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A highly selective and sensitive spectrofluorometric method for quantification of meropenem in its dosage form and fresh human plasma

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Abstract: The present study aims to develop and validate a robust, fast, simple, and sensitive spectrofluorimetric approach for the estimation of meropenem (MRP) in its dosage form and fresh human plasma. The developed method is mainly depending on a nucleophilic substitution reaction of MRP with 4-Nitro-7-chlorobenzofurazan (NBD-Cl) in alkaline media (pH 9.0), which results in a strongly fluorescent yellow adduct measured at 536 nm when excited at 471 nm. The variables that influence the stability and development of reaction product were thoroughly investigated and optimized. Calibration curve is rectilinear within the concentration range of 25-650 ng/mL of MRP with a linear correlation coefficient (r =0.9981). Detection and quantification limits were estimated to be 9.55 ng/mL and 3.15 ng/mL respectively. The presented approach was successfully used to the analysis of commercial meropenem vials and meropenem-containing fresh human plasma with good results.

Keywords: NBD-Cl; meropenem; human plasma; spectrofluorimetry.

1. Introduction

Meropenem is an intravenous beta-lactam antibacterial with an ultra-broad spectrum of activity against both gram-positive and gram-negative bacteria Figure S1 [1-4]. Meropenem reveals good stability toward β-lactamases and is used as a last-resort antibiotic, particularly in intensive care units, to treat intra-abdominal infection, peritonitis, bacterial meningitis, febrile neutropenia, gynaecological, pneumonia, anthrax, and sepsis. Meropenem is a new antibiotic from the carbapenem family of antibacterial that has a truly extended spectrum when used alone [5-8]. Meropenem can be used to treat various infections that are caused by multiple drug-resistant organisms, as well as infections due to mixed aerobic and anaerobic organisms [9]. Meropenem, like other beta-lactam antibiotics, penetrates the cell wall of bacteria and inhibits the enzymes known as penicillin-binding proteins (PBPs), which catalyze the cross-linking of glycopeptides that form the bacterial cell wall. Consequently, preventing cell wall synthesis [10]. Despite having a similar structure to imipenem, meropenem has some advantages over it,

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including a lower seizure proclivity and being stable against human renal damage caused by dehydropeptidase I (DHP-I), So it does not require to be combined with cilastatin [11-13].

Various methods were reported for the quantitation of meropenem medication in real samples. Of these, we can mention: capillary zone electrophoresis [14-16], HPLC [17-23], LC–MS–MS [24,25], microbiological assay [26], and spectrophotometric methods [27,28]. However, Chromatographic methods are sophisticated and require expensive solvents and instruments as well as difficulty in analysis. Spectrofluorimetric and spectrophotometric methods are regarded as the most convenient and popular analytical techniques due to their simplicity, specificity, inexpensive, and availability in most laboratories of quality control. whereas the spectrophotometric method has lower sensitivity than the spectrofluorimetric method.

Literature survey showed that no fluorometric approach has been published for the quantification of meropenem till now. Thus, the presented approach was regarded as the first fluorometric method for quantification of meropenem (MRP) in its pharmaceutical vials and in human plasma. The suggested method depends on a nucleophilic substitution reaction of MRP with 4-Nitro-7-chlorobenzofurazan (NBD-Cl) in alkaline media (pH 9.0), which results in a strongly fluorescent yellow adduct measured at 536 nm when excited at 471 nm.

2. Experimental

2.1. Apparatus

Spectrofluorometric measurements were carried out using a Shimadzu (RF-5301 PC, Kyoto, Japan) equipped with a xenon lamp (150 watt) together with 1 cm quartz cuvette. Slit width set at 5 nm for both excitation and emission monochromator. Thermostatically controlled water bath (LCB-22D, daihan Labtech CO., Korea) was utilized for heating purposes. pH meter wtw (inolab pH 720, Germany) for pH adjustment. Sensitive digital balance (BP 3015, Sartorius Germany).

