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Thermal and alkaline stability of meropenem: Degradation products and cytotoxicity

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Abstract

The stability of the broad-spectrum antibiotic meropenem was investigated in order to isolate and elucidate the mean degradation products involved in thermal and alkaline decomposition of meropenem in solution. The purification of thermal degradation product (45 °C) involved a combination of preparative chromatographic techniques. The degradates were characterized by NMR and ESI-MS. The thermal degradation product was a result of several chemical reactions, with modification of side chain and β-lactam ring, resulting in a pyrrolic derivative. Under alkaline conditions (NaOH 0.1 N), meropenem was converted totally to the corresponding β-lactam ring-opened derivative, in sodium salt state. The degraded samples of meropenem reconstituted solution, powder for injection and alkaline solution was also studied in order to determine the preliminary cytotoxicity *in vitro* against mononuclear cells. The results obtained indicated that samples could be toxic in high concentration (2.0 mg/mL) after 48 h of incubation. The present study confirms the lability of the drug in aqueous solution, specially when submitted to thermal and alkaline conditions. Thus, it is necessary attention during the handling and storage of this antibiotic.

Keywords: Meropenem; Thermal and alkaline degradation; Identification of degradation products; Cytotoxicity *in vitro* assay

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 synthetic 1β-methylcarbapenem that is structurally related to β-lactam antibiotics such as penicillins and cephalosporins (Blumer, 1997; Dalhoff et al., 2006). It has activity against a wide range of Gram-negative and Gram-positive aerobic and anaerobic bacteria. Meropenem shows stability against both β-lactamase and human renal dehydropeptidase I (DHP-I), and therefore is not combined with inhibitors such as cilastatin, for protection against enzymatic hydrolysis (Wiseman et al., 1995; Blumer, 1997; Pfaller and Jones, 1997). Meropenem is supplied as a 0.5 or 1.0 g sterile lyophilized powder for either i.v. or intramuscular injection after reconstitution with appropriate infusion solutions.

Stability studies of carbapenem antibiotics have been examined and the degradation under variable conditions has been performed (Takeuchi et al., 1995; Sajonz et al., 2001; Mendez et al., 2005; Zajac et al., 2007). Hydrolysis of the strained ring system is a very important characteristic of the molecule that accounts for the instability of carbapenem antibiotics in water and leads to the ring-opened hydrolysis degradation product (Takeuchi et al., 1995; Sajonz et al., 2001; Cai and Hu, 2005). Like all β-lactam antibiotics, aqueous solution of meropenem is unstable and subject to hydrolytic degradation (Takeuchi et al., 1995; Cai and Hu, 2005). The identified degradation products of meropenem were the β-lactam hydrolyzed product (meropenemic acid) and the polymers products (dimers and trimers) resulting from intermolecular aminolysis of β-lactam ring by the amine of the second molecule (Cai and Hu, 2005). To separate meropenem and its degradation products gel filtration chromatography and RPLC–MS were used. In a recent

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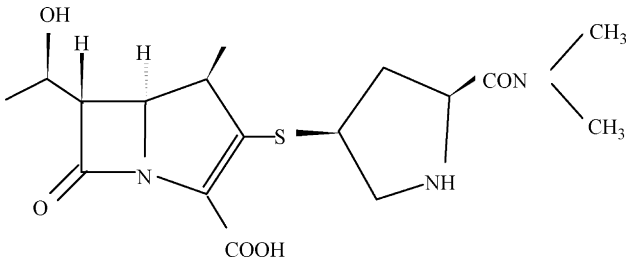


Fig. 1. The chemical structure of meropenem.

report, the thermal degradation kinetics of meropenem in aqueous solution and in solid state was studied. The degradation of meropenem followed first-order kinetics, and the drug was found to degrade extensively after reconstitution, with formation of some degradation products (Mendez et al., 2006).

The study of degradation products formation in the pharmaceutical formulations, their isolation, and characterization is a very important area, since it can help to understand the decomposition patterns of the drug molecule, what is a valuable information about its stability. Such information is used for determining storage and packaging conditions of the bulk and formulated drug substance. According to ICH (ICH, 2000), the stress testing of the drug substance can help to identify the likely degradation products, which can help establish the degradation pathways.

