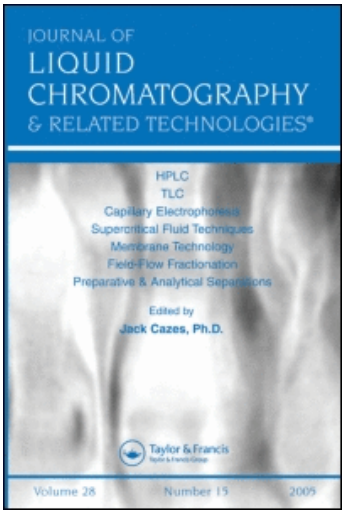

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PREPARATION, ISOLATION, AND CHARACTERIZATION OF DIMERIC DEGRADATION PRODUCTS OF THE 1β-METHYLCARBAPENEM ANTIBIOTIC, ERTAPENEM

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PREPARATION, ISOLATION, AND CHARACTERIZATION OF DIMERIC DEGRADATION PRODUCTS OF THE 1β-METHYLCARBAPENEM ANTIBIOTIC, ERTAPENEM

P. Sajonz,[1,*] T. K. Natishan,[1] Y. Wu,[1] J. M. Williams,[1] B. Pipik,[1] L. DiMichele,[1] T. Novak,[1] S. Pitzenberger,[2] D. Dubost,[2] and Ö. Almarsson[†]

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ABSTRACT

Dimeric degradates of ertapenem, a novel broad-spectrum 1β-methyl carbapenem antibiotic currently in development, have been prepared by controlled degradation of the bulk drug substance in concentrated solutions and isolated by preparative HPLC. The degradates were characterized by LC/MS and NMR and result from various bimolecular reactions of the carbapenem leading to dimers and dehydrated dimers. The development of selective degradation procedures was critical in facilitating isolation of the degradates, and provided a better understanding of the degradation process. This report will focus on the development of

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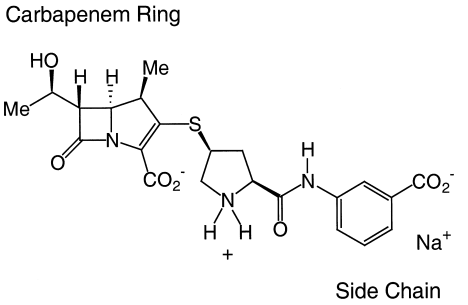
preparative chromatographic methods for isolation of these highly unstable compounds.

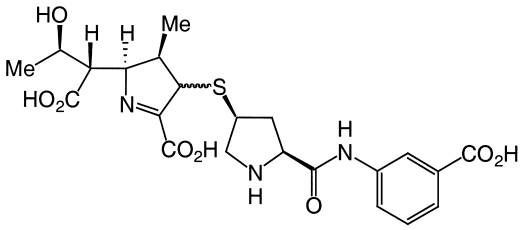
INTRODUCTION

Ertapenem (The bulk drug substance ertapenem sodium is a monosodium salt. Ertapenem is formulated as a di-sodium salt and is known under the name INVANZ™, which is a trademark of Merck & Co., Inc., Whitehouse Station, NJ, USA.) is a novel long-acting synthetic broad-spectrum 1β-methyl carbapenem antibiotic that is currently in development. It is structurally related to β-lactam antibiotics such as penicillins and cephalosporins, and shows activity against a wide range of Gram-negative and Gram-positive aerobic and anaerobic bacteria.(1,2) In clinical trials, ertapenem has been shown to be effective and well tolerated over the course of therapy in the treatment of a variety of moderate to severe bacterial infections. The antibacterial activity of ertapenem exceeds that of several cephalosporins and compares favorably to other carbapenems such as imipenem and meropenem.(1-4)

The structure of ertapenem sodium is shown below:



The molecule consists of a carbapenem ring and a thiaproline amide side chain. Hydrolysis of the highly strained ring system accounts for the instability of carbapenem antibiotics in water at high and low pH, and leads to the ring-opened hydrolysis degradate shown below(5-7):



In addition to the hydrolysis degradate, other degradates appear at low levels in aqueous solution. These degradates tend to form at higher ertapenem sodium concentrations. Initial characterization by LC/MS showed that these impurities are dimers and dehydrated dimers(throughout the manuscript dimers and dehydrated dimers will be referred to as "dimers")of ertapenem. We have identified six dimers in ertapenem sodium drug substance samples and solutions. The levels of the dimers in the drug substance can be controlled and are typically very low. The dimers that were observed in ertapenem sodium were named dimers I+II (dimer I and dimer II inter-convert in solution and cannot be isolated independently. The isolated equilibrium mixture of dimer I and dimer II is referred to as dimers I+II in the manuscript), dimer III, dimer $-H_2Oa$, dimer $-H_2Ob$, and dimer V (names for the dimers were assigned in the order of their discovery with increasing roman numbers for dimers and $-H_2O$ followed by small case letters for dehydrated dimers. An initial observed dimer IV was later found to be a dehydrated dimer and it was re-named dimer $-H_2Ob$).

