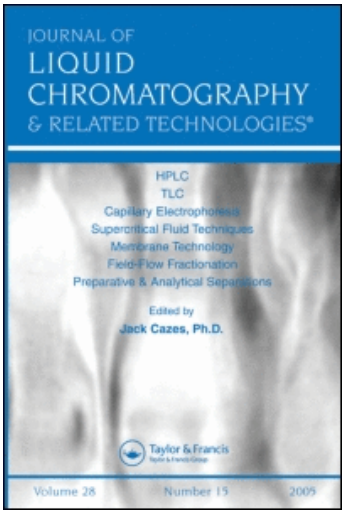

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HPLC-UV METHOD DEVELOPMENT AND IMPURITY PROFILING OF THE MARINE ANTICANCER AGENT APLIDINE IN RAW DRUG SUBSTANCE AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

The development and validation of a reversed-phase high performance liquid chromatographic (RP-HPLC) method with ultraviolet (UV) detection for the quantification and purity determination of aplidine in raw drug substance and pharmaceutical dosage form is described. Using this method, the aplidine rotamers present as a consequence of cis-trans isomerism of the peptide bond

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between the proline and pyruvoyl moieties in the molecule, elute as one single peak. Linear calibration curves in the range of 12.5-300 μg/mL of aplidine with correlation coefficients > 0.999 were obtained. Within-run and between-run precisions were ≤ 2.2% and accuracy was within 100.6-101.4%. A profile of recurrent impurities was drawn up from several lots of aplidine raw drug substance manufactured thus far. Major impurities were identified as didemnin-A, acetyldidemnin A, and noraplidine using liquid chromatography-mass spectrometry (LC-MS). No significant differences in chromatographic profile between aplidine raw drug substance and its pharmaceutical dosage form were found.

INTRODUCTION

Aplidine (dehydrodidemnin B, MW 1109, Figure 1) is a novel representative of an evolving group of anticancer agents derived from marine sources.(1) This naturally occurring cyclic depsipeptide is isolated from the Mediterranean tunicate *Aplidium albicans* and belongs to the didemnin family, a class of marine-derived compounds which exhibit antiviral, antitumor, and immunosuppressive activity. Didemnin B, the most potent representative of the didemnin class up to now, was the first marine-derived anticancer compound to enter clinical trials in the early 1980's.(1) The dehydro-derivative of didemnin B, dehydrodidemnin B, or aplidine, displayed even more potent *in vitro* and *in vivo* antitumor activity against various solid human tumor xenografts. After initial extraction of the

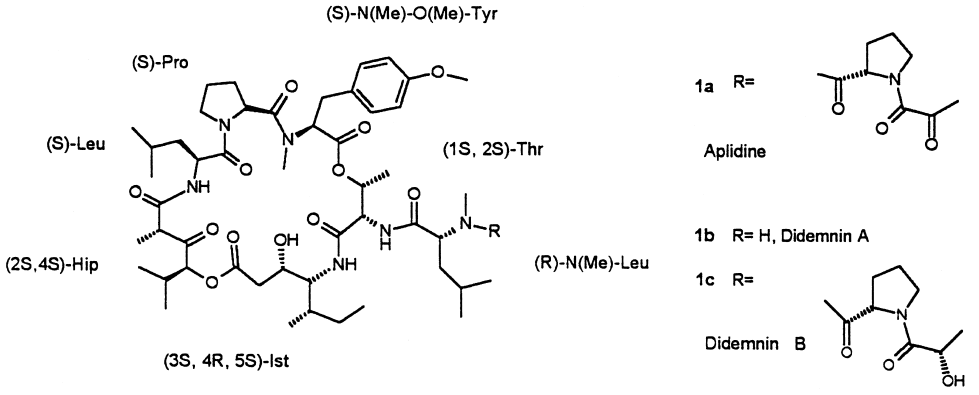


Figure 1. Chemical structures of aplidine, didemnin A, and didemnin B (Hip: hydroxyisovalerylproprionyl; Ist: isostatine; Leu: leucine; Pro: proline; Thr: threonine; Tyr: tyrosine).