Considering the extensive degradation of meropenem in aqueous solutions and the possibility to identify novel degradation products, the aim of the present work was to study the stability of meropenem under forced degradation conditions. Decomposition of meropenem under thermal and alkaline conditions was conducted, and the main degradation products were identified using MS and NMR techniques. From the final structures of isolated products, a degradation route with probable reactions was proposed. The degraded samples were also evaluated to determine the preliminary cytotoxicity *in vitro* against mononuclear cells. In agreement with ICH (ICH, 2006), it is important to evaluate the biological safety of an individual impurity or a given impurity profile, including degradation products.

2. Materials and methods

2.1. Materials and instrumentation

Meropenem reference standard was kindly supplied by Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan) and AstraZeneca (São Paulo, Brazil). Pharmaceutical dosage form (Meronem®) 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. Acetonitrile for chromatography, analytical grade potassium dihydrogenphosphate, orthophosphoric acid, sodium hydroxide, methylene chloride and methanol were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore).

The HPLC method (Mendez et al., 2003) was performed on a Shimadzu LC 10 AD VP pump, SCL-10A VP system controller, autosampler, CTO-10AC VP column oven, and SPD-M10A diode array detector (Shimadzu, Kyoto, Japan). The method was conducted using a reversed-phase technique. Meropenem was eluted isocratically with a flow rate of 1.0 mL/min 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 DAD detector was set to 298 nm (for quantitative analysis) and 220 nm (for qualitative analysis). The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter (Millipore) and sonicated before use. A MetaChem® LC RP-18 column (250 mm × 4.6 mm i.d., 5 μm particle size) (MetaChem Technologies, Torrance, USA) was used. The HPLC system was operated at 25 ± 1 °C.

Nuclear magnetic resonance (NMR) spectra were recorded using a Varian INOVA 500 spectrometer (Palo Alto, CA, USA), operating at 500 MHz. Samples for NMR were dissolved either in methanol-*d* or DMSO-*d* (Aldrich) and spectra were obtained using tetramethylsilane as internal reference. Mass spectrometry (MS) analyses were conducted using a Micromass Quattro spectrometer equipped with an electrospray ion (ESI) source operating in positive ion mode and MassLinx 3.5 data processor.

Silica aluminized plates GF_{254}, silicagel for column chromatography (0.062–0.200 mm) and silicagel for thin layer chromatography were obtained from Merck (Darmstadt, Germany).

2.2. Isolation of degradation products

2.2.1. Thermal degradation

Meropenem aqueous solution (50 mg/mL) was stored at 45 °C for 36 h. The degradation product was isolated by column chromatography and preparative thin layer chromatography. The degraded samples were dried by concentrator centrifuge Speed Vac Univapo 100H and the solid was transferred to the chromatographic column top. Methylene chloride was used as the first eluent. The separation was carried out by the addition of 100 mL portions of mixtures of methylene chloride and methanol (increasing 4% of methanol in each portion until reaching the composition 40:60, v/v). Each fraction was analyzed by thin layer chromatography (TLC) applying methylene chloride –methanol, 95:05 (v/v), as mobile phase and revelation by UV 254 nm light. Those which showed to have degradation products were analyzed also by HPLC. The fractions containing the main degradation product were mixed and purified by preparative TLC, using the same conditions described above. The spot corresponding to the degradation product (Rf = 0.71) was removed and extracted with mixture of methylene chloride–methanol, 50:50 (v/v) (three portions of 10 mL). The solvent of this final solution was distilled to dryness under reduced pressure, and the solid was stored.

2.2.2. Alkaline degradation

Meropenem aqueous solution (1.0 mg/mL) was prepared using sodium hydroxide 0.1 N as solvent. The samples were stored at 25 °C. Aliquots were collected in different times and analyzed by HPLC. For the alkaline degradation product, it was not necessary to procedure the isolation step, since only one

degradation product was observed in the chromatographic analysis. So, the identification was performed directly in the total degraded sample.

2.3. Identification of degradation products

The identification of the isolated degradation products was carried out by ^{1}H and ^{13}C NMR spectroscopy. Two-dimensional spectra COSY and HSQC were also obtained. For basic degradation product, the identification was also performed by MS analysis. The NMR and MS spectra of meropenem reference standard were also determined under the same conditions.