The study of formation of impurities in the drug substance, their isolation, and characterization is very important because it can help to understand the degradation patterns of the drug substance. This gives valuable information about the drug stability under various conditions. Such information is vital for determining storage and packaging conditions of the bulk and formulated drug substance. Furthermore, improvements in the manufacturing process of the bulk drug substance are difficult to achieve without knowledge of possible degradation pathways.(7-8)

The goal of this work was the preparation, isolation, and characterization of dimeric ertapenem degradates. The preparation of these degradates was accomplished under controlled conditions with isolation by preparative HPLC and structural characterization by LC/MS and NMR.(8)

EXPERIMENTAL

Chemicals and Materials

Ertapenem sodium drug substance samples were supplied by Merck Process Research Department (Merck Research Laboratories, Rahway, NJ, USA). The water used was distilled and purified by a HYDRO System (Garfield, NJ, USA). Acetonitrile was HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). Sodium and ammonium hydroxide were 50% concentrated and ortho phosphoric acid was 75% concentrated (Fisher Scientific, Fair Lawn PA, USA).

Instrumentation and Procedures

Analytical HPLC Analysis

An Agilent 1100 Series HPLC system equipped with an auto injector, a quaternary pump, a column oven, and a diode array detector (Agilent Technologies, Palo Alto CA) was used for analytical analyses of ertapenem sodium samples. Impurity profiles were obtained using a gradient HPLC method. The chromatographic conditions of this method are shown in Table 1. The analytical method used was able to separate the main component, ertapenem, the hydrolysis degradates and the other degradates of interest, and provided area-% results. The wavelength of the detector was set to 230 nm. At this wavelength, the response of the detector was linear in the concentration range analyzed for all components investigated.

Preparative HPLC Separation

Two preparative HPLC instruments were used for the dimer isolation procedures. A Varian system (Palo Alto, CA, USA) with a Dynamax Model SD-1 pump, model 410 auto-sampler, and a model UV-1 detector was used for the iso-

Table 1. Experimental System and Conditions for the Analytical HPLC Impurity Profile Method

Gradient Profile		
Time (min)	%B	%A
0	2	98
3	5	95
25	15	85
35	25	75
45	25	75
47	2	98
62	2	98

HPLC System: HP1100.
Column: Metachem Technologies (Torrance, CA, USA), Inertsil Phenyl, 10 x 2.5 cm.
Flow rate: 1 mL/min, Injection volume: 10 µL.
Mobile phase: A: 0.1 % sodium phosphate buffer pH = 8.0, B: acetonitrile.
Detector: UV, wavelength 230 nm.
Temperature: ambient.

lation of dimers I+II. A Waters DeltaPrep 4000 system (Waters, Milford, MA, USA) was employed to simultaneously isolate dimer III, dimer-H_2Oa, dimer-H_2Ob, and dimer V. Detailed experimental conditions for the preparative HPLC separation are shown in Tables 2 and 3.

Lyophilization

The collected dimer fractions were very unstable at ambient temperature. Therefore, the fractions were frozen immediately using dry ice, and the frozen solutions were subsequently lyophilized. Fractions containing dimers I+II were lyophilized at ambient temperature. This procedure took 1 to 2 days. Prior to lyophilization, the amount of acetonitrile in the sample was reduced under vacuum at subambient temperatures using a rotary evaporator. Collected fractions containing dimer III, dimer -H_2Oa, dimer -H_2Ob, and dimer V were lyophilized at a controlled temperature of -15°C without prior removal of excess acetonitrile. This procedure took 3 to 7 days. A Virtis Genesis 25XL Freeze Dryer (Gardinier, NY, USA) was used to lyophilize samples at a controlled temperature. For the lyophilization at ambient temperature, a Virtis Freezemobile 12SL Freeze Dryer (Gardinier, NY, USA) was used. After lyophilization was complete, the samples were stored at –70°C.

Table 2. Experimental System and Conditions for the Preparative HPLC Separation A: Dimers I+II

Gradient Profile		
Time (min)	%B	%A
0	0	100
20	0	100
40	5	95
50	7	93
70	40	60
71	0	100
80	0	100

HPLC System: Pump: Dynamax Model SD-1, Autosampler: Varian Model 410, Detector: Dynamax Model UV-1, wavelength 310 nm.
Column: Vydac (Hesperia, CA, USA).
Protein & Peptide C-18, 25 x 5cm, 10–15 μm particle size, 300 Å pore size.
Flow rate: 60 mL/min, Injection volume: 2.5 mL.
Mobile phase: A: 0.1 % ammonium acetate buffer pH=8.0, B: acetonitrile.
Temperature: ambient.