2.2. Reagent and Chemicals

Meropenem trihydrate was provided as a gift from SDI company (Samarra, Iraq). 4-Chloro-7-nitrobenzofurazan (NBD-Cl) 98% (purchased from Baoji Guokang Bio-Technology Co.,Ltd (Baoji, china), Fifty milligrams of the NBD-Cl reagent were dissolved using 50 mL of methanol to prepare a solution labeled to contain 1 mg/mL of reagent. Aluminum foil was used to protect the solution from light. A borate buffer solution (0.2 M) of pH (7.0 – 11.0) was prepared by dissolving 1.237 gram of boric acid (H_3BO_3) together with 1.5 gram of potassium chloride (KCl) using 100 mL of distilled water. 0.2 M of sodium hydroxide was utilized to adjust the pH of the solution. Chemicals used were boric acid, sodium hydroxide, hydrochloric acid, potassium chloride, methanol, ethanol, butanol, 1,4-dioxan, acetonitrile, acetone, ethyl acetate, DMF, DMSO they were all brought from Merck. Meropenem trihydrate® 1 g vial was purchased from a local pharmacy and labeled to be manufactured by biomedics pharma Ltd. London. United Kingdom.

2.3. Standard Solution

A stock MRP solution with concentration 100 μg/mL was freshly prepared by dissolving 10 mg of MRP in distilled water in a 100 mL volumetric flask, followed by dilution to the mark using D.W. The working standard solutions containing 10 μg/mL were prepared from stock solutions by dilution. There is a six-day shelf-life of the stock solution when stored at 4 °C.

2.4. Recommended Procedure

To a series of 10 mL volumetric flasks, appropriate aliquots of the working standard solution (10 μg/mL) were accurately transferred using a micropipette to prepare solutions with drug concentration ranging from (25-650 ng/mL), Then, 0.75 mL of borate buffer solution (pH 9.0) and 0.5 mL of NBD-Cl reagent were added. The flask contents were gently mixed before being heated at 80 °C for 15 min in a thermostatically controlled water bath, then allowed to cool in an ice bath. After that, the cooled solution was acidified using 0.5 mL of concentrated HCl and then diluted to the mark using acetone. The FI of the

formed fluorophore was measured at 536 nm when excited at 471 nm against blank solution. Calibration curve was constructed by plotting the increase in FI as a function of meropenem concentration. The regression equations of the corresponding calibration curve were derived.

2.5. Procedure for Vials

A quantity of meropenem® vial powder equivalent to 10 mg meropenem trihydrate was precisely weighed and then dissolved in 25 mL distilled water in 100 mL calibrated flask, followed by dilution to the mark using distilled water to prepare a stock solution labeled to contain 100 μg/mL of MRP. This solution was further diluted with D.W to obtain a solution with final MRP concentrations of 100 ng/mL in the recommended procedure detailed in section (2.4). MRP concentrations were calculated using the linear regression equation.

2.6. Procedure for Spiked Human Plasma

Five milliliters of blood specimens were collected from five healthy human volunteers into a sodium heparin tube, which was then centrifuged for 30 minutes at 4000 rpm. Then, one milliliter of MRP standard solution was spiked with 1 mL of plasma in centrifuged tubes containing 3 mL acetonitrile as a proteins precipitant agent [29,30]. The obtained solution was completed with acetone to 10.0 mL, and then centrifuged again for 20 min at 4000 rpm. The obtained supernatant was further diluted to obtain a solution containing final MRP concentration within the range (50-200 ng/mL) in the recommended procedure detailed in section (2.4).

3. Results and Discussion

3.1. Fluorescence Spectra

In order to estimate the concentration of non-fluorescent pharmaceutical products spectrofluorometrically, a variety of fluorescent labeling compounds is used. NBD-Cl is one of the most fluorescence derivatizing reagents that has been utilized for quantifying a wide range of compounds with a secondary or primary amine moiety. Meropenem is a non-fluorescent drug that acts as a source of the secondary amino group. Under heated alkaline conditions, a nucleophilic aromatic substitution reaction occurred in which meropenem acted as a nucleophile which attacked an electron-poor aromatic molecule (NBD-Cl), resulting in the substitution of a leaving group (Cl) Figure 2. The reaction produces a highly fluorescent yellowish-colored adduct measured at 536 nm when excited at 471 nm Figure S3.