2.4. Cytotoxicity assay

The degraded samples of meropenem aqueous solution (50 mg/mL, stored at 45 °C for 36 h), powder for injection (500 mg, stored at 90 °C for 90 days) and alkaline solution (1.0 mg/mL, stored at 25 °C for 10 min) were diluted in RPMI 1640 medium (Sigma) immediately before use at 1.0, 2.0 and 4.0 mg/mL.

Human mononuclear cells were separated from the peripheral blood of three healthy donors, after receiving their written informed consent. Heparinized venous blood was diluted 4:3 with Hank's medium (Sigma). Mononuclear cells were isolated by plasma separation on Ficoll-Paque (Amersham) gradient and washed twice in Hank's medium. The total cells were counted in Neubauer chamber by tripan blue exclusion. Mononuclear cells were washed and resuspended in RPMI to a concentration of 10^6 viable cells in 1.0 mL. These cell suspensions were dispensed in 96-well plates (100 μL in each well), and the samples were immediately added (100 μL in each well) on that cells. In each well the final concentration analyzed of each sample was 0.5, 1.0, 2.0 mg/mL, in triplicate. Controls with mononuclear cells and meropenem standard were included. After addition of the samples, the plates were cultivated in a humidified 5% CO_2 incubator at 37° for 24, 48 and 72 h. Cell viability was determined by flow cytometry after addition of propidium iodide. FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) using CellQuest Software. At least 5000 events were collected. WinMDI 2.8 software was used to obtain the final results. In this work, it will be considered cytotoxic the samples that present a minim reduction of cell viability of 50%.

3. Results and discussion

Stability studies have received considerable attention in recent years because of their importance in development and quality control of pharmaceutical products. They make part of a development strategy under ICH requirements and should be carried out to establish the inherent or intrinsic stability characteristics of the molecule in a variety of suggested degradation conditions. Moreover, the degradation forced study may be extended to establish the degradation pathways and to identify the mean degradation products (ICH, 2000).

In this study, thermal and alkaline stability of meropenem in aqueous solution was carried out through the employment of stress conditions. It is well known that carbapenem antibiotics are unstable in solution because of the highly strained fused ring system. Nucleophiles can attack the carbapenem ring and lead to its opening, primarily forming the ring-opened hydrolysis product when in aqueous solution (Rovatti et al., 1998)

In agreement with previously reported data (Mendez et al., 2006), meropenem undergoes extensive degradation in thermal conditions, producing a large number of compounds as seen by HPLC detection (Fig. 2). As described in Section 2, several attempts should be carried out in order to find appropriate techniques for the isolation and purification of the thermal degradation products. Column chromatography and preparative TLC were applied using different mixtures of methylene chloride–methanol as eluent. Although the collected fractions were impure, the posterior purification by preparative TLC allowed the isolation of the thermal degradation product (DP1). The purity of this one was evaluated by TLC and HPLC analysis. The chromatogram obtained by HPLC assay demonstrates

