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Degradation kinetics of aplidine, a new marine antitumoural cyclic peptide, in aqueous solution

Joop C.M. Waterval[a], Jeroen C. Bloks[a], Rolf W. Sparidans[a], Jos H. Beijnen[a], Ignacio M. Rodriguez-Campos[b], Auke Bult[c], Henk Lingeman[c], Willy J.M. Underberg[d,*]

[a]*Department of Pharmacy and Pharmacology, The Netherlands Cancer Institute/Slotervaart Hospital Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

[b]*Department Synthetic Processes, PharmaMar, Tres Cantos, Madrid, Spain*

[c]*Free University Amsterdam, Department of Analytical Chemistry and Applied Spectroscopy, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

[d]*Utrecht University, Faculty of Pharmacy, Department of Biomedical Analysis, PO Box 80082, 3508 TB Utrecht, The Netherlands*

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Abstract

The degradation kinetics of aplidine were investigated using reversed-phase high-performance liquid chromatography combined with UV detection. Aplidine consists of at least two isomers that undergo interconversion at a low rate. Influences of pH, temperature, buffer ions and ionic strength on the degradation kinetics were studied. The log k_{obs}–pH profile can be divided into three parts, a proton, a solvent and a hydroxyl-catalysed section. The stability-indicating properties of the used analysis technique as well as the identities of the main degradation products were checked using gradient liquid chromatography and mass spectrometric detection. The overall degradation rate constant as a function of the temperature under acidic and alkaline conditions obeys the Arrhenius equation. No catalytic influences were observed with phosphate and carbonate buffers and, in addition, the ionic strength showed no substantial effect on the stability, as expected. Results from gradient LC–MS indicated that hydrolysis of the ester groups present in the ring structure was the main degradation route. There is no difference in degradation rate constants for the individual isomers.

Keywords: Aplidine

1. Introduction

Didemnins are cyclic peptides, isolated from Caribbean tunnicates (*Tridemnum* genus), that have been shown to be immunomodulators, inhibitors of viral replication in vitro and with in-vivo antitumour activity. The didemnin of interest for this kinetically orientated study is aplidine, or dehydrodidemnin B, which was isolated from the Mediterranean tunnicate *Aplidium albicans*. Interest in this compound arose as a result of its therapeutic similarity to didemnin B, a potent anticarcinogen, which is, in contrast to aplidine, limited in its use by its severe secondary effects.

Evaluation of the bioactivity of 42 depsipeptides

*Corresponding author. Tel.: +31-30-253-6952; fax: +31-30-253-5180.

E-mail address: w.j.m.underberg@pharm.uu.nl (W.J.M. Underberg).

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revealed that the native cyclic depsipeptide core is an essential structural requirement for most of the bioactivities of the didemnins, especially for cytotoxic and antiviral activities. The linear side-chain portion of the peptide can be altered with a gain, in some cases, of bioactivity. In particular, aplidine, which was tested against several types of tumour cells and in in-vivo studies in mice, showed remarkable gains in its in vitro and in vivo activities compared to didemnin B [1].

Both didemnins behave as strong antitumoural agents, probably primarily based on the inhibitory effect on the synthesis of proteins that are essential for cell proliferation. Daily administration of aplidine (2.5 μg/mouse) almost doubled the life-span of mice, and the total number of Ehrlich carcinoma cells decreased by 70–90% [2].

Other in-vitro studies with aplidine demonstrated strong antiproliferative effects on rapidly dividing human colon carcinoma cell lines [3] and on prostate cancer cells [4] compared with the effects of vincristine, vinorelbine and taxol. In the latter study, some caution was urged in the clinical use of these agents because of potential neurotoxic side-effects.

In a study evaluating the antiproliferative action of aplidine against a variety of freshly explanted human tumour specimens, it was found that especially breast, melanoma and non-small-cell lung cancer appear to be sensitive to low concentrations of aplidine [5]. The mild bone marrow toxicity observed on continuous exposure to active concentrations of aplidine indicated that a therapeutic window at marginally myelotoxic concentrations might exist.

The first clinical data on aplidine were published recently [6]. Two methods have been published for the analysis of aplidine in biological fluids, based on chromatographic separation followed by either fluorescence [7] or tandem mass spectrometric detection [8]. In order to use fluorescence as a detection technique, aplidine had to be derivatised with *trans*-4′-hydrazino-2-stilbazole. For practical reasons, the stability and adsorption were briefly investigated after reconstitution and dilution in infusion bags [9].

