparing the peak area of the drug obtained from the serum with peak area obtained by the direct injection of pure aqueous drug standard. The mean recovery of the drug at three concentration levels (4, 120 and 200 µg/mL) was calculated by comparing the concentration obtained from the drug supplemented serum to the actually added concentration.

Stability. The stability of MEM standard solutions was tested at 4°C for 1 week. The freeze–thaw stability of MEM in serum samples was evaluated over three freeze–thaw cycles. Stability control serum samples in triplicate at the levels of 4, 120 and 200 µg/mL were immediately frozen at −20°C and thawed at room temperature three consecutive times. After that, the samples were processed and assayed. The stability of MEM in spiked serum stored at 4°C for 72 h and −20°C for 2 weeks was evaluated as well. Long-term stability was assessed using samples stored at −20°C over a period of 8 weeks.

Results

Method development

A rapid and sensitive HPLC method has been developed for the determination of MEM in human serum. DOR, imipenem and ertapenem were used for IS trials. Sharp peaks with good resolution and suitable retention time were obtained using DOR. Therefore, DOR was chosen as IS for this study.

The chromatographic conditions, especially the composition of mobile phase and its pH, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. For this purpose, at various flow rates different solvents of mixtures, such as methanol, acetonitrile, o-phosphoric acid and acetic acid, were tested.The best results were obtained by using methanol:acetic acid as the mobile phase consisting of two components and was applied gradient program. The retention times of IS and MEM were 4.06 and 5.30 min, respectively, and the total analysis run time was 8 min. No interfering peaks were observed. Representative chromatograms of (a) drug-free serum, (b) the serum spiked with MEM (200 µg/mL) and IS (120 µg/mL) and (c) the serum obtained at 2.5 h from Patient II who was taking 1000 mg (for 3 h intravenous administration) three times a day of MEM are given in Figure 2.

Sample preparation

Various preliminary experiments such as liquid–liquid extraction and protein precipitation with different solvents have been done for extracting of MEM from serum and avoid possible interference of serum proteins. The recovery rate was very low with doing chloroform–methanol liquid–liquid extraction. When acetonitrile–chloroform system was used, the peak splitting was observed. When acetone–chloroform system was used, the peak splaying was observed. For these reasons, it was decided to try protein precipitation.

For this purpose, different organic solvents (methanol, acetonitrile and acetone) and various acidic reagents (acetic acid, o-phosphoric acid and hydrochloric acid) were tested for highest recovery with the cleanest extracts. At first, methanol was chosen for precipitation. The peaks splitting of MEM and IS was observed. With that, literature research was investigated about carbapenems and has been reported to delay degradation of carbapenems by adding NaCl solution. Thus, it was decided to increase the concentration of salt solution to prevent degradation. The experiment was repeated by adding 1%, 5%, 20%, 50% and saturated NaCl solutions, respectively. As a result of this experiments, recovery value was still low, the experiments have been continued with using different organic solvents (acetonitrile, acetone, etc.) as precipitant. After precipitation, centrifuge and evaporate under nitrogen process was repeated. Evaporating under nitrogen process was given up because the recovery was still low. The precipitate was centrifugated and filtrated respectively. And then, it was injected directly. The mobile phase–methanol mix was used as a precipitant in new experiments. Due to spooling of peaks in the chromatograms,in order to protein precipitation is closer to the mobile phase it was decided to add various acidic solutions (acetic acid, phosphoric acid, hydrochloric acid) at different concentrations. The best recovery values were obtained using ortho-phosphoric acid solution in methanol (75 µL/10 mL) for MEM extraction from serum samples.

The calibration curve was found as linear over the concentration ranges of 4–240 µg/mL for serum. The regression equations were as follows: $A = 0.037\ C - 0.0219$ ($r^2 = 0.9985$), where A is the peak area ratios (A_{MEM}/A_{IS}) and C is the concentration of MEM (µg/mL). The limit of detection (LOD) and LOQ were expressed as the equations between the standard deviation of the response and the slop. The slope S may be estimated from the calibration curve of the analyte:

$$\mathrm{LOD} = 3.3\ \sigma/\mathrm{S}$$

$$\mathrm{LOQ} = 10\ \sigma/\mathrm{S}.$$

Method validation

Recovery. As shown in Table II, the mean absolute recoveries of MEM were found as 91.5–98.4% for serum.

Precision and accuracy. The values of precision and accuracy of MEM are summarized in Table III. The results were determined analyzing the samples spiked with MEM at three different concentrations.