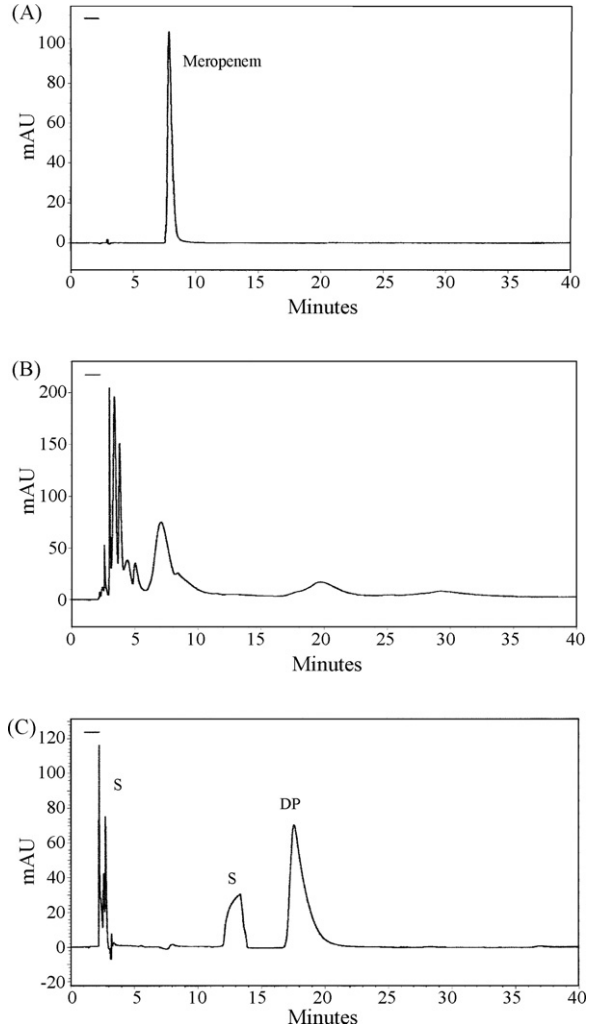


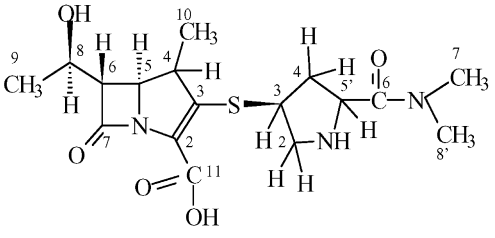
Fig. 2. Chromatoghrams showing decomposition of meropenem in reconstituted sample and isolated thermal product (detection at 220 nm). *Key*: (A) meropenem control; (B) meropenem reconstituted sample (50 mg/mL) exposed at 45 °C for 36 h; (C) Isolated thermal product (DP1) (S: extraction solvents – methanol and chloroform).

one peak in retention time of 19 min (Fig. 2), indicating that compound is relatively lipophilic.

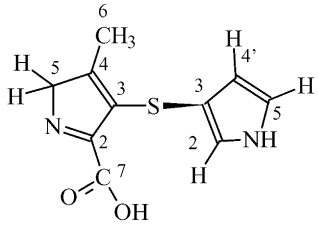
The structure of thermal degradation product was proposed according to the results obtained by NMR spectroscopy. Two-dimensional spectra (COSY and HSQC) were very helpful for the identification. The ^{1}H and ^{13}C NMR spectra were interpreted by comparing the chemical shifts of meropenem standard with those of degradation product, as shown in Table 1. The chemical shifts are in good agreement for the proposed structure. The thermal decomposition applied in this present work allowed a drastic molecular modification. The absence of many signals present in meropenem spectra confirms this purpose. Fig. 3 shows the proposed structure of degradation product (4-methyl-3-(1H-pyrrol-3-ylsulfanyl)-5H-pyrrole-2-carboxylic acid) and the possible route of decomposition. The intermediate reactions are suggested considering the susceptibility of chemical bonds. The modification in side chain of meropenem could be explained by descarboxylation and aromatization of pyrrolidinic ring, resulting in a pyrrolic ring. In the β-lactam ring, a first hydrolysis is suggested, following the elimination of alcoholic chain by cleavage of carbanion ion (enolate anion) formed in an intermediate equilibrium.

In alkaline conditions, the degradation of meropenem was immediate and only one degradation product (DP2) was formed, which have short retention time (3.0 min, Fig. 4) compared with the meropenem peak, suggesting it is a highly polar (hydrophilic) compound. A preliminary assay using acid solution (chloridric acid 0.1 N) indicates a formation of the same degradation product. Considering these results, the identification of the basic degradation product was performed in the degraded sample, without isolation procedure. The ring-opened compound was expected to be the basic and acid product because its formation involved the cleavage of the labile β-lactam bond. In specific study about quantification of meropenem metabolites, it was found a ring-opened metabolite with the same retention time (Blumer, 1997). This degradation product was also detected in a study about development of HPLC method for quantification of meropenem in pharmaceuticals, when the sample was treated with sodium hydroxide 0.01 N (Mendez et al., 2003). From the ^{1}H and ^{13}C NMR data (Table 2), and COSY and HSQC correlation spectra, it was possible to elucidate the basic degradation product. The comparison of the chemical shifts with meropenem spectra indicates that structure is similar, with a short modification, probably in the sensible β-lactam ring. In order to confirm the structure of basic compound, the MS measurements were performed. The analyses were conducted using the direct insertion technique, without coupling with LC method. The mass spectrum of

