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Microbiological assay for the determination of meropenem in pharmaceutical dosage form

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Abstract

Meropenem is a highly active carbapenem antibiotic used in the treatment of a wide range of serious infections. The present work reports a microbiological assay, applying the cylinder-plate method, for the determination of meropenem in powder for injection. The validation method yielded good results and included linearity, precision, accuracy and specificity. The assay is based on the inhibitory effect of meropenem upon the strain of *Micrococcus luteus* ATCC 9341 used as the test microorganism. The results of assay were treated statistically by analysis of variance (ANOVA) and were found to be linear ($r = 0.9999$) in the range of 1.5–6.0 $\mu g\,ml^{-1}$, precise (intra-assay: R.S.D. = 0.29; inter-assay: R.S.D. = 0.94) and accurate. A preliminary stability study of meropenem was performed to show that the microbiological assay is specific for the determination of meropenem in the presence of its degradation products. The degraded samples were also analysed by the HPLC method. The proposed method allows the quantitation of meropenem in pharmaceutical dosage form and can be used for the drug analysis in routine quality control.

Keywords: Meropenem; Microbiological assay; Cylinder-plate method; Pharmaceutical dosage form

1. Introduction

Meropenem (Fig. 1), chemically (4R,5S,6S)-3-[[(3S,5S)-5-dimethylcarbamoyl pyrrolidin-3-yl]thio]-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3,2,0]hept-2-ene-2-carboxylic acid, is a new parenteral carbapenem antibiotic. It has a very broad spectrum of antibacterial activity against the majority of gram-positive and Gram-negative pathogens [1]. Meropenem is more active in vitro than imipenem against Enterobacteriaceae and *Pseudomonas aeruginosa*, but less active against Gram-positive cocci [2]. This antibiotic is more stable to ring opening by human renal dehydropeptidase I (DHP-I) than imipenem and consequently does not require concomitant administration of a DHP-I inhibitor. Meropenem has shown clinical efficacy in the treatment of a wide range of serious infections such as intra-abdominal infections and lower respiratory tract infections [3,4].

Several methods have been reported in the literature for the determination of meropenem and its main metabolite (ICI-213689) in biological fluids, including high performance liquid chromatography (HPLC) [5–8] and capillary zone electrophoresis [9,10]. Meropenem has been measured in pharmaceutical dosage form only by HPLC method [7,11,12]. Since this antibiotic is very used in the antimicrobial therapy, it is important that alternative methods for its determination in pharmaceutical dosage form are developed. Antibiotics can be measured by microbiological assay, evaluating their inhibitory effects on growth of the test microorganisms [11].

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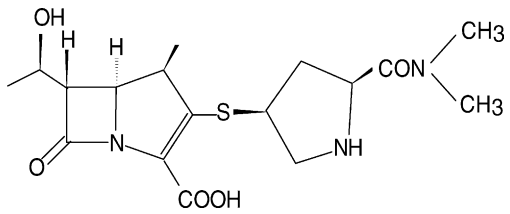

Fig. 1. The chemical structure of meropenem.

The microbiological assay can be an alternative method to HPLC. This assay can reveal subtle changes not demonstrable by conventional chemical methods [11]. Moreover, microbiological assay requires not only no specialized equipment but also no toxic solvents. The present study reports the development and validation of a stability indicating microbiological assay, applying the cylinder-plate method, for the quantitation of meropenem in powder for injection. A high performance liquid chromatographic (HPLC) method developed and validated in our laboratory [12] was chosen as a comparison method for the determination of meropenem in degraded samples.

2. Experimental

2.1. Chemicals

Meropenem reference standard was kindly supplied by Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan) and AstraZeneca (São Paulo, Brazil). Pharmaceutical dosage form containing meropenem was obtained commercially and was claimed to contain 500 mg (as anhydrous base) of the drug and 104 mg of the anhydrous sodium carbonate as excipient. Grove Randall number 11 agar and Grove Randall number 1 agar were obtained from Merck (Darmstadt, Germany). Sodium chloride was obtained from Quimibrás (Rio de Janeiro, Brazil). Distilled water was used to prepare all solutions in all experiments.