compound from its natural source, aplidine raw substance is currently obtained by a hemisynthetic process from natural didemnin A and synthetic enantiopure pyruvyl-proline-hydroxide (Pyr-Pro-OH). Recently, a total synthesis route has been developed. Aplidine is pharmaceutically formulated as a lyophilized powder for intravenous use containing 500 μg aplidine per dosage unit,(2,3) and has recently entered Phase I clinical trials in the UK, Spain, France, and Canada.(4,5,6)

The pharmaceutical development of aplidine, warranted the availability of an assay for the quantification of aplidine and determination of related impurities and degradation products in both raw drug substance and the pharmaceutical dosage form.(7,8) A complicating factor in the development of a suitable assay was, however, the two chromatographic peaks observed for the compound, caused by *cis-trans* isomerism of the peptide bond between the proline and pyruvoyl moieties in the side chain of the molecule (Figure 2).(9) In this study, the development and validation of a stability-indicating RP-HPLC method with UV-detection, to be used in the quality control of both aplidine raw drug substance and pharmaceutical dosage form, is presented. Furthermore, the impurities found in aplidine raw drug substance were identified using liquid chromatography-mass spectrometry (LC-MS). Based on these findings, an impurity profile of aplidine raw drug substance was defined.

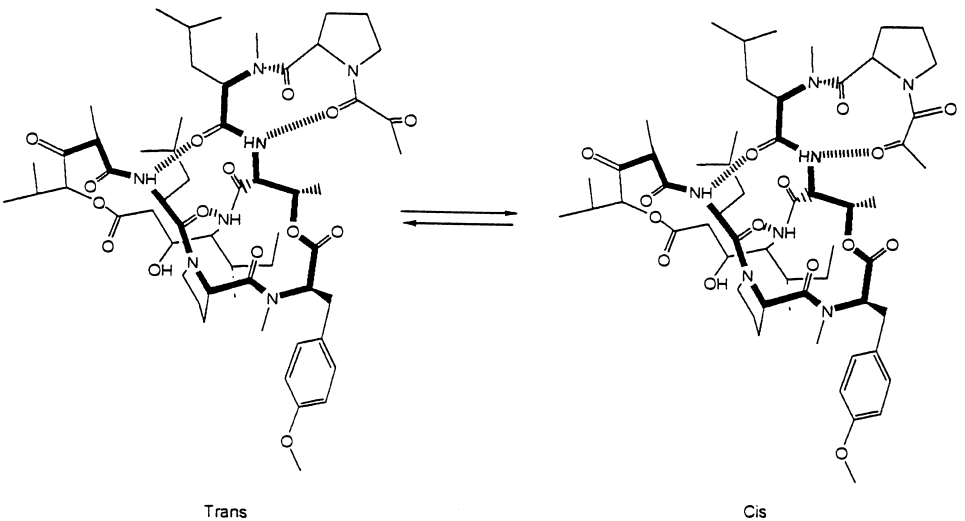


Figure 2. *Cis-trans* isomerism of aplidine at the peptide binding between the proline and pyruvoyl moieties.

EXPERIMENTAL

Chemicals

Aplidine was obtained from natural didemnin A and Pyr-Pro-OH by a hemisynthetic route under the responsibility of Pharma Mar SA (Tres Cantos, Madrid, Spain). Aplidine 500 μg/vial lyophilized powder for intravenous use was manufactured in-house (Department of Pharmacy of the Slotervaart Hospital/The Netherlands Cancer Institute). All reagents were of analytical grade and used without further purification. Acetonitrile was purchased from Biosolve (Amsterdam, The Netherlands). Trifluoroacetic acid was obtained from Merck (Amsterdam, The Netherlands). Distilled water was used throughout.

Equipment

Chromatographic analyses were performed on the following configuration: A Model SP8800 ternary pump (Thermo Separation Products (TSP), Fremont, CA, USA), a Model 996 photodiode array (PDA) detector (Waters, Milford, MA, USA), and a Model SP8880 autosampler (TSP) equipped with a 100 μL-sample loop. The column was thermostated using a Model 7971 column heater (Jones Chromatography Inc., Lakewood, CO, USA). Chromatograms were processed using Millennium® software (Waters). ^{1}H NMR spectra were recorded with a Gemini 300 BB instrument (Varian Assoc., Palo Alto, CA USA) at 300.1 MHz.