Table 1
^{1}H and ^{13}C NMR chemical shift assignment for meropenem and its thermal degradation product



Meropenem



Thermal degradation product

Assignments	Chemical shifts (ppm)			
	Meropenem		Thermal degradation product	
	^{1}H	^{13}C	^{13}H	^{13}C
Solvent	DMSO	DMSO	DMSO	DMSO
2	–	135.82	–	131.57
3	–	132.99	–	129.48
4	2.25 (m)	44.70	Absent	128.63
5	3.85 (m)	66.22	3.30 (s)	38.05
6	2.65 (m)	52.95	1.23 (s)	28.95
7	–	167.39	–	163.18
8	4.00 (m)	72.26	Absent	Absent
9	1.10 (d)	20.20	Absent	Absent
10	0.82 (d)	14.61	Absent	–
11	–	174.41	–	–
2′	2.90 (m); 3.05 (m)	55.37	6.86 (m)	120.96
3′	3.90 (m)	65.10	Absent	124.69
4′	1.24 (m); 2.40 (m)	37.28	6.11 (m)	108.47
5′	3.80 (m)	57.48	6.53 (m)	112.22
6′	–	164.44	–	Absent
7′	2.85 (s)	35.18	Absent	Absent
8′	2.95 (s)	36.13	Absent	Absent

s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

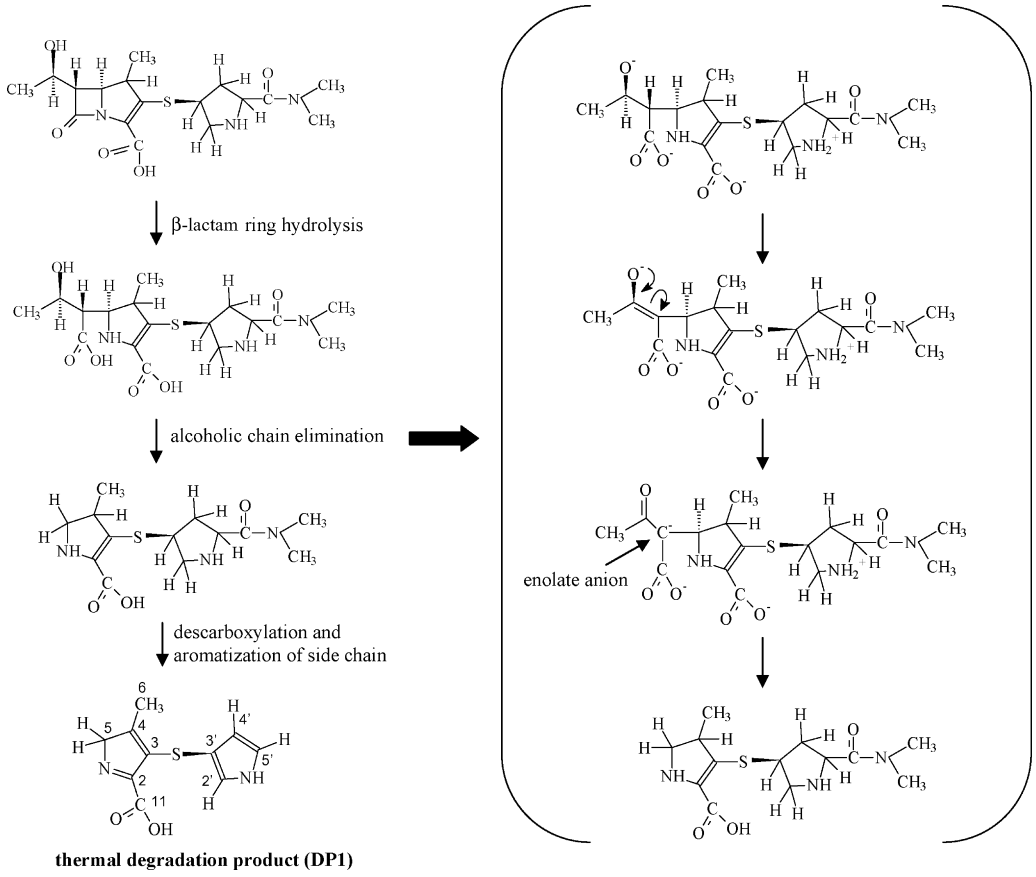


Fig. 3. Thermal decomposition route of meropenem aqueous solution and the chemical structure of identified degradation product (DP1).