The purpose of the present study is to extend the insight into the degradation mechanisms and the influences of pH, temperature, buffer ions and ionic strength on the reaction kinetics of aplidine. This study is useful as a reference in the research of the stability of other cyclic peptides from the didemnin group.

2. Experimental

2.1. Chemicals

Aplidine originated from PharmaMar (Tres Cantos, Madrid, Spain) and was obtained via the Slotervaart Hospital, Amsterdam, The Netherlands. All other chemicals used were of analytical grade, and demineralized water (by reversed osmosis) was used throughout the study.

2.2. Liquid chromatography

To obtain the kinetic degradation data, an isocratic LC system was employed using a Waters HPLC pump, model M6000A. Samples (20 μl) were injected using a Perkin-Elmer ISS-100 autosampler (Uberlingen, Germany). Separation was accomplished at 25°C using a Waters Symmetry® reversed-phase C_{18}, 3.5 μm, 100×4.6 mm column (Waters, Milford, MA, USA), provided with a SecurityGuard™ C_{18} precolumn, 4×3 mm, from Bester, Amstelveen, The Netherlands. A UV absorbance detector from Applied Biosystems, Model 785 A (Separations, H.I. Ambacht, The Netherlands), was used for detection. The mobile phase consisted of acetonitrile–water (65:35, v/v), the flow-rate was set at 1 ml/min and detection was performed at 208 nm. Data acquisition was performed using Gynkosoft V5.30 software (Gynkotek, Munich, Germany).

2.3. Gradient liquid chromatography mass spectrometry

A high pressure gradient system, using two LC-10 AD micro-LC pumps (Shimadzu, 's Hertogenbosch, The Netherlands), in combination with a Supersphere 100 reversed-phase C_{18} (5 μm) column, 125×2 mm I.D. from Merck (Darmstadt, Germany) was used for separation of all components. The gradient system started with acetonitrile–water–trifluoroacetic acid (20:80:0.04, v/v), increasing linearly to acetonitrile–water–trifluoroacetic acid (70:30:0.04, v/v) over 30

min. The flow-rate was set at 200 μl/min, a 10-μl sample was injected and the VG platform II mass spectrometer (Fisons, Altricham, UK), operated in the positive ion mode, was used for detection. The scanned mass ranged from 150 to 1300 m/z, scan time was set at 10 s, and low and high mass resolution were both 15.5 (instrumental units). Data acquisition and calculation of the m/z values was done using Masslynx version 2.1 (Fisons).

2.4. Standard degradation conditions

Degradation experiments were performed with 0.400 ml sample solutions in flame-sealed 1 ml glass ampoules, which were kept in a thermostated water bath at the appropriate temperature. Sampling of the ampoules was performed over a range of at least two half-lives. Every ampoule that was withdrawn from the bath was immediately placed in a freezer at −80°C for storage until analysis. Storage at this low temperature was chosen since, at this temperature, no degradation could be observed. To minimise degradation during HPLC analysis, the samples were mixed with 0.200 ml of a 1-*M* phosphate buffer (pH 3.2), giving a sample pH of 3–4. In a pilot study, this pH range was shown to be the pH range with the lowest degradation rate. For each degradation curve, at least eight measurement points were used and all experiments were performed in triplicate. The aplidine concentration was 10 μg/ml. Standard degradation conditions were 60°C, an ionic strength of 0.100 and a buffer concentration of 25 m*M*.

2.5. Influence of pH on k_{obs}

The influence of pH on k_{obs} was determined between H_0/pH 0 and 10.7. The buffers used were perchloric acid, below pH 3, phosphate in the pH range 3–8, and carbonate in the pH range 9–10.7.

2.6. Arrhenius plot

The influence of temperature on k_{obs} was determined at pH values of 1.0 and 8.0 using perchloric acid solutions and phosphate buffer solutions, respectively. At pH 1, the reaction rate constant was determined at 40, 50, 60 and 70°C, while at pH 8, it was determined at 30, 40, 50 and 60°C.

2.7. Influence of buffer ions on k_{obs}

The influence of buffer ions on k_{obs} was determined for phosphate and carbonate by performing experiments at buffer concentrations between 25 and 200 m*M* at pH values of 7 and 10, respectively, at a constant ionic strength (μ=0.3). For phosphate buffer solutions, the pH was 7.0 and for carbonate, pH 10 was employed, with degradation temperatures of 60 and 40°C, respectively.