NMR Procedure and Conditions

Aplidine (1 mg, Lot APL#297) was dissolved in 0.65 mL deuterochloroform ($CDCl_3$) or hexadeuterodimethylsulfoxide (DMSO-d_6). In $CDCl_3$, TMS was used as internal reference, in DMSO-d_6 the central DMSO line was set at 2.50 ppm.

Chromatographic Conditions

Twenty-μL injections were made on a Zorbax SB-C_{18} column (150 x 4.6 mm i.d., particle size 3.5 μm) (Rockland Technologies Inc., Newport, DE, USA). The column temperature was kept at +80°C. The eluent was comprised of a linear gradient of acetonitrile containing 0.04% trifluoroacetic acid, starting at 35% up to 70% in 15 minutes and water containing 0.04% trifluoroacetic acid. The flow rate was 0.6 mL/min and UV-detection was performed at 225 nm. A total run time of 30 minutes was employed.

LC/MS Conditions

LC-conditions were similar to those described under "*Chromatographic conditions*". The eluent flow at a rate of 0.6 mL/min was led directly into the electrospray interface operating in the positive ion mode. Settings for mass range, drying gas rate, gas temperature, and HED were 750-1250; 10 L/min; 330°C and 10,000 V, respectively.

Validation Procedure

Linearity

From a 500 μg/mL stock solution of aplidine in acetonitrile, calibration curves at six concentration levels (12.5, 25, 50, 100, 200, and 300 μg/mL aplidine in acetonitrile) were prepared and analyzed in duplicate, in three separate runs. Least-squares regression analysis of concentration, weighted by [1/(concentration)^2], versus the area of the aplidine peak was applied. The *F* test for lack of fit was used to evaluate the linearity of the calibration curves.

Accuracy and Precision

Accuracy, between-run, and within-run precision of the method was determined by assaying samples at three concentration levels (15, 150, and 250 μg/mL aplidine in acetonitrile), in triplicate, in three separate analytical runs. Samples were prepared from a second stock solution of 500 μg/mL aplidine in acetonitrile. Accuracy was measured as the percent deviation from the nominal concentration. The within-run and between-run precision was obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Specificity

The stability indicating capability of the HPLC method was tested by subjecting aplidine stock solutions to several stress test conditions (heat, oxidation, acidic, and alkaline environment). Heat: To 1 mL of a 0.5 mg/mL aplidine solution in acetonitrile 1 mL of distilled water was added. The resulting solution was subsequently autoclaved at 121°C for 30 minutes at a pressure of 1 Atm. 20 μL of the solution was injected into the HPLC system. Oxidation: To 1 mL of a 0.5 mg/mL aplidine solution in acetonitrile, 1 mL of a 30% hydrogen peroxide solu-

tion was added. After 24 hours, 20 µL of the solution was injected into the HPLC system. Acidic: To 1 mL of a 0.5 mg/mL aplidine solution in acetonitrile, 0.5 mL of a 1 N hydrochloric acid (HCl) solution was added. The mixture was allowed to stand for 30 minutes at room temperature, after which period 0.5 mL of a 1 N sodium hydroxide (NaOH) solution was added. 20 µL of the resulting solution with neutral pH was injected into the HPLC system. Alkaline: Similar to the method described under "Acidic", using 1 N NaOH as alkalizer and 1 N HCl to quench the reaction.

Sample Preparation

Raw Drug Substance

Aplidine raw drug substance sample was prepared by accurately weighing 500 µg of drug substance and subsequent dissolution in 1.00 mL of acetonitrile, resulting in a sample concentration of 0.5 mg/mL of aplidine.

Pharmaceutical Product

Aplidine 500 µg/vial lyophilized powder for intravenous use was dissolved in 2.0 mL of a 1:1, v/v mixture of acetonitrile and water, resulting in a theoretical concentration of 250 µg/mL of aplidine.