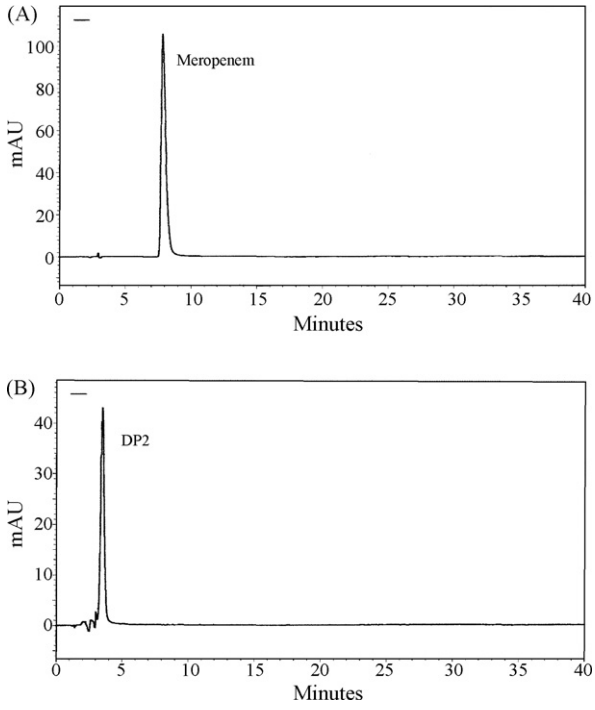
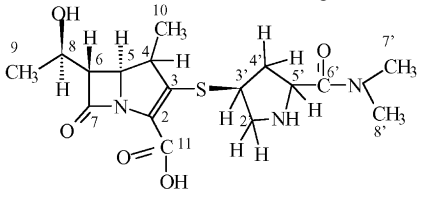


Fig. 4. Chromatoghrams showing decomposition of meropenem in aqueous solution submitted to alkaline catalysis (NaOH 0.1 N). *Key*: (A) meropenem control; (B) meropenem aqueous solution after alkaline treatment (DP2: alkaline degradation product).

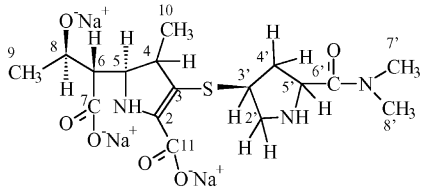
meropenem had an ion at 384 Da, which agrees with the molecular weight $[M+H^+]$. For the described basic degradation product, the ESI-MS gave an abundant $[M+H^+]$ ion at *m/z* 468 (Fig. 5), suggesting an unexpected structure. The complete examination of the MS and NMR spectra data indicates that the structure should be a sodium salt (3-(5-dimethylcarbamoyl-pyroolidin-3-ylsulfanyl)-4-methyl-5-(2-propyl-sodium alcoxyde)-4,5-dihydro-1H-pyrrol-5-(1)-2-carboxylic acid disodium salt) formed after the hydrolysis of β-lactam ring. The hydrolytic reaction is possible because meropenem is in alkaline solution, providing a nucleophilic attack lactam bond. The ionization in carboxylic and alcoholic groups is allowed because the presence of sodium hydroxide in the sample during the degradation and the spectroscopy analysis. The sodium salt in the position 8 can be formed in high concentration of alkaline reagent, as occurred in this assay. Compared with thermal degradation product, it was observed that the side chain in the alkaline compound was maintained intact. Although the aqueous medium provides hydrolytic reactions, the ambient temperature (25 °C) used in decomposition did not allow the extensive degradation of the drug.