Table 3. Experimental System and Conditions for the Preparative HPLC Separation B: Dimer III, Dimer-H_2Oa, Dimer-H_2Ob and Dimer V

Gradient Profile		
Time (min)	%B	%A
0	5	95
10	5	95
45	15	85
50	90	10
60	90	10
61	5	95
71	5	95

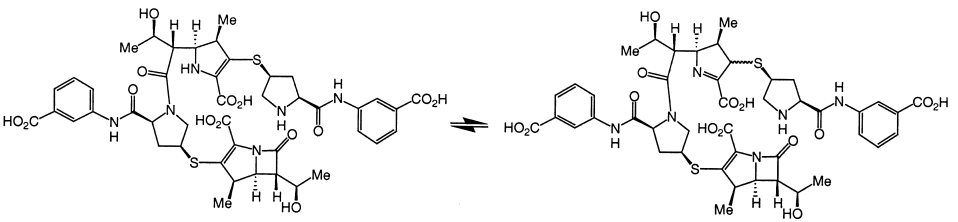
HPLC System: Waters Deltaprep 4000.
Column: Waters (Milford, MA, USA).
Symmetry 10 x 2.5 cm.
Flow rate: 15 mL/min, Injection volume: 10 mL.
Detector: UV, wavelength 230 and 254 nm.
Mobile phase: A: 0.1 % ammonium acetate buffer pH=7.0, B: acetonitrile.

RESULTS AND DISCUSSION

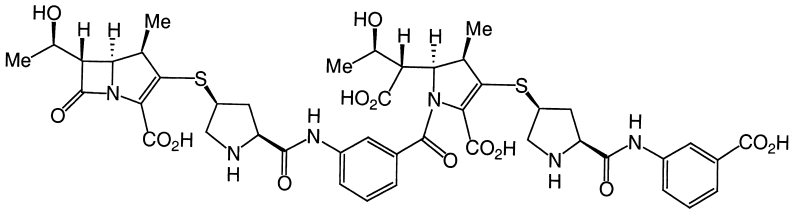
Structures of the Ertapenem Dimer Degradates

The structures of the dimer degradates have been assigned on the basis of NMR and mass spectra, and the proposed degradation pathways. The details of the degradation pathways and pertinent discussion of the spectral results will be provided in another publication.(8)

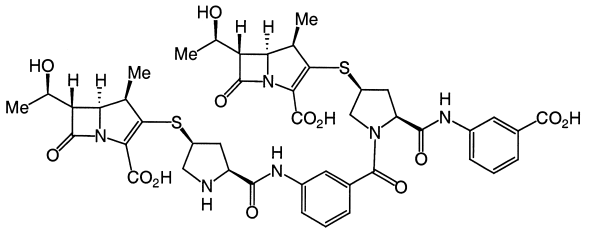
Dimers I+II are a pair of inter-converting tautomeric isomers, which are in equilibrium in solution and arise from intermolecular aminolysis in a manner similar to dimer production observed in meropenem.(6) Dimers I+II arise from opening the carbapenem ring of one ertapenem molecule and linkage with the proline amine group of another molecule. The structure of dimers I+II is shown below:



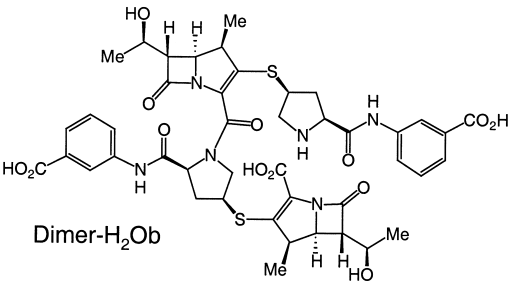
Dimer III arises from the carbapenem ring opening of one molecule by the meta amino benzoic acid carboxylate (MABA) group from a second molecule, followed by acyl transfer. The structure of dimer III is shown below:



Dimers $-H_2O$ a and b are internal amides between a carboxylate group of one molecule (either the carbapenem carboxylate or MABA carboxylate) with the proline amine of a second molecule. Both carbapenem rings are intact. The structures of dimer $-H_2O$ a and b are shown below:

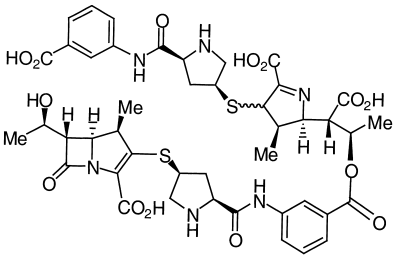


Dimer-H_2Oa



Dimer-H_2Ob

Dimer V arises from the carbapenem ring opening of one molecule by the MABA carboxylate group of a second molecule. Acyl transfer to the secondary alcohol gives the ester. The structure of dimer V is shown below:



Impurity Profile of a Typical Ertapenem Sodium Drug Substance Sample

The ring opened hydrolysis product and dimers are always observed in the final drug substance. An HPLC impurity profile of a typical bulk ertapenem sodium drug substance sample is shown in Figure 1. The hydrolysis product is usually the largest impurity, followed by a process impurity, dimers I+II, dimers $-H_2O$a+b and dimer III. The ring opened hydrolysis product elutes early in the

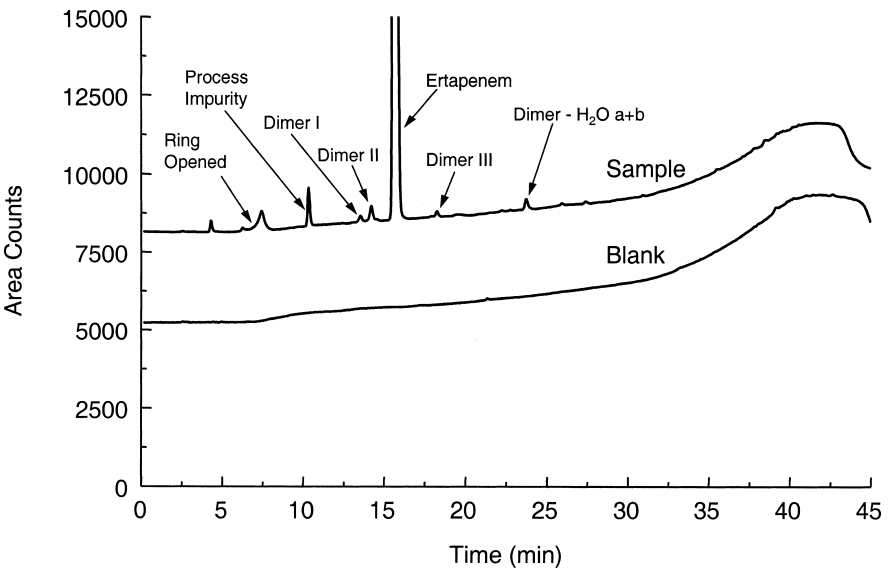


Figure 1. HPLC impurity profile of a typical ertapenem sodium sample.

gradient as a broad peak. Dimers I and II are well separated, however, they cannot be isolated as independent compounds. Inter-conversion occurs in aqueous solution and, therefore, isolated dimer I always contains dimer II, and vice versa.

Degradation of Ertapenem in Solution

Degradation of ertapenem in aqueous solution was studied to find conditions for the selective formation of individual dimers, while reducing the formation of the hydrolysis product and other degradates not observed in the drug substance. The other degradation products are further breakdown products of the unstable dimers.

Dimers are formed when the ertapenem sample concentration is high, i.e. ≥ 100 mg/mL. In diluted aqueous sample solutions (<1 mg/mL) the formation of dimers is insignificant. The major degradate in diluted solutions is the ring opened hydrolysis product. The degradation of ertapenem in diluted solutions follows a pseudo-first order kinetics, similar to the degradation of imipenem and meropenem.(4) The degradation of a 0.2 mg/mL ertapenem solution at 30°C for 12 h is shown in Figure 2 (HPLC area-% data vs. time). The level of hydrolysis product increases from 1% to 3.5%, but only a very slight increase from 0.3% to 0.4% in total dimers is observed.

Dimerization products are formed at higher sample concentrations. Therefore, solutions with high sample concentrations have been prepared. It was possible to dissolve as much as 2500 mg bulk drug sample in 1 mL of water, resulting in a viscous mixture. The degradation of this 2500 mg/mL solution at

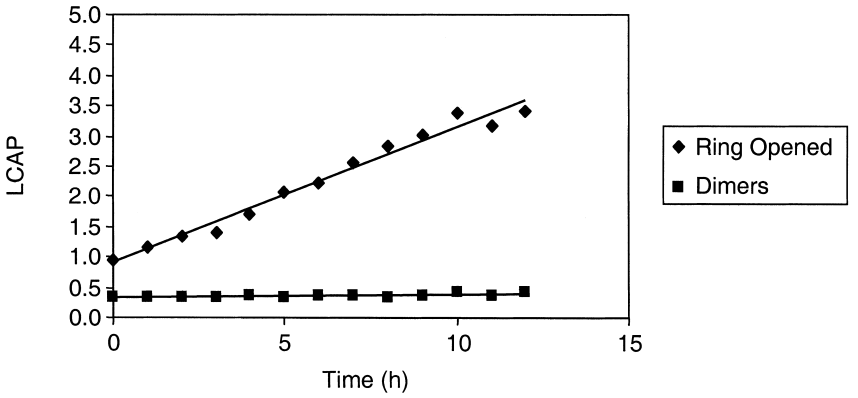


Figure 2. Degradation of ertapenem sodium in a diluted aqueous solution. Sample concentration: 0.2 mg/mL. Temperature: 30°C.