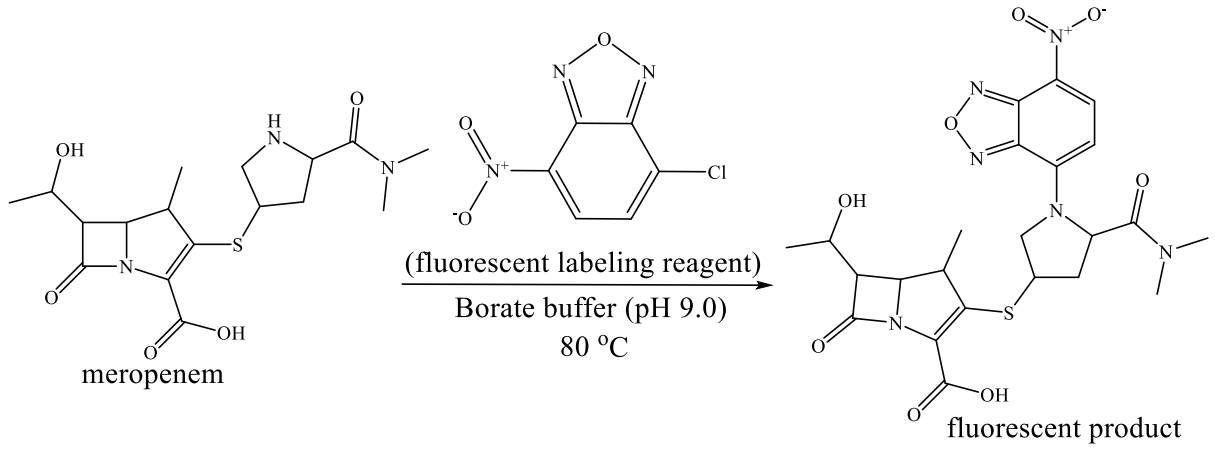

Figure 2. The proposed reaction route between meropenem and NBD-Cl reagent

3.2. Optimization of Experimental Conditions

The experimental parameters of the suggested method that affect the stability and intensity of the NBD-MRP product were carefully estimated and optimized. Each factor was investigated separately while the other parameters remained constant.

3.2.1. Effect of Reagent Volume

Different volumes of 1 mg/mL NBD-Cl (0.1-2.5 mL) were used and, as observed in Figure 4 (a), the fluorescence intensity increased with respect to the reagent volume until reaching a steady state point (0.5 mL) and there was no further increase in fluorescence intensity. As a result, 0.5 mL of reagent was chosen for subsequent experiments.

3.2.2. Effect of Buffer's Volume and pH

Different borate buffer solutions in the pH range (6.5-11.5) were used to check the influence of pH value upon the formation of the fluorescent product. As shown in Figure 4 (b), the intensity of the fluorescent product increased gradually with pH until reaching an optimal point at pH value 9.0, after which it decreased significantly owing to an increase in hydroxide ion concentration, which increased background signal through NBD-OH formation and hold-back the nucleophilic reaction between NBD-Cl and MRP. Different borate buffer volumes of pH 9.0 covering the range (0.2-2.0 mL) were employed. It was obvious from Figure 4 (b), that 0.75 mL of borate buffer volume exhibited a maximum fluorescence intensity. Decrease or increase in the buffer's volume leads to decrease in the FI of the fluorescent product, thus, 0.75 mL of buffer solution was considered as the optimum volume.

3.2.3. Effect of the Warming Time and Temperature

The temperature effect on the intensity of the fluorescent product was studied at various temperatures ranging from (40-90 ^{o}C) over a period of time. Figure 4 (C) shows that the maximum fluorescence intensity was observed at 80 ± 2 ^{o}C followed by a steady state region until 90 ^{o}C, above which there was a slight decline in fluorescence intensity. At 80 ^{o}C, various time intervals ranging from (2 to 40 min) were used to determine the optimal time required to complete the reaction. As shown in Figure 4 (C), the optimal intensity of the fluorescent product was achieved after 15 minutes at 80±2 ^{o}C.

3.2.4 Effect of Concentrated HCl Volume

It has been reported that NBD-Cl hydrolysis under alkaline heated conditions to yield a side product known as 4-hydroxy-7-nitro-2,1,3-benzoxadiazole (NBD-OH) [31], that has high fluorescence intensity at λ_{emi} 500-550 nm. Therefore, acidification of the reaction mixture before dilution was required to reduce the fluorescence intensity of the interfering background without affecting the NBD-MRP product and, as a result, improve sensitivity. Different volumes of concentrated HCl were used. The results reveal that the ideal HCl volume was 0.5 mL.

3.2.5 Effect of Dilution Solvents

Several dilution solvents were tested, including methanol, acetone, ethanol, butanol, 1,4-dioxan, acetonitrile, ethyl acetate, DMF, and DMSO, to find the solvent that reveals the highest intensity of the fluorescent product. The results show that the maximum intensity value was obtained after dilution with acetone, as shown in Figure 4 (d).