The cytotoxicity assay against mononuclear cells was developed in order to evaluate the probable cytotoxic potential of thermal and alkaline degraded samples. This assay also includes the analysis of meropenem powder for injection submitted to degradation in solid state. The experiments against mononuclear cells and polymorphonuclear cells have been performed

Table 2
^{1}H and ^{13}C NMR chemical shift assignment for meropenem and its alkaline degradation product



Meropenem



Alkaline degradation product

Assignments	Chemical shifts (ppm)			
	Meropenem		Alkaline degradation product	
	^{1}H	^{13}C	^{1}H	^{13}C
Solvent	MeOH	MeOH	MeOH	MeOH
2	–	74.30	–	129.21
3	–	68.13	–	129.91
4	3.18 (m)	43.84	2.51 (m)	47.18
5	4.10 (m)	57.55	4.32 (m)	74.30
6	3.21 (m)	61.18	2.55 (m)	57.11
7	–	174.75	–	175.04
8	4.01 (m)	66.67	4.02 (m)	69.75
9	1.18 (d)	21.85	1.26 (d)	22.09
10	1.11 (d)	16.76	1.05 (d)	15.75
11	–	175.46	–	180.33
2′	3.20 (m); 3.56 (dd)	53.09	3.09 (m); 3.18 (m)	54.90
3′	3.84 (m)	42.44	3.67 (m)	45.66
4′	1.66 (m); 2.87 (m)	35.42	1.43 (m); 2.62 (m)	38.82
5′	4.60 (t)	59.60	3.94 (m)	58.91
6′	–	168.85	–	169.30
7′	2.90 (s)	36.21	2.94 (s)	36.09
8′	2.96 (s)	37.03	3.04 (s)	37.05

s: singlet; d: doublet; dd: double doublet; t: triplet; m: multiplet.

to evaluate the effects of meropenem in the bactericidal activity and immunomodulation (Cornacchione et al., 2000; Novelli et al., 2000). These effects are observed for β-lactams antibiotics (Labro, 1998).

The results obtained (Table 3) indicate that the degraded samples analyzed did not present cytotoxic effects against the assayed mononuclear cells after 24 h of exposure. In contrast, the degraded solid sample and alkaline solution at 1.0 mg/mL, after 48 h of incubation, present cytotoxic effects with a reduction of cell viability in 41.17 and 16.12%, respectively. This effect was also observed for the degraded reconstituted sample at 2.0 mg/mL, after 48 h of incubation. Considering that the factors absorption, distribution and excretion are not present in *in vitro* tests (ICH, 1995), the absence of cytotoxic effect in the

Table 3
Results of cell viability obtained by *in vitro* cytotoxic assay for meropenem and degraded samples (powder for injection, reconstituted sample and alkaline solution) against mononuclear cells

Concentration (mg/mL)	Cell viability (%)[a] ± S.D. (incubation time 24 h)				
	Meropenem	Degraded solid	Degraded reconstituted sample	Degraded alkaline sample	Control
0.5	98.40 ± 0.61	98.16 ± 0.28	98.15 ± 0.45	98.25 ± 0.38	98.38 ± 0.53
1.0	98.47 ± 0.56	96.80 ± 1.12	98.09 ± 0.27	97.34 ± 1.01	
2.0	97.71 ± 0.81	59.83 ± 6.67	95.06 ± 2.07	84.82 ± 10.73	
Concentration (mg/mL)	Cell viability (%)[a] ± S.D. (incubation time 48 h)				
0.5	96.79 ± 0.50	94.45 ± 0.88	95.62 ± 0.97	89.89 ± 5.07	96.74 ± 0.64
1.0	95.91 ± 0.68	41.17 ± 12.22	69.32 ± 9.77	16.72 ± 7.42	
2.0	83.23 ± 11.29	4.04 ± 2.47	20.55 ± 3.00	3.69 ± 1.19	
Concentration (mg/mL)	Cell viability (%)[a] ± S.D. (incubation time 72 h)				
0.5	92.51 ± 2.64	75.47 ± 6.70	84.22 ± 7.01	19.98 ± 6.69	95.01 ± 0.92
1.0	63.98 ± 15.26	8.41 ± 2.18	26.82 ± 6.94	3.60 ± 2.08	
2.0	14.33 ± 1.92	1.62 ± 0.45	4.40 ± 2.16	2.63 ± 1.36	

[a] Mean of three analyses.