2.2. Microorganism and inoculum

The cultures of *Micrococcus luteus* ATCC 9341 were cultivated on Grove Randall number 1 agar at freezer and pealed to another Grove Randall number 1 agar (24 h before the assay) that was kept in stove at 37 °C. The bacteria were suspended in sodium chloride 0.9% using a glass homogenizer. A culture suspension of 25 ± 2% turbidity (transmittance) were obtained at 580 nm, using a spectrophotometer (Analyser-Model 800, São Paulo, Brazil) and a 10 mm diameter test tube as absorption cells against sodium chloride 0.9% as blank. Portions of 1 ml of the inoculated sodium chloride 0.9% were added to 100 ml of Grove Randall number 11 agar at 47 ± 2 °C and used as inoculated layer.

2.3. Preparation of the standard solutions

Accurately weighed 30 mg of meropenem reference standard was transferred to 100 ml volumetric flask and dissolved in distilled water (final concentration of 300 μg ml^{-1}). Aliquots of this solution were diluted in distilled water at concentrations of 1.5, 3.0 and 6.0 μg ml^{-1}, which were used in the assay.

2.4. Preparation of the sample solutions

The samples of powder for injection were prepared by the same procedure used for the reference standard.

2.5. Cylinder-plate assay

The agar was composed of two separate layers. Twenty millilitres of Grove Randall number 11 agar was poured into 100 mm × 20 mm petri dish as the base layer. After solidification portions of 5 ml of the inoculated layer was poured onto the base layer. Six stainless steel cylinders of uniform size (8 mm × 6 mm × 10 mm) were placed on the surface of inoculated medium. Three cylinders were filled with 200 μl of standard solutions (three concentrations) and the other three cylinders with the sample solutions (three concentrations). After incubation (37 °C for 24 h) the zone diameters of the growth inhibition were measured (mm) using an electronic digital caliper (Starret®). Six assays were performed (three assays a day) using eight plates in each one.

2.6. Calculation

The potency of meropenem in powder for injection was calculated by Hewitt equation [13]. The assay was treated statistically by the linear parallel model and by linear regression analysis. Analysis of variance (ANOVA) was used to verify the validity of the method.

2.7. Method validation

The method was appropriatelly validated by determination of the parameters linearity, precision, accuracy and specificity [14].

2.7.1. Linearity

The calibration curve was obtained with three doses of the reference standard. The linearity was evaluated by linear regression analysis, which was calculated by the least squares regression method.

2.7.2. Precision

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability was evaluated by assaying samples, at same concentration and during the same day. The intermediate

precision was studied by comparing the assays on different days.

2.7.3. Accuracy

The accuracy was determined by recovery of known amounts of meropenem reference standard (0.15, 0.3 and 0.6 μg ml^{-1}) added to the samples at the beginning of the process. An accurately weighted amount of powder for injection equivalent to 30 mg of meropenem was transferred to 100 ml volumetric flask and dissolved in distilled water (final concentration of 300 μg ml^{-1}). Aliquots of 1.0, 2.0 and 4.0 ml of this solution were transferred, respectively, into 200 ml volumetric flasks containing 1.0, 2.0 and 4.0 ml of meropenem standard solution (30 μg ml^{-1}) and distilled water was added to make up to volume to give final concentrations of 1.65, 3.3 and 6.6 μg ml^{-1}. These solutions were assayed and the percentage recovery of added meropenem standard was calculated.

2.7.4. Specificity

To show that the microbiological assay is specific, it was necessary to subject the analyte to specific conditions for degradation. The stability of meropenem in aqueous solution was checked by microbiological assay and HPLC method. For the microbiological assay, commercial samples (triplicate) of meropenem (500 mg) were reconstituted in 10 ml of ultrapure water (final concentration of 50 mg ml^{-1}) and stored at 25 °C and 40 °C for 12, 24, 36 and 48 h. Aliquots of these solutions were diluted in distilled water at concentrations of 6.0 μg ml^{-1} (samples stored at 25 °C) and 10 μg ml^{-1} (samples stored at 40 °C), which were assayed against freshly prepared solutions of reference standard and sample at concentrations of 1.5, 3.0 and 6.0 μg ml^{-1}. Each sample was analysed eight times. For the analysis by HPLC method, the degraded samples were diluted in ultrapure water at concentration of 50 μg ml^{-1}, which were assayed against freshly prepared solutions of reference standard and sample at same concentration.