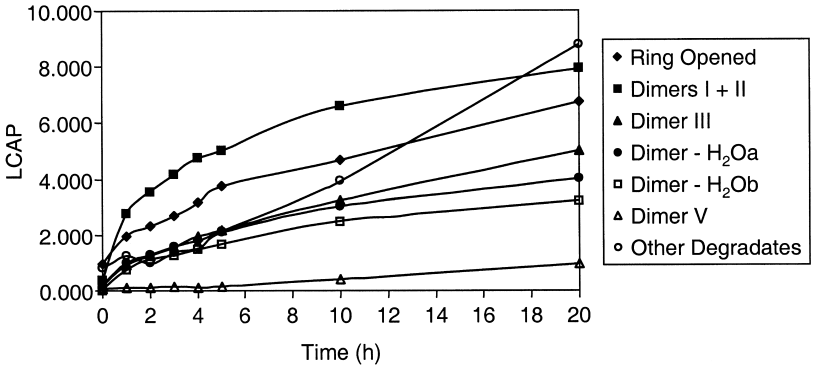


Figure 3. Degradation of ertapenem sodium in a concentrated aqueous solution.

8°C with time, is shown in Figure 3. After 20 h, the level of dimers I+II increased to 8% while the level of hydrolysis product increased to 7%. The levels of the other dimers increased by 1-5%. A significant increase in other degradates, which are not of interest, was observed after 10 h. The level of these degradates rose more sharply with time than the hydrolysis product and dimers, and after 20 h these degradates surpassed the level of the hydrolysis product and the dimers. Controlling the level of these degradates is important, because they will interfere with a simultaneous separation of dimers using a single preparative HPLC method.

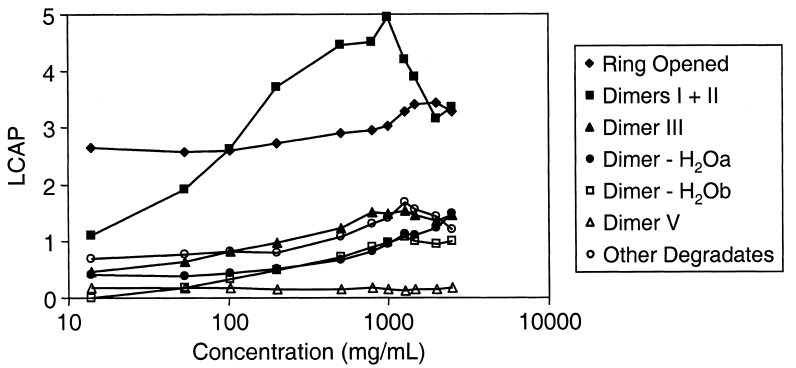


Figure 4. Concentration dependence of the degradation of ertapenem sodium. Degradation time: 2h. Temperature: 8°C.

The concentration dependence of the dimer formation is shown in Figure 4. The plot shows ertapenem sodium samples in aqueous solution at various concentrations, stored for 2 h at a controlled temperature of 8°C. As expected, the level of dimers increases with rising sample concentration; however, the formation of dimers I+II is significant. Furthermore, dimers I+II formation declines with concentrations greater than 1000 mg/mL. This observation does not apply to the other dimers, the hydrolysis product, and other degradates.

The dependence of the formation of dimers at different temperatures is shown in Figure 5. In this case, 2500 mg of sample was dissolved in 1 mL of water and stored for 2 h at various temperatures. This plot shows that the amount of the hydrolysis product and other degradates increases significantly for temperatures greater than 10°C and at temperatures above 25°C these impurities are above 10 area-%.

The pH dependence of dimer formation is shown in Figure 6. Here a pH-adjusted solution of 200 mg/mL of ertapenem sodium was stored for 24 h at 8°C. Figure 6 shows that a pH of 6.5-7.0 is beneficial for the formation of dimers I+II, however, this is not the case for the other dimers, which are more easily formed at lower pH. Above pH 7.5, the hydrolysis degradate is formed at an appreciable rate. To improve selectivity in the formation of dimers, it is best to maintain the pH below 7.5.

Based on the degradation study results, it makes sense to use two different degradation procedures to generate ertapenem dimers, one procedure for the generation of dimers I+II and a second procedure for the generation of the other dimers.