2.8. Influence of ionic strength on k_{obs}

The ionic strength (μ) was varied between 0.05 and 1.3 using sodium perchlorate while keeping the pH and buffer concentration constant. At pH 1.0 and pH 7.2, perchloric acid and 25 m*M* phosphate buffer were used, respectively.

2.9. pH measurement

All pH measurements were performed on a Consort P514 pH meter (Salm and Kipp, Breukelen, The Netherlands) equipped with a Slim-trode electrode (Hamilton, Darmstadt, Germany). The pH was measured before and after degradation at the temperatures employed.

3. Results and discussion

3.1. Analytical procedures

At ambient temperature, two conformations of aplidine (Fig. 1) can be observed in solution, tentatively attributed to *cis–trans* isomerisation at the proline peptide bonds [10,11]. The existence of these forms is visualised as two completely separated peaks in the chromatogram (Fig. 2). The plateau between the peaks is caused by the interconversion of the aplidine isomers during the chromatographic run. In cases where the column temperature is cooled to 5°C, the interconversion rate decreases to such an extent that two baseline-separated peaks are obtained, without the mentioned plateau. This extreme column temperature was not used for the kinetic degradation experiments, since, at low temperatures,

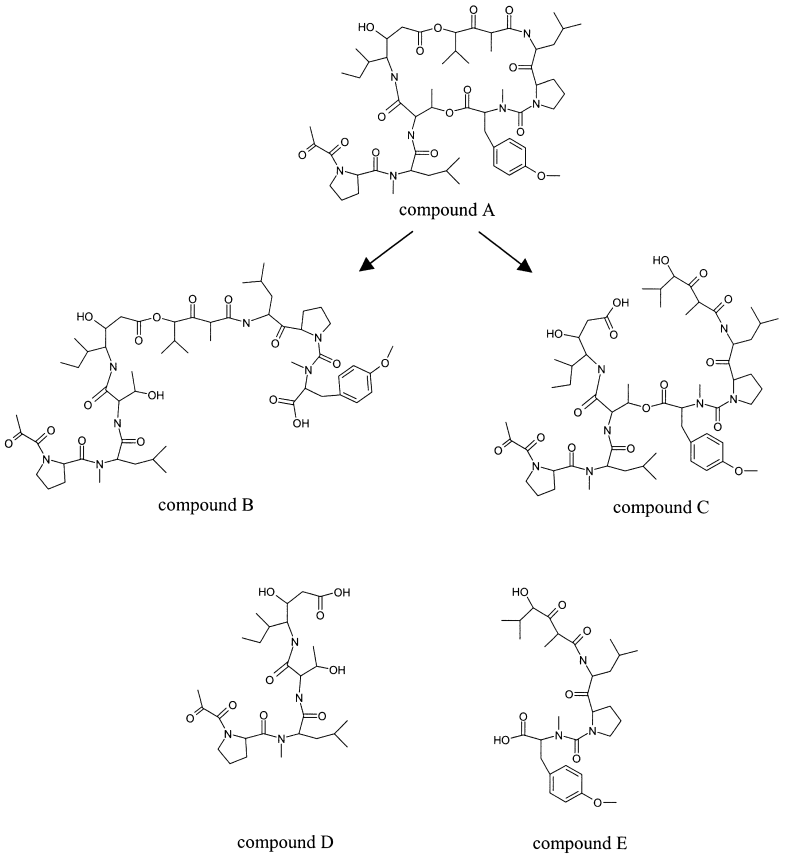

Fig. 1. Structure of aplidine (A) and the degradation products presumed to be obtained by hydrolysis of the ester bonds.

a high backpressure was observed due to the increased viscosity of the mobile phase.

Initial gradient LC runs indicated that all peaks of degradation products eluted prior to the peaks of intact aplidine. For the study of the degradation kinetics, a fast separation run was preferred, which could be used to determine the decrease in intact aplidine. Therefore, an isocratic LC separation, thermostated at 25°C, was chosen, which proved to be stability-indicating, as was also demonstrated with LC–MS results (discussed later). Aplidine eluted within 6 min and, in contrast to gradient LC runs, re-equilibration between runs was not necessary. Not all degradation products were retained by the column when acetonitrile–water (65:35, v/v) was used as mobile phase; instead, they eluted as one peak in the dead volume. Since only the decrease in aplidine has to be monitored to determine the degradation kinetics, no attention was paid to the separation or quantitation of the degradation products. For elucidation of the structure of the degradation products, a gradient LC–MS run was employed.