2.8. Chromatographic conditions

The HPLC method was performed on a Shimadzu SCL-10A HPLC system, equipped with a model LC-10AD pump, ultraviolet–visible detector SPD-10A, Rheodyne injector fitted with a 20 μl loop and a integrator C-R6A chromatopac model (Shimadzu, Kioto, Japan). The method was conducted using a reversed-phase technique. Meropenem was eluted isocratically with a flow rate of 1.0 ml min^{-1} using a mobile phase consisting of 30 mM monobasic phosphate buffer and acetonitrile (90:10; v/v), adjusted to pH 3.0 with orthophosphoric acid. The wavelength of the ultraviolet–visible detector was set to 298 nm. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter (Millipore) and sonicated before use. A LiChrospher® 100 RP-18 column (250 mm × 4.0 mm i.d., 5 μm particle size) (Merck, Darmstadt, Germany) was used. The HPLC system was operated at 25 ± 1 °C.

3. Results and discussion

The development of analytical methods for the determination of drugs has received considerable attention in recent years because of their importance in pharmaceutical analysis. A microbiological assay was proposed as a suitable method for the determination of meropenem in pharmaceutical dosage form. The experimental conditions were adjusted to accurately determine the performance of the assay. A strain of *M. luteus* was found to be an appropriate test microorganism allowing quantitation of meropenem. The potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a reference standard [11]. The assay of antibiotics must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the two-log dose–response line of the preparations to be examined and the standard preparation must be linear over the range of doses used in the calculation [15]. The microbiological assay described in this work was performed in 3 × 3 design (three doses of standard and three doses of sample), according to the European Pharmacopoeia (2002). A direct relationship between the observed zone diameter and logarithm of applied dose normally is verified in the calculation. The mean zone diameters (mm) for standard solutions were: 17.39 (R.S.D. = 0.82) for dose of 1.5 μg ml^{-1}; 21.23 (R.S.D. = 0.44) for dose of 3.0 μg ml^{-1} and 25.04 (R.S.D. = 0.61) for dose of 6.0 μg ml^{-1} (Table 1). The calibration curve of meropenem was constructed by plotting log of concentrations (μg ml^{-1}) versus zone diameter (mm) and shows good linearity in the range of 1.5–6.0 μg ml^{-1} (Fig. 2). The representative linear equation was $y = 12.708x \pm 15.158$, where x = log of concentration and y = zone diameter. The correlation coefficient ($r = 0.9999$) was highly significant for the method. The validity of the assay was verified by means of the ANOVA. According to ANOVA, there is no deviation from parallelism ($F_{calculated} < F_{critical}$; $p = 0.01$) and there is linearity ($F_{calculated} < F_{critical}$; $p = 0.01$). The experimental values obtained for the determination of meropenem in samples are present in Table 2. The precision of the assay was deter-

Table 1
Experimental values of diameter zone of inhibition for meropenem standard solutions obtained by microbiological assay

Concentration (μg ml^{-1})	Range of zone size	Mean diameter zone of inhibition[a] (mm) ± R.S.D. (%)
1.5	17.2–17.6	17.39 ± 0.82
3.0	21.1–21.3	21.23 ± 0.44
6.0	24.9–25.3	25.04 ± 0.61

[a] Mean of six assays with eight plates in each one.

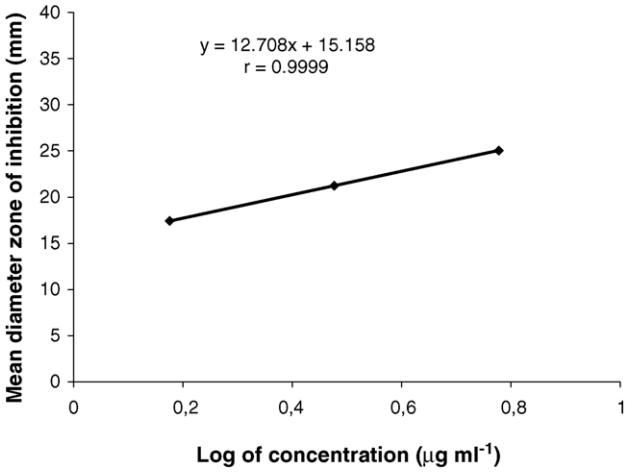

Fig. 2. Calibration curve for meropenem obtained by the microbiological assay. The best line calculated by the method of least squares is shown.