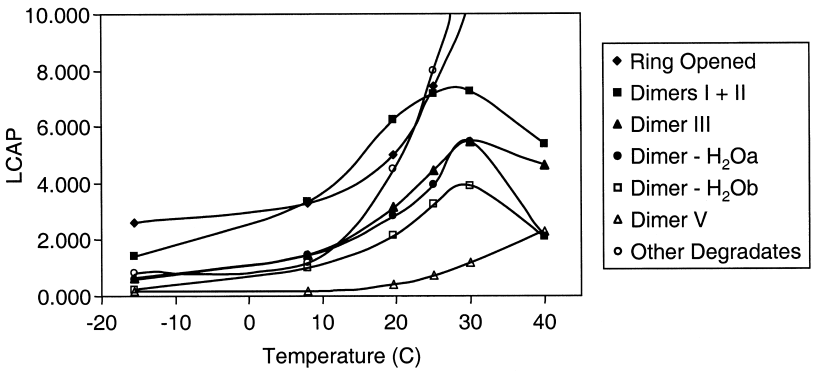


Figure 5. Temperature dependence of the degradation of ertapenem sodium. Sample concentration 2500 mg/mL. Degradation Time: 2 h.

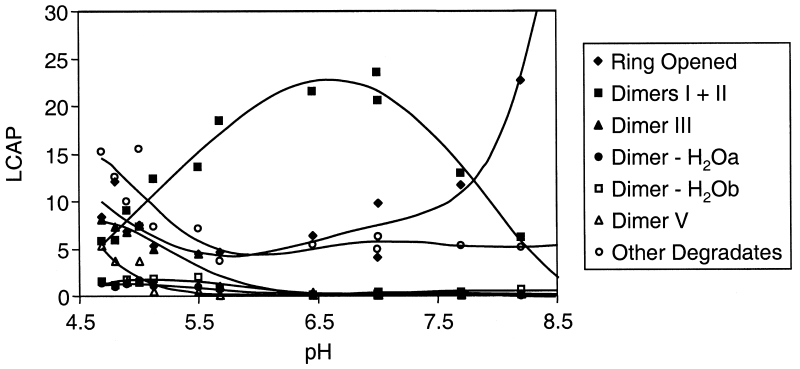


Figure 6. pH dependence of the degradation of ertapenem sodium. Sample concentration: 200 mg/mL. Degradation time: 24h.

Preparation and Isolation of Dimers I+II

Controlled Degradation of Ertapenem

The best conditions to form dimers I+II, while avoiding the generation of other dimers and ring opened hydrolysis product, are at an adjusted pH of 6.5-7, ambient temperature, and a high sample concentration. Therefore, in order to prepare dimers I+II, a concentrated solution of 200 mg/mL ertapenem sodium in water was prepared, the pH was adjusted to 7 and the solution was stored for 24 h at ambient temperature (degradation method A). The composition of the initial and degraded ertapenem solution is shown in Table 4. Using higher concentrations than 200 mg/mL was not practical because of the difficulty in measurement of pH values in the resulting viscous solutions. The degradation procedure produced a mixture of ertapenem with over 20% dimers I+II and less than 5% ring opened hydrolysis product.

Isolation of Dimer Degradates by Preparative HPLC

The isolation of dimers I+II from the degraded ertapenem solution was achieved by preparative HPLC on a Vydac Protein C-18 column. Other C-18 stationary phases were also attempted, but co-elution of the main component, ertapenem sodium with dimers, occurred when overloading the column. The main challenge in the separation was to enhance the resolution between the ertapenem and dimers I+II.

Table 4. HPLC Area-% Levels of Dimers in the Aqueous Ertapenem Solution Before and After Degradation

	Initial, Undegraded Sample	Degradation method A:Selective Formation of Dimers I+II	Degradation method B: Selective Formation of Dimer III, -H_2Oa, -H_2Ob and V
Ring Opened	0.976	4.116	2.324
Dimers I+II	0.372	23.479	3.543
Dimer III	0.174	0.293	1.239
Dimer –H_2Oa	0.222	0.371	1.307
Dimer –H_2Ob	0.010	0.186	1.109
Dimer V	0.082	0.069	0.113
Other Degradates	0.813	4.949	1.008

The experimental conditions for the optimized preparative separation are shown in Table 2. The scale-up of the HPLC method is shown in Figure 7, where chromatograms with two different injection sizes are depicted, 100 and 500 mg. Ertapenem and dimers I+II elute at approximately 50 and 53 min, respectively. Higher injection amounts resulted in partial co-elution of the ertapenem and dimers I+II peak, however, the high degree of overloading permitted a higher isolation yield per injection. The dimers I+II fractions were collected as tail of the partially co-eluting ertapenem sodium-dimer peak, starting immediately after a shoulder was visible (see Figure 7). Each single fraction had to be carefully analyzed for purity to confirm that the cut-points were chosen correctly. The average yield per injection was 50 mg of dimers I+II and their purity by HPLC was >95 Area-%. The recovery of dimers I+II was ca. 10% based on the initial amount of ertapenem sodium drug substance used. The main impurity in isolated dimers I+II was ertapenem. By comparison, an injection size of 100 mg doubled the recovery, but yielded only 20 mg of dimers I+II per injection. Since the supply of ertapenem sodium drug substance was not an issue, the higher loading with 500 mg per injection was chosen for the isolation of dimers I+II.