3.2. Degradation kinetics

3.2.1. Reaction order

In the H_0/pH range 0 to 10.7, the aplidine concentration decreased over the first two half-lives according to pseudo first-order kinetics, providing the reaction rate constant, k_{obs}, as the slope of plots of the natural logarithm of the residual drug concentration versus time [12].

3.2.2. Influence of pH on k_{obs}

The log k_{obs}–pH profile is shown in Fig. 3. In the pH region 2 to 6, solvent-catalysed degradation

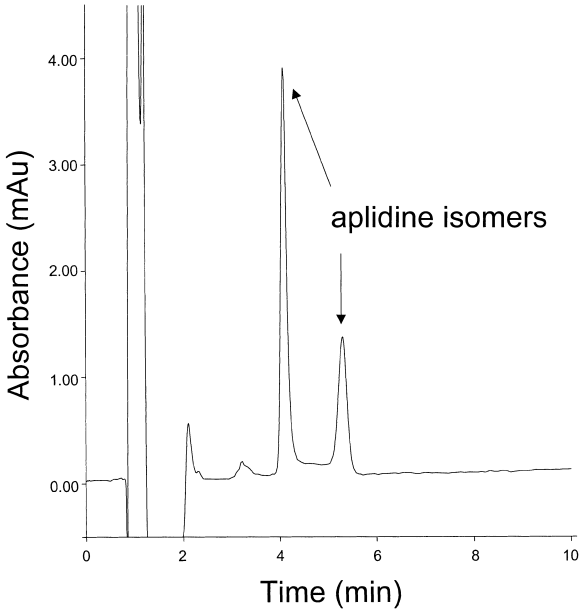

Fig. 2. Reversed-phase chromatogram of a typical aplidine $t=0$ min sample (10 μg/ml in perchloric acid buffer, pH 2, 20 μl injected; see Experimental for further conditions).

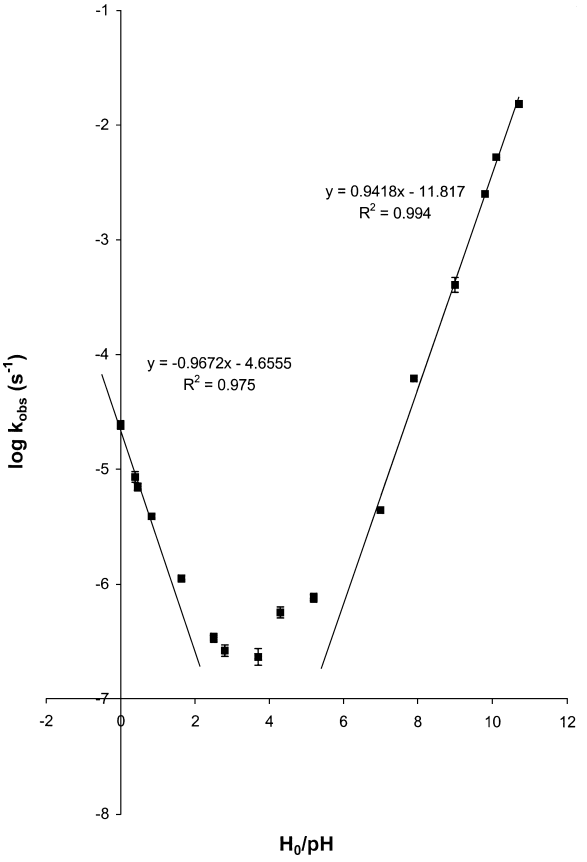

Fig. 3. Plot of the degradation rate constant, k_{obs}, of aplidine versus the pH of the sample. Each point represents the mean of three measurements (see Experimental for conditions).

dominates and optimal stability is obtained, yielding a half-live of more than 2 weeks at 60°C. In the pH area below and above this region, clear proton-catalysed and hydroxyl-catalysed hydrolyses can be observed, respectively. As expected, no points of inflection are observed in the plot, since aplidine contains no prototropic groups in the pH range used.

The reaction rates were calculated using the total peak area of all isomers. From the literature, it is known that reaction rates are influenced by the secondary and tertiary structure of peptides and proteins [13]. When either the peak area of the first-eluting peak or the second peak is used, the same degradation rates are obtained (Table 1). Apparently, the *cis–trans* isomerisation at the proline peptide bond has no influence on the degradation sites.