Stability. MEM in the spiked serum samples was found to be stable for 1 week at 4°C and minimum three freeze–thaw cycles. Furthermore, the solutions showed good stability when stored at −20°C for 60 days to test them for long-term stability. Overall, no stability-related problem was observed during the course of analysis under different conditions, which suggests that the sample and standard solutions can be evaluated without any significant loss (the acceptance criteria is ±15%).

Application to therapeutic drug monitoring in patients

The proposed HPLC method with diode array detection was applied to the analysis of serum samples from patients with different infections who were taking 1000 mg of MEM (three times a day). The chromatogram of a serum sample from Patient II who was taking 1000 mg a day of MEM is shown in Figure 2. In Patient I (79 year, female, pulmonary infection), serum concentrations of taking 1000 mg of MEM (for 0.5 h intravenous administration) were

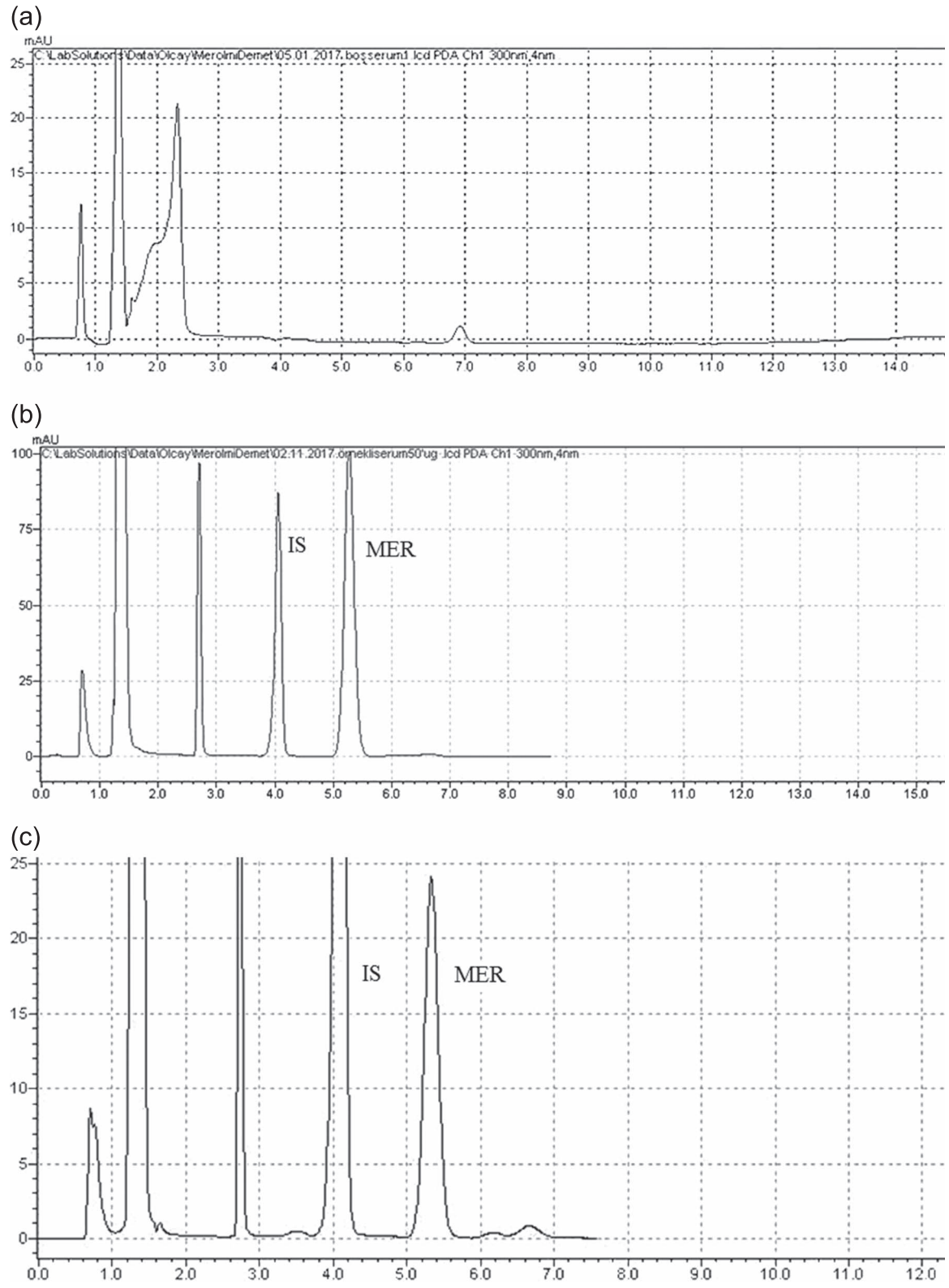

Figure 2. HPLC chromatograms of a (a) blank serum, (b) serum spiked with MEM (200 µg/mL) and IS (120 µg/mL) and (c) serum sample from Patient II who was taking 1000 mg MEM three times a day.