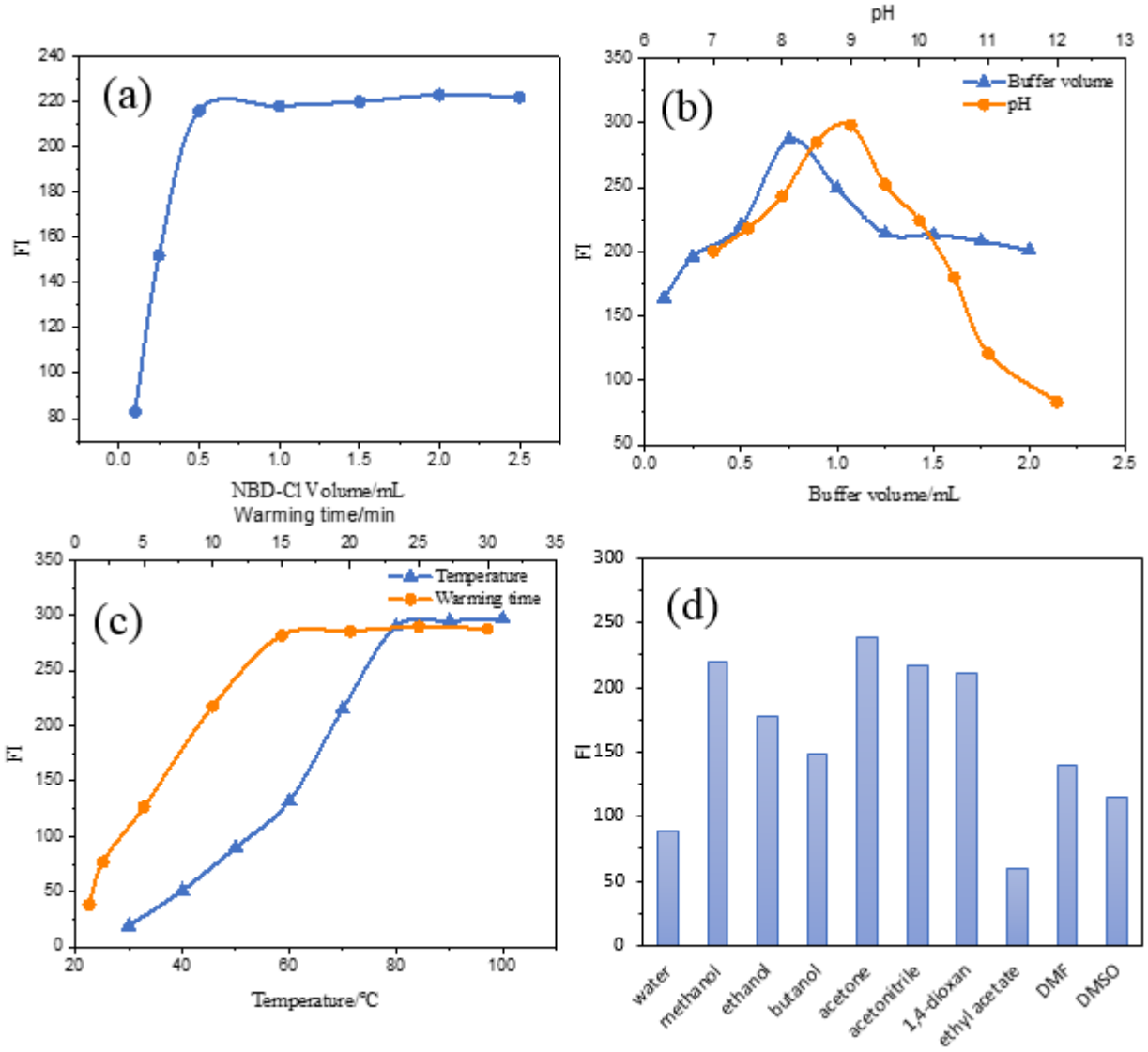

Figure 4. Effect of various experimental parameters on the FI of the NBD-MRP fluorophore

3.3. Validation of Method

The suggested approach has been validated according to International Conference on Harmonization (ICH) guidelines [32,33].