3.2.3. Arrhenius equation

The influence of the temperature on the reaction rate constant is given by the Arrhenius equation.

$$\ln k_{obs} = \ln A - \frac{E_a}{RT}$$

in which A represents the frequency factor while E_a is the energy of activation, R is the gas constant and T is the temperature in Kelvin. Both in the proton- and hydroxyl-catalysed sections, the influence of temperature was determined. The data obtained were fitted with the equation and the results are shown in Table 2. The linear correlation allows extrapolation of the observed degradation kinetics to typical storage conditions at more ambient temperatures.

3.2.4. Influence of ionic strength/buffer ions

Since the compound under investigation is non-ionic in the applied pH range, no effect was expected. On the other hand, buffer ions can act as catalysts and, therefore, can influence the reaction

Table 1
Calculated degradation rates in the pH region used, calculated using the total peak area or using the individual isomer peak areas

pH buffer solution	Log k_{obs} based on total amount of isomers	Log k_{obs} based on first-eluting isomer	Log k_{obs} based on second-eluting isomer
0.0	−4.61	−4.60	−4.51
0.4	−5.07	−5.02	−5.12
0.5	−5.16	−5.15	−5.16
0.8	−5.41	−5.42	−5.38
1.6	−5.95	−5.98	−5.86
2.5	−6.47	−6.48	−6.44
2.8	−6.58	−6.66	−6.37
3.7	−6.63	−6.64	−6.63
4.3	−6.24	−6.26	−6.18
5.2	−6.11	−6.12	−6.13
5.7	−6.11	−6.13	−6.13
7.0	−5.35	−5.35	−5.33
7.9	−4.20	−4.19	−4.21
9.0	−3.38	−3.39	−3.35
9.8	−2.58	−2.60	−2.52
10.1	−2.26	−2.29	−2.21
10.7	−1.80	−1.80	−1.78

Table 2
Influence of temperature on the degradation rate constant, k_{obs}, of aplidine

pH	E_a [J/mole]	A [s^{-1}]	R^2
1	7.74×10^4	4.89×10^6	0.994
8	1.01×10^5	4.76×10^{11}	0.998

rate. In Fig. 4, both the results of the experiments concerning the influence of ionic strength and buffer ions on the degradation rate are presented. The ionic strength has no substantial effect on the degradation rate, and the buffer ions are free of catalytic influences.

3.2.5. Determination of the main degradation products

Gradient LC–MS revealed the identity of the most abundant peaks observed on analysis of degraded aplidine samples. In Fig. 5, a total ion chromatogram (TIC) is presented, accompanied by several selected ion chromatograms (SICs) of a degraded sample of aplidine, stored in a carbonate buffer solution at pH 9, 60°C for 69 h. Since aplidine virtually disappears under these conditions, the peak does not exceed the baseline noise of the TIC. However, in the selected ion chromatogram, the peaks of the *cis* and *trans* isomers of aplidine are still visible. Since the proline residue is present in all hydrolysis degradation products (Fig. 1), the corresponding SICs show the characteristic double peaks. Compounds B and C seem to form a triple peak, but, in fact, it is a combination of both sets of double peaks, which are not fully separated chromatographically. Theoretically, hydrolysis of the amide bonds in the ring structure would yield compounds with the same mass as that from compounds B and C. However, this is not likely to occur since the amide bond is considered as stable, as long as no Asp residues are present in the molecule [13]. This assumption is also in agreement with the fact that no degradation products were observed, resulting from hydrolysis of the amide bonds in the linear side-chain portion of the peptide. In addition to the products formed after hydrolysis of the ester bonds, other degradation