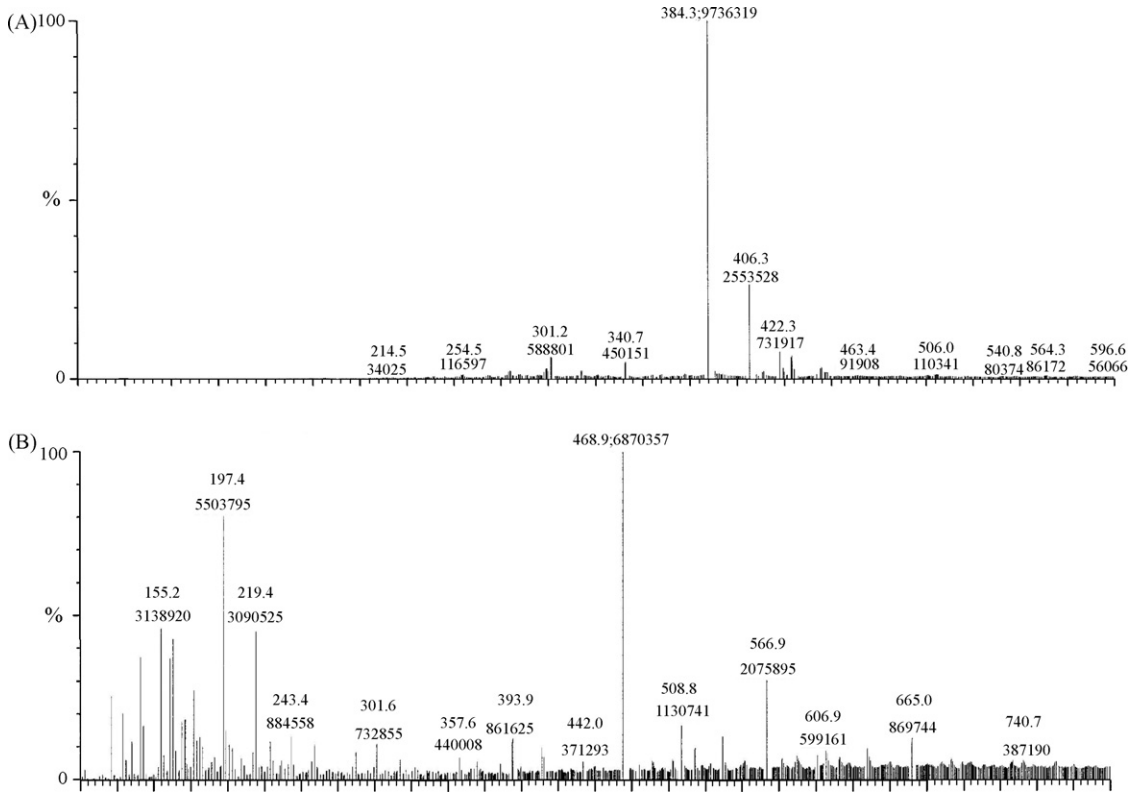


Fig. 5. Mass spectra (ESI) of meropenem (A) and alkaline degradation product-DP2 (B).

concentrations tested at 24 h suggests the same result *in vivo*. Despite of this result, it is important to conduct these biological assays in stability studies.

4. Conclusions

Meropenem demonstrated to be unstable in aqueous solution when submitted to thermal and alkaline treatment. The thermal degradation product isolated by preparative TLC and column chromatography was the *n*-pyrrol derivative originated from the hydrolysis, descarboxylation and alkyl chain elimination reactions. The alkaline degradation product, identified by NMR and MS analysis, was a sodium salt derivative formed from β-lactam ring hydrolysis and ionization of carboxyl and alcoholic groups. The *in vitro* cytotoxicity assay against mononuclear cells demonstrated that thermal and alkaline degraded samples must be better evaluated for their safety. The instability of meropenem showed by the present studies indicates that special care must be taken to avoid exposure of the drug to the degradation conditions during the handling and storage of the pharmaceutical preparation.

Acknowledgements

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