3.3.1. Linearity

The Calibration curve of the proposed method was constructed by plotting the fluorescence intensity at 536 nm (after excitation 471 nm) against its corresponding meropenem concentration. The calibration curve is linear in the concentration range of 25 to 650 ng/mL with an excellent correlation coefficient (r = 0.9981). Furthermore, the obtained results were statistically analyzed and the regression parameters were summarized in Table 1.

3.3.2. Limit of Quantitation (LOQ) and Limit of Detection (LOD)

LOQ and LOD of the proposed spectrofluorometric method were determined using the formula provided by the ICH Q2 (R1) recommendation. The determined values of LOQ and LOD indicate that the developed method was high sensitivity, as observed in Table 1.

Table 1. The Analytical parameters of the suggested spectrofluorimetric method.

Parameter	Validated Method
λ_{exc} (nm)	471
λ_{emi}(nm)	536
Concentration range (ng/mL)	25-650
Determination coefficient (r^2)	0.9964
Correlation coefficient (r)	0.9981
Intercept	45.96
Slope	0.8618
S.D of Intercept	8.74
S.D of slope	0.0145
LOD[a] (ng/mL)	3.15
LOQ[b] (ng/mL)	9.55

[a]LOD: Limit of detection; [b]LOQ: Limit of quantitation.

3.3.3. Accuracy

Three concentrations (75, 150, 250 ng/mL) have been used to verify the accuracy of the proposed method using five replications for each concentration. The data was expressed as percent recovery (R %) as illustrated in Table 2. The obtained results exhibit a high degree of agreement between calculated and true values. As a result, the suggested method is highly accurate.

Table 2. Accuracy data of the suggested spectrofluorimetric method

Sample	Concentrations ng/mL	%Recovery[a]± SD
1	75	99.69 ± 1.81
2	150	99.20 ± 1.92
3	250	99.34 ± 1.87

[a]Mean of five replicates

3.3.4 Precision

The analytical method precision was expressed as intermediate precision (inter-day precision) and repeatability (intra-day precision). The precision of the proposed method was checked by analyzing three distinct concentrations of MRP (100, 200,300 ng/mL) at successive time intervals on the same day and different day for intra-day precision and inter-day precision respectively, followed by measuring the fluorescence intensity of the solution whose concentration was calculated using the previously computed regression equation. The value of the calculated relative standard deviation (%RSD) was found to be less than 2 %, indicating acceptable precision as detailed in Table 3.

Table 3. Precisions results of the suggested spectrofluorimetric method.

Concentration (ng/mL)	Intra-day precision		Inter-day precision	
	%Recovery[a]	RSD	%Recovery[a]	RSD
100	98.90	0.99	99.83	1.19
200	99.00	0.52	100.16	0.52
300	99.80	0.77	100.27	0.42

[a]Mean of five replicates

3.3.5. Robustness

The proposed method's robustness was checked by measuring the fluorescence intensity after making a little variation in some experimental parameters such as pH, borate buffer volume, and reagent volume. The data was presented as relative standard deviation (RSD) and percent recovery (% R) as illustrated in Table 4, the results reveal that the low value of RSD (less than 2%) indicates that the little variation in experimental parameters has no significant effect on the determination of MRP.

Table 4. Robustness evaluation for the suggested spectrofluorimetric method

Experimental parameters	% Recovery[a] ± RSD
NBD-Cl Volume (mL)	
0.4	97.98 ± 0.87
0.5	98.44 ± 0.63
0.6	98.90 ± 0.99
Buffer pH	
8.8	99.83 ± 0.75
9	99.60 ± 0.63
9.2	99.14 ± 1.02
Warming time (min)	
12	97.51 ± 0.76
15	99.60 ± 0.98
18	100.30 ± 0.86

[a]Mean of five replicates

3.3.7. Estimation of Uncertainty

All the analytical procedures suffer from certain errors arising from different experimental sources that vary according to the analytical method. These sources include (experimental conditions, instrumentation, calibration, sampling, preparation of solutions, etc.). Estimation of uncertainty indicates how large these errors could be. Therefore, incorporating uncertainty together with the reported results is highly recommended. The main uncertainty contributions of the current method were considered as the standard preparation, slope of the calibration curve, recovery, and repeatability. Uncertainty measurement and sources were determined according to the EURACHEM/CITAC guide and reported methods [34-37]. The combined uncertainty was calculated using equation (1).

$$U_{combined} = C_m \times \sqrt{u^2{}_{standard} + u^2{}_{calibration} + u^2{}_{Recovery} + u^2_{repeatability}} \quad (1)$$

Where C_m represents drug concentration.