measured at 0, 2, 6, 16, 18 and 24 h after starting the infusion. In Patient I, the drug concentration in serum at 2, 6, 16, 18 and 24 h was 3.42, 3.35, 2.59, 2.55 and 0.75 µg/mL, respectively (Figure 3). In Patient II (50 year, female, cholangiocarcinoma with pericholangitis abscess), serum samples were collected from this patient taking 1000 mg of drug (for 3 h intravenous administration because she

Table II. Recovery of MEM from Serum Samples by the Proposed Method

Added (μg/mL)	Found (μg/mL) ± SD	% Absolute recovery	% RSD
4	3.71 ± 0.119	91.5 ± 0.029	3.3
120	114.7 ± 1.979	93.6 ± 0.016	1.7
200	196.8 ± 5.982	98.4 ± 0.029	3.0

Table III. Intra-day and Inter-day Precision and Accuracy of MEM in Serum ($n = 6$)

Serum sample	Added conc. (μg/mL)	Found conc. (μg/mL) ± SD	Relative Standart Deviation (RSD) (%)	Relative Mean Error (RME) (%)
Intra-day	4	3.7 ± 0.129	3.5	−6.43
	120	115.5 ± 2.242	1.9	−3.75
	200	197.3 ± 3.563	1.8	−1.33
Inter-day	4	3.7 ± 0.120	3.2	−7.04
	120	115.2 ± 2.006	1.7	−3.97
	200	197.1 ± 3.581	1.8	−1.43

had allergy) after 0 and 2.5 h. In Patient II, the drug concentration in serum at 2.5 h was 43.12 μg/mL. In Patient III (72 year, female, metastatic pancreas cancer with pulmonary infection), serum concentrations of taking 1000 mg of MEM (for 0.5 h intravenous administration) were measured after 0 and 40 min and 1 h. In Patient III, the drug concentration was found to be 12.77 μg/mL in serum. In Patient IV (47 year, male, cholangiocarcinoma with *Psedumenos aureuginosa* in blood), serum samples were collected from this patient taking 1000 mg of drug (for 0.5 h intravenous administration) after 0 and 2 h. In Patient IV, the drug concentration in serum was determined 10.59 μg/mL at 2 h. In Patient V (28 year, male, pancreas abscess), serum concentrations of taking 1000 mg of MEM (for 0.5 h intravenous administration) were measured at 0 and 1.5 h. In Patient V, the drug concentration in serum was found to be 2.28 μg/mL. The results of Patient II, III, IV and V are given in Table IV. This study was approved by the Ethics Committee at Bezmialem Vakif University Hospital.

Discussion

In the literature, there have been many studies for determining MEM from pharmaceutical preparations and biological fluids. For these studies, a few UV spectrophotometer assays (18,19), several HPLC (8) and LC–MS assays (14,16,17) have been done. In general, plasma samples were purified by extraction column (10,11,13) within the system and analyzed in these HPLC studies. This system enables the recovery range ~91.5–98.4% while offering a great convenience in sample preparation steps but it cannot be found in every laboratory. By considering the other HPLC methods, specific centrifugal filter device was used for extracting of MEM from plasma samples (7,12).The studies were also achieved wide linearity range such as 0.05–100 μg/mL with using these devices and provides quick analyze and save time in sample preparation steps. However, these devices cannot be applied in every laboratory, because of their high cost. The LC–MS assays can provide the analysis MEM from plasma with 77.6% recovery rate (20). Also, solid-phase extraction and back extraction in sample preparation steps were used with other LC methods (21). The recovery rates of these methods are 62% and 59%, respectively (14,16). Some of HPLC method was used protein precipitation and drying under nitrogen process for sample preparation step (22). The retention time of MEM is about 20 min in this method. The sample preparation process and analysis time is taken a long time. Although HPLC–MS or LC–MS/MS techniques are generally more sensitive and precise compared to other techniques, they are quite expensive and still cannot be found in most of the laboratories.

Considering all the literature studies, HPLC-DAD technique was chosen for determining MEM of human serum in this study. First of all, various experiments have been done to determine the optimum conditions for HPLC analysis. Among the above systems, the best separation is achieved by a C18 column (3.9 × 150 mm, particle size 5 μm) as stationary phase at a flow rate of 1 mL/min with 1.75 mM acetic acid: methanol as the mobile phase consisting of two components and was applying a gradient program. Under these conditions, different drug molecules were tested as IS. The best result in the developed chromatographic conditions was given by DOR.