A long run time at the initial gradient conditions (B=0%) was necessary to achieve a good loading capability of the column. By reducing the time at the initial gradient of 0%B, the separation deteriorated significantly and a visible shoulder between ertapenem sodium and dimers I+II disappeared, and subsequently, the purity of the collected fractions was less than 90%. The main impurity in the dimer fractions was again ertapenem.

The lyophilized dimers I+II sample was fully characterized. The purity was 54.2 weight-% expressed as free acid based on 10.8 area-% impurities by HPLC, 4.9% water, 0.2% total organic solvents, and 34.1% residual buffer salts. The ratio of dimer I to dimer II was 1:6.6.

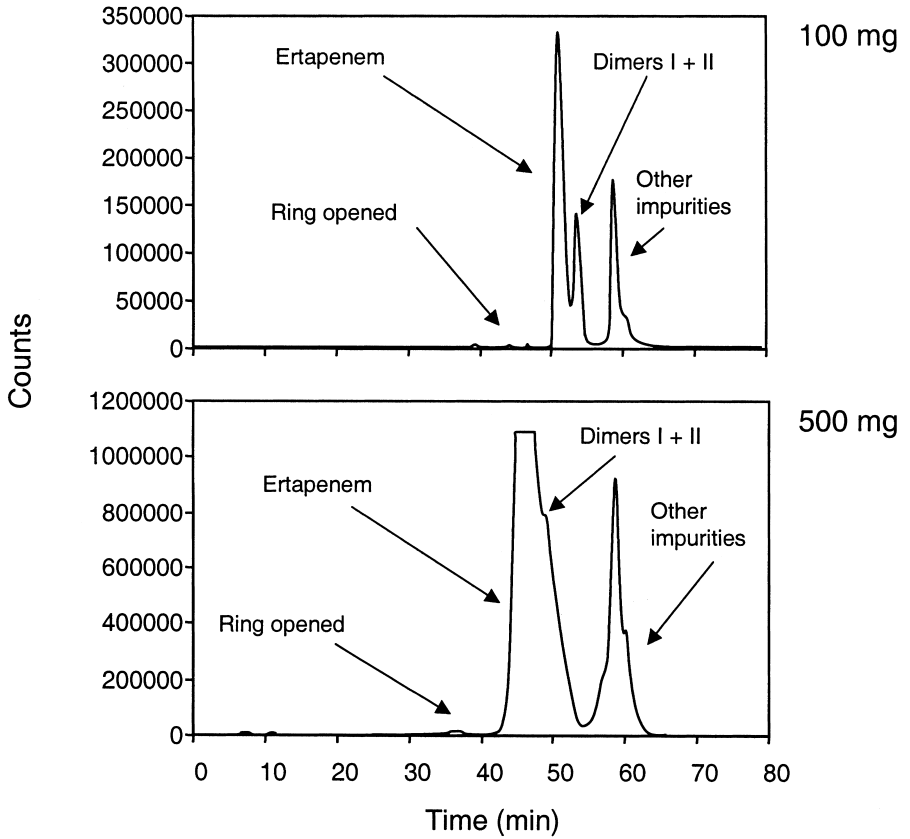


Figure 7. Preparative HPLC separation of dimers I+II.

Preparation and Isolation of Dimer III, Dimer -H_2Oa, Dimer-H_2Ob, and Dimer V

Controlled Degradation of Ertapenem

For the preparation of dimer III, dimer -H_2Oa, dimer-H_2Ob, and dimer V, pH adjustment was unnecessary, however, an increase in the sample concentration was useful. A concentrated solution of 2500 mg in 1 mL ertapenem sodium in water was prepared and stored at 8°C for 2 h (degradation method B). The composition of the initial and degraded solution is shown in Table 4. A longer degradation time or a higher temperature, yielded more dimers in the degradation mixture; however, the levels of other degradates were significantly increased and this deteriorated the separation and the purity of the collected dimers.