3.3.7.1. Uncertainty Associated to the Standard Preparation

Uncertainty related to the standard meropenem preparation is categorized into four terms: purity of meropenem; mass determination; molecular weight; and dilution. It has been found that the main uncertainty of the standard arises from the purity of meropenem, which is calculated according to equation (2).

$$U_{standard}\% = \frac{(100 - P\%)}{\sqrt{3}} = \frac{(100 - 98.00)}{\sqrt{3}} = 1.15\% \quad (2)$$

Where $P\%$ represents the purity of meropenem which is equal to 98.00% pure as reported in the certificate provided from the manufacture. Since the remaining three uncertainties of standard were of very small values, they were neglected.

3.3.7.2. Uncertainty Associated to the Slope of Calibration Curve

Origin 2018 software was used to plot the calibration curve and to determine the slope and standard error. The relative standard uncertainty ($U_{calibration}$) in calibration curve slope was calculated using equation (3).

$$U_{calibration}\% = \frac{standard\ error \times 100}{slope} = \frac{2.11}{0.8618} = 2.45\% \quad (3)$$

3.3.7.3. Uncertainty Associated to Recovery

To evaluate the uncertainty of recovery, meropenem was quantified in five individual aliquots of standard meropenem solution. The mean of the obtained recovery was found to be 98.67%. Therefore, the relative standard deviation, that is, relative standard uncertainty ($U_{Recovery}$) was 1.2%.

3.3.7.4. Uncertainty Associated to Repeatability

The relative standard deviation of data resulting from the determination of meropenem in pharmaceutical vials (section 3.3.7) was considered as the uncertainty of repeatability, $U_{repeatability} =$ 1.68%.

3.3.7.5. Expanded Uncertainty

According to equation 1, the uncertainty of the method was estimated as 3.40 %. Thus the Expanded uncertainty was calculated at 95% confidence level by multiplying the combined uncertainty $k = 2$ (coverage factor). The expanded uncertainty, $u_{expanded}$, is estimated as 6.8%.

3.3.7. Application in the Pharmaceutical Vial

The developed approach was successfully utilized for the quantification of MRP concentration in commercial vials (Meropenem trihydrate® 1 g vial). The obtained data were compared statistically to those obtained from the reported and official method according to t and F- tests at the 95% confidence level [38]. Table 5 shows that the values of t and f-test were lower than the corresponding critical (tabulated) values, denoting there was no statistically significant difference between the official or reported method and the suggested methods.

Table 5. Statistical comparison of data obtained by suggested spectrofluorometric method with the reported and official methods for quantification of meropenem in pharmaceutical vials

Parameter	Proposed Method	Reported Method	Official Method
% Recovery	99.48	100.55	100.18
S.D	1.67	1.56	1.32
Variance	2.81	2.44	1.75
Observation (***n***)	5	5	5
t test	$t_{\text{ critical}}$ = 2.3 (*p*= 0.05)	1.05	1.60
f test	$f_{\text{ critical}}$ = 6.38 (*p*= 0.05)	1.15	0.73

3.3.8. Application of the Method in the Spiked Human Plasma Samples

In order to prove the ultra-sensitivity of the proposed spectrofluorimetric method, different MRP concentrations (50, 75, 100, 150, and 200 ng/mL) were assayed in spiked human plasma. The data was presented as a percentage of recovery (% R), as shown in Table 6.

Table 6. Quantification of MRP in fresh human plasma by the proposed method

Added Concentration (ng/mL)	Found Concentration (ng/mL)	% Recovery[a] ± SD
50	48.78	98.02 ± 1.30
75	73.46	97.84 ± 1.64
100	98.90	98.90 ± 2.16
150	147.18	98.12 ± 1.30
200	196.84	98.42 ± 1.14

[a]Mean of five replicates

In conclusion, the presented paper describes the first spectrofluorimetric approach that has been utilized for quantification of meropenem in its pharmaceutical formulation and in human plasma. The obtained results of the suggested method, such as recovery (97.5-100), detection limit (3.15 ng/mL), warming time (15 min) and linearity (25-650 ng/mL), exhibit that the proposed approach is very sensitive, rapid, selective, reproducible and free of excipient interference. Furthermore, unlike chromatographic methods, which require costly hazardous solvents and sample pretreatment, these procedures are simple and low cost, allowing them to be used in routine analysis of meropenem in the quality control and clinical laboratories.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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