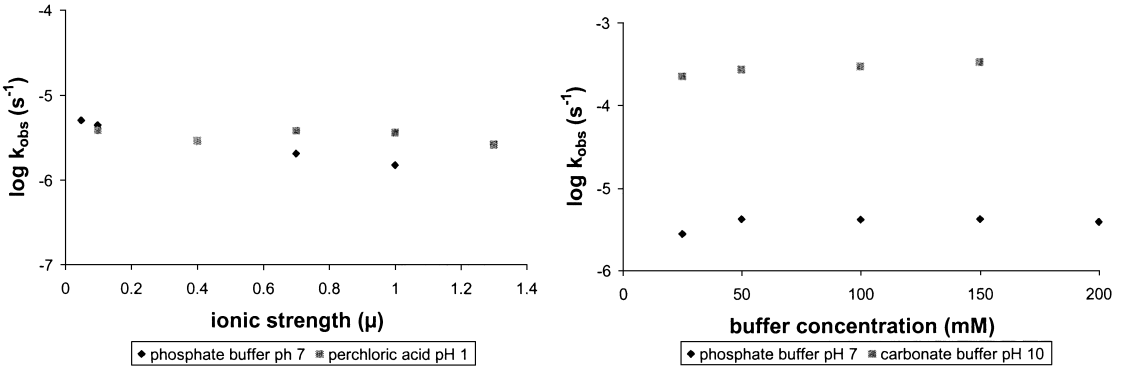

Fig. 4. Influence of ionic strength/buffer ions on the degradation rate constant, k_{obs}, of aplidine.

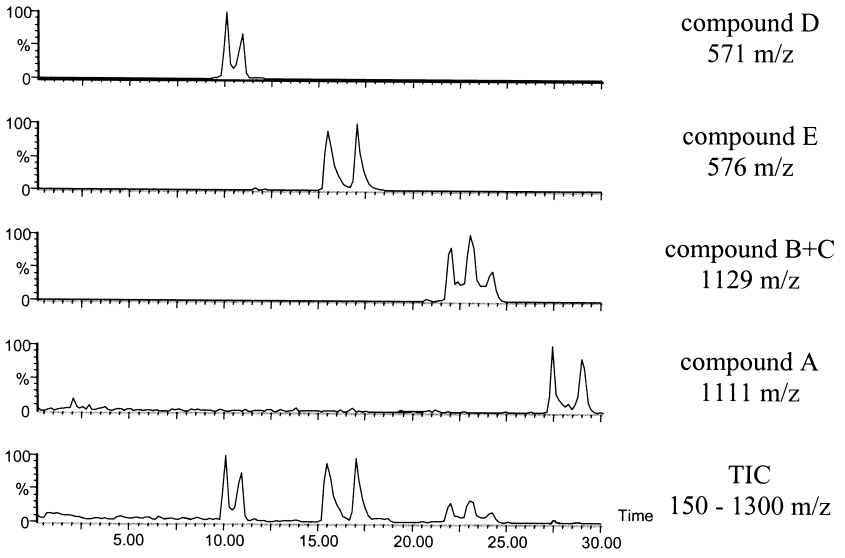

Fig. 5. Total ion chromatogram (TIC) obtained using gradient LC–MS analysis of a degraded aplidine sample (10 μg/ml aplidine in 0.25 *M* carbonate buffer, pH 9; $t = 69$ h). The selected ion chromatograms given are extracted from the TIC using the theoretical masses of the degradation products.

reactions are possible. Also, oxidation of the proline groups to glutamyl semialdehyde or 2-pyrrolidone has been described [13,14]. Furthermore, since aplidine contains a methylated tyrosine amino-acid residue, racemisation followed by β-elimination of the methoxyphenyl group is a possible degradation route [13,14]. However, thorough investigation of fully degraded aplidine samples did not prove the existence of these degradation products. In fact, the ester hydrolysis products seem to account for all peaks in the chromatogram of both alkaline and acidic degradation samples. The observed masses of the degradation products are in good agreement with the calculated masses (Table 3).

Table 3
Comparison of the observed mass versus the theoretically calculated mass of aplidine and its major degradation products

Compound	$[M+H]^+$ (m/z)	Theoretical $[M+H]^+$ value (m/z)
Aplidine (compound A)	1110.8	1110.63
1 of 2 ester groups hydrolysed (Compound B or C)	1128.5	1128.64
Compound D	571.3	571.33
Compound E	576.4	576.33

4. Conclusions

The presented isocratic LC–UV system is a stability-indicating, simple and effective method for determination of the degradation kinetics of aplidine. The gradient micro LC–MS system revealed the identity of the degradation products obtained after hydrolysis of the ester bonds located in the ring structure of this peptide. No traces of other degradation products were found in either acidic or alkaline samples. The degradation of aplidine can be divided into three types of mechanism: proton-, solvent- and hydroxyl-catalysed degradation. Below pH 2, proton-catalysed hydrolysis dominates, while, starting from pH 6, hydroxyl-catalysed hydrolysis is the main degradation route. No catalytic effects from the used buffers were observed and, as expected, no effects of sample ionic strength were observed.

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