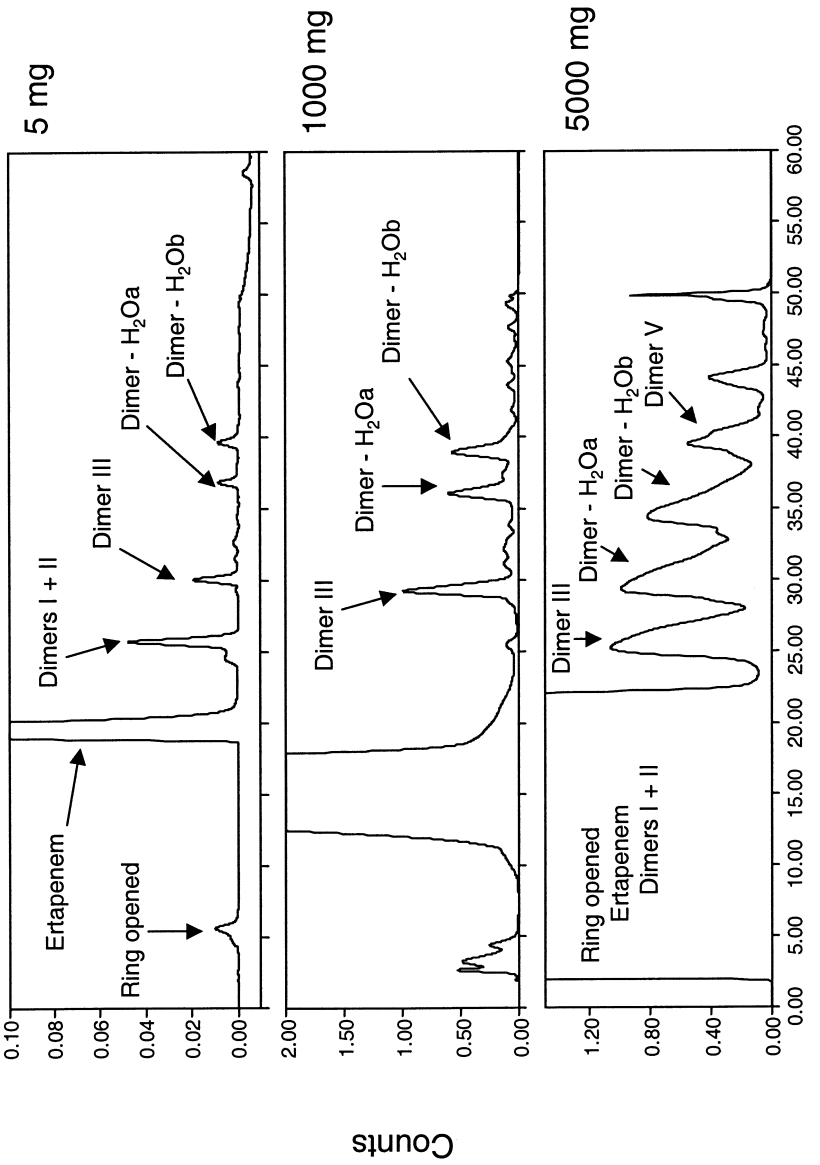


Figure 8. Preparative HPLC separation of dimers III, dimer -H_2Oa, dimer -H_2Ob and dimer V.

Isolation of Dimer Degradates by Preparative HPLC

The isolation of the dimers from the degraded solution was achieved by preparative HPLC using a Waters Symmetry C-18 column. Prior to injection, the viscous mixture was diluted to 10 mL and the pH of this solution was adjusted to 7. Although pH adjustment was not necessary for the controlled degradation, dilution and pH adjustment was important for the isolation procedure, because the buffer capacity of the mobile phase at pH 7 was not optimal. The experimental conditions for the optimized separation are shown in Table 3.

The scale-up of the preparative method is illustrated in Figure 8. The dimers are well separated at a loading of 5 mg. An increase of the amount injected to 1000 mg results in co-elution of dimers I+II with the main peak ertapenem; the retention times of the other dimers do not change. Further scale-up to 5000 mg shifts the retention times of the dimers but the separation remains adequate. The dimer recoveries for the 5000 mg injection were between 0.1% to 0.6%, based on the initial amount of bulk ertapenem sodium used. The purities and yield recoveries of dimers III to V are shown in Table 4. Each single run took 80 minutes, including equilibration time.

The lyophilized dimer samples were characterized by HPLC. The HPLC area-% results are shown in Table 5. The isolated dimers contained 5 to 10% water, 1-2% total organic solvents, and 20 to 30% residual buffer salts.

CONCLUSION

The current work presents the selective preparation and isolation of ertapenem sodium dimers. An understanding of the degradation of ertapenem under various conditions was necessary for the preparation of these thermally labile compounds in suitable quantities. Dimer degradates were selectively prepared with two different controlled degradation procedures and two preparative HPLC methods. The preparation of dimers I+II was achieved at an adjusted pH

Table 5. Yield and Purity (by HPLC) of Isolated Dimers Fractions

	Yield Per Injection (mg)	HPLC Area-%	Yield Recovery (%)
Dimers I+II	50	96	10
Dimer III	30	83	0.6
Dimer $-H_2Oa$	20	76	0.4
Dimer $-H_2Ob$	10	84	0.2
Dimer V	5	70	0.1

7, and the separation was possible on a Vydac Protein and Peptide C18 column with a recovery of 10%. The other dimers (dimer III, dimer -H_2Oa, dimer -H_2Ob, and dimer V) could be prepared without pH adjustment and isolated simultaneously. A Waters Symmetry C18 column was used for the separation and the recoveries were 0.1 to 0.6%.

ACKNOWLEDGMENTS

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