Table 2
Results of the determination of meropenem in powder for injection by microbiological assay

Sample (mg) powder for injection	Experimental amount[a] (mg)	Purity (%)	R.S.D. (%) Intra-assay	R.S.D. (%) Inter-assay
500	501.40	100.28	0.29	0.94
	487.95	97.59		
	494.10	98.82		
	499.20	99.84		
	496.95	99.39		
	496.50	99.30		

[a] Mean of eight determinations.

mined by repeatability (intra-assay) and intermediate precision (inter-assay) and was expressed as the relative standard deviation (R.S.D.) of a series of measurement. In the microbiological assay, the number of replications per dose must be sufficient to ensure the required precision. Furthermore, the assay may be repeated and the results combined statistically to obtain the required precision [15]. The repeatability was studied by determination of the samples in three assays, at the same concentration, during the same day under the same experimental conditions. The result obtained shows R.S.D. of 0.29 indicating good intra-assay precision. Inter-assay variability was calculated from assays on 2 days and shows R.S.D. of 0.94. The accuracy of the assay was studied. The mean recovery was calculated and was found to be 102.01% (Table 3). The mean potency of the sample determined by the microbiological assay (496 mg) was compared statistically with the mean potency determined by the HPLC method (497.85 mg) [12] using the Student's *t*-test, which indicated there is no significant difference between two methods at 5% significant level.

Table 3
Experimental values obtained in the recovery test for meropenem in powder for injection by microbiological assay

Sample concentration ($\mu g\,ml^{-1}$)	Concentration of added standard ($\mu g\,ml^{-1}$)	Percentage recovery[a] ± R.S.D. (%)
1.5	0.15	101.93 ± 2.17
3.0	0.3	102.49 ± 1.27
6.0	0.6	101.63 ± 1.94

[a] Mean of ten determinations.

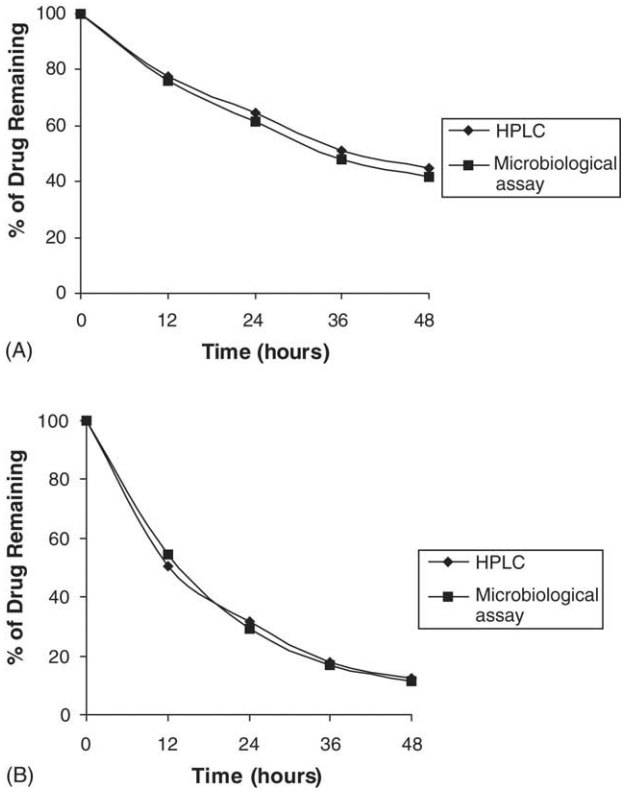

Fig. 3. Degradation profile of meropenem commercial samples (500 mg) reconstituted in aqueous solution and stored at 25 °C (A) and 40 °C (B). Initial potency of meropenem: 50 mg ml^{-1}. The samples were stored in triplicate.