For the sample preparation, protein precipitation method was used. Different solvents (methanol, acetonitrile, acetone and salt

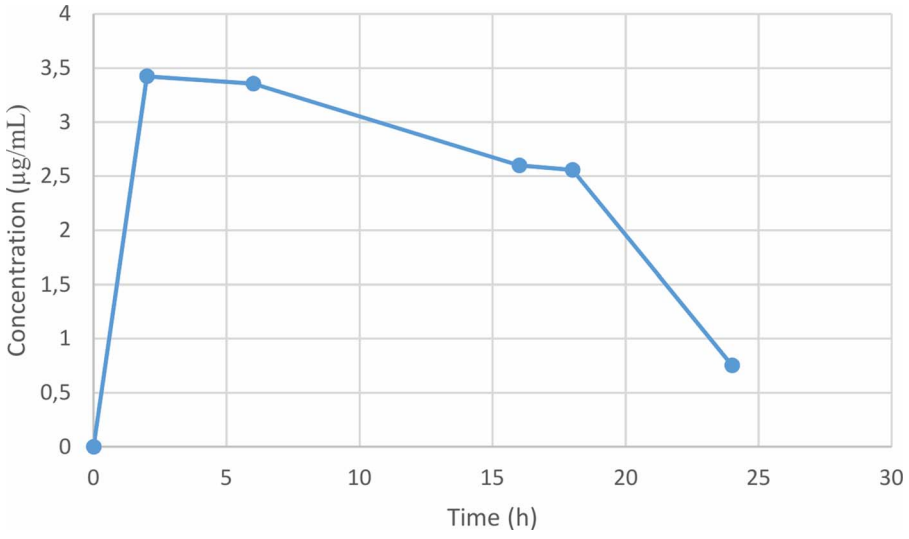

Figure 3. Time course of MEM serum concentrations in Patient I.

Table IV. MEM Concentrations of Patients II–V

Patient	Age and sex	The type of infection	Time	Serum concentration (μg/mL)
P-II	50, F	Pericholangitis abscess (cholangiocarcinoma)	2.5 h	43.12
P-III	72, F	Pulmonary infection (metastatic pancreas Cancer)	1 h and 40 m	12.77
P-IV	47, M	*Psedumenos aureuginosa* (cholangiocarcinoma)	2 h	10.59
P-V	28, M	Pancreas abscess	1 h and 30 m	2.28

solution with different percentage) and acidic reagents (acetic acid, phosphoric acid, hydrochloric acid) were tested for protein precipitation. As a result of all these trials, o-phosphoric acid:methanol mixture was defined as the best optimal extraction method of MEM and IS from serum. Mean absolute recovery range of this method is from 91.5% to 98.4%. In the literature, some of LC–MS methods showed that recovery range is from 60% to 72% (14).

When we analyzed the results that MEM drug concentrations (rapidly excreted in urine from the body) in the patient's blood in a short time to reach C_{max} value, subsequently drug concentrations in body begin to fall rapidly in the 1–2 h range. The serum concentration of patients has been shown in Table III. The concentrations of Patients II–IV have been found higher than the other patients; in other words, excretion of MEM from body is slower. These patients have different gender, age and various types of infections while their only common properties are cancer. Due to the usage of chemotherapeutic agents for cancer treatment, changes in drug elimination mechanism of cancer patients may occur. In the literature, there are also studies that investigate the metabolism of cancer patients and drug resistance (23,24). The concentration of Patient II is the highest; she received MEM longer than 3 h intravenously because she had allergy. Patients I and V have no serious illness except infection. Although they have different genders and different age ranges compared to the group of cancer patients, because of the excretion of drug in the body is faster, the drug levels in body have been found in low concentrations. Referring to the results, both of patient groups were found to be conform with each other. It was difficult to find the patient volunteers that have the same infections at similar ages. The next investigations can be performed on these patients using our method.

Conclusion

In this study a new, simple and high selective HPLC method using diode array detection has been developed for the assay of MEM in serum samples. This new method was successfully applied for the determination of MEM in human serum samples that were obtained from volunteer patients. Results showed that the developed method can be used for therapeutic drug monitoring of MEM.

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online.

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Compliance with ethical standards

The approval for human experiments was obtained from Bezmialem Vakif University Human Ethics Committee numbered 71306642/050-01-04/305 (see Supplementary Data).

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