The specificity of the proposed microbiological assay was studied by analysis of degraded samples, using the HPLC method as a comparison method. The results obtained demonstrate that occur a rapid degradation of meropenem in aqueous solution at 25 and 40 °C. The results were expressed as percentage of drug remaining. Meropenem was less stable at 40 °C with a mean degradation of 70% after 24 h, whereas a

Table 4
Results of mean potency of meropenem in commercial samples reconstituted in aqueous solution after storage at 25 °C and 40 °C obtained by microbiological assay and HPLC method

Time (h)	Mean potency[a] of samples stored at 25 °C (%)		Mean potency[a] of samples stored at 40 °C (%)	
	HPLC	Microbiological assay	HPLC	Microbiological assay
0	100	100	100	100
12	77.48	76.03	50.4	54.3
24	64.73	61.69	31.53	29.38
36	51.1	48.07	17.91	16.61
48	44.58	41.88	12.52	11.26

Initial potency of meropenem: 50 mg ml^{-1}.
[a] The samples were analysed in triplicate.

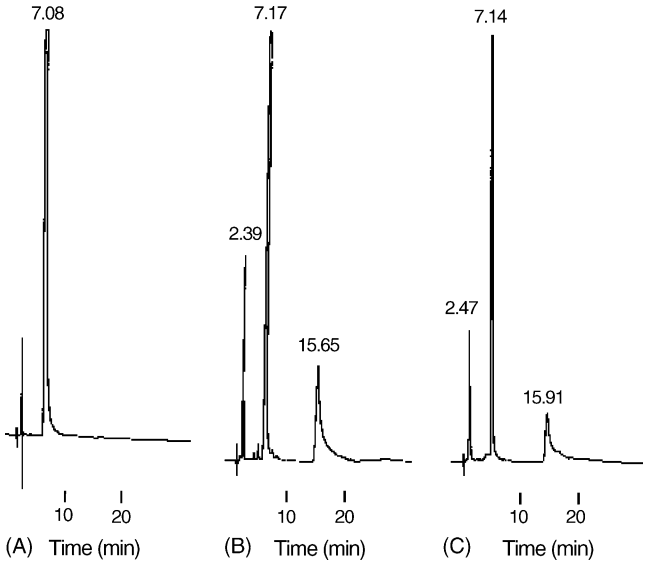

Fig. 4. Chromatograms obtained from the analysis of fresh and degraded samples of meropenem using the HPLC method. (A) Analysis of a fresh sample, (B) analysis of a reconstituted sample stored at 25 °C for 48 h, (C) analysis of a reconstituted sample stored at 40 °C for 48 h. The samples were assayed at concentration of 50 μg ml^{-1}. The peaks of degradation products can be observed at retention time of 2.5 and 15 min, approximately, separated from meropenem (retention time of 7.0 min, approximately).

mean degradation of 30% was observed after 24 h at 25 °C. The degradation profile (Fig. 3 and Table 4) verified by the two methods was very similar, suggesting that degradation products are inactive. Thus, the microbiological assay described is specific for the determination of meropenem in the presence of degradation products and can be used alternatively to HPLC method. Fig. 4 shows representative chromatograms obtained from the analysis of fresh and degraded samples of meropenem using the HPLC method. As shown in this figure, two major degradation products are observed at retention time of 2.5 and 15 min, approximately, well separated from meropenem (retention time of 7.0 min). These degradation products were observed in the two conditions of storage related. The results obtained in this preliminary stability study reveal that meropenem is very suscetible to thermal degradation. More studies should be developed for isolation and structural elucidation of these degradation products in order to establish a probable degradation route.

Although the biological assays have a high variability, the results obtained in this assay were very satisfactory. Performed validation proved that microbiological assay is a good method for pharmaceutical analysis of meropenem in powder for injection.

4. Conclusions

The proposed microbiological assay for the determination of meropenem in pharmaceutical dosage form is linear, precise and accurate. The results obtained in the preliminary stability study shows that no degradation products interfere with the determination of the drug, indicating that the proposed method is specific for the determination of meropenem in powder for injection. Hence, this assay allows reliable quantitation of meropenem in pharmaceutical dosage form and can be an alternative method for the drug analysis in routine quality control.

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