RESULTS AND DISCUSSION

NMR

Table 1 gives the assignments for the ^{1}H NMR spectrum in $CDCl_3$, based on 1D proton and 2D HH-COSY spectra. Two conformers appear to be present in an approximately 1:1 ratio (NH protons and methyl protons of the pyruvoyl group). The existence of two conformers relating to *cis-trans* isomerism of the pyruvoyl-proline amide bond was also shown with ^{13}C NMR.(9) The spectrum in DMSO-d_6 shows the presence of even four conformations in approximately a 35:35:15:15 ratio (methyl protons of the pyruvoyl group). The additional signals probably originate from *cis-trans* isomerism of the second proline amide bond. This hypothesis was further examined by carrying out temperature experiments in DMSO-d_6 at +105°C, which showed that only the signals for the pyruvoyl methyl group are still present at this elevated temperature. In the $CDCl_3$ spectrum, the threonine NH signal of one conformer is more shielded than that of the other con-

Table 1. ^{1}H NMR-Analysis ($CDCl_3$) of Aplidine Raw Drug Substance (Lot APL#297)

Proposed Assignments:
Amino acid residue: *Proline (1)*: δ 4.70 H-α; 2.10 H-β_1; 1.95 H-β_2; 3.66 H-δ_1; 3.84 H-δ_2; 2.54/2.52 CH_3CO; *N-Me-Leucine:* δ 3.15/3.11 *N*-CH_3; 4.62 H-α; 2.15 H-β_1; 1.78 H-β_2; 1.28 H-γ; 0.90 CH_3; *Threonine*: 7.61/7.05 NH_4; 4.57/4.65 H-α; 5.29/5.17 H-β; 1.41/1.40 γ-CH_3; *4-Amino-3-hydroxy-5-methylheptanoic acid*: 7.20/7.19 NH_4; 3.25 H-α_1; 2.62 H-α_2; 4.08 H-β; 4.08 H-γ; 1.80 H-δ; 1.64 δ-CH_3; 0.90 H-ε; 0.90 ε- CH_3; 2,5-*diMe-hydroxy-3-ketohexanoic acid*: 4.23/4.19 H-α; 1.33/1.32 α-CH_3; 5.40 H-γ; 1.70 H-δ; 0.90 δ-CH_3; *Leucine*: 7.86/7.81 NH_4; 4.80 H-α; 1.60 H-β_1; 1.20 H-β_2; 0.90 CH_3; *Proline (2)*: 5.10 H-α; 2.90 H-β_1; 1.90 H-β_2; *N,O-diMe-tyrosine*: 3.80 *O*-CH_3; 2.54 *N*-CH_3; 3.57 H-α; 3.36 H-β_1; 3.16 H-β_2; 7.08 H-1,4; 6.85 H-2,3

former. This indicates that the two conformers arise, indeed, as a result of *cis-trans* isomerism at the pyruvoylproline residue (Figure 2). From the literature, the occurrence of more than one conformation in proline-containing peptides is well known.(10) The proline residue and *cis-trans* isomerization is extremely important in the folding, denaturation, and renaturation of polypeptides and proteins, and might also be a factor in the biological activity of aplidine.

HPLC-UV Method Development

The existence of multiple conformations of aplidine complicated the development of a quantitative, stability indicating HPLC method. The initial assays, both normal phase, isocratic and gradient reverse phase HPLC methods, showed the presence of two peaks in the chromatograms referring to the equilibrium between the *cis* and *trans* isomers of the pyruvoyl-proline amide bond in the aplidine molecule (Figure 2). At ambient temperature (+20-25°C), this equilibrium reaction appeared too slow to elute both isomers as one peak, but on the other hand, too fast to elute them as two baseline-separated peaks (Figure 3b). Collection of one of the individual conformers at the detector outlet and re-injection of the component, resulted again in a double-peak, indicating a continuous inter-conversion of both conformers.

Quantitation of aplidine concentrations based on the total area of both isomer peaks was satisfactory in terms of linearity and reproducibility. However, due to the broad elution-profile of the aplidine peak-complex, co-elution of structure-related impurities and degradation products might occur, thus, resulting in low specificity. Therefore, attempts were made to develop a chromatographic system in which both conformers examined: influence of type of mobile phase, type of solvent, buffer pH, stationary phase, column temperature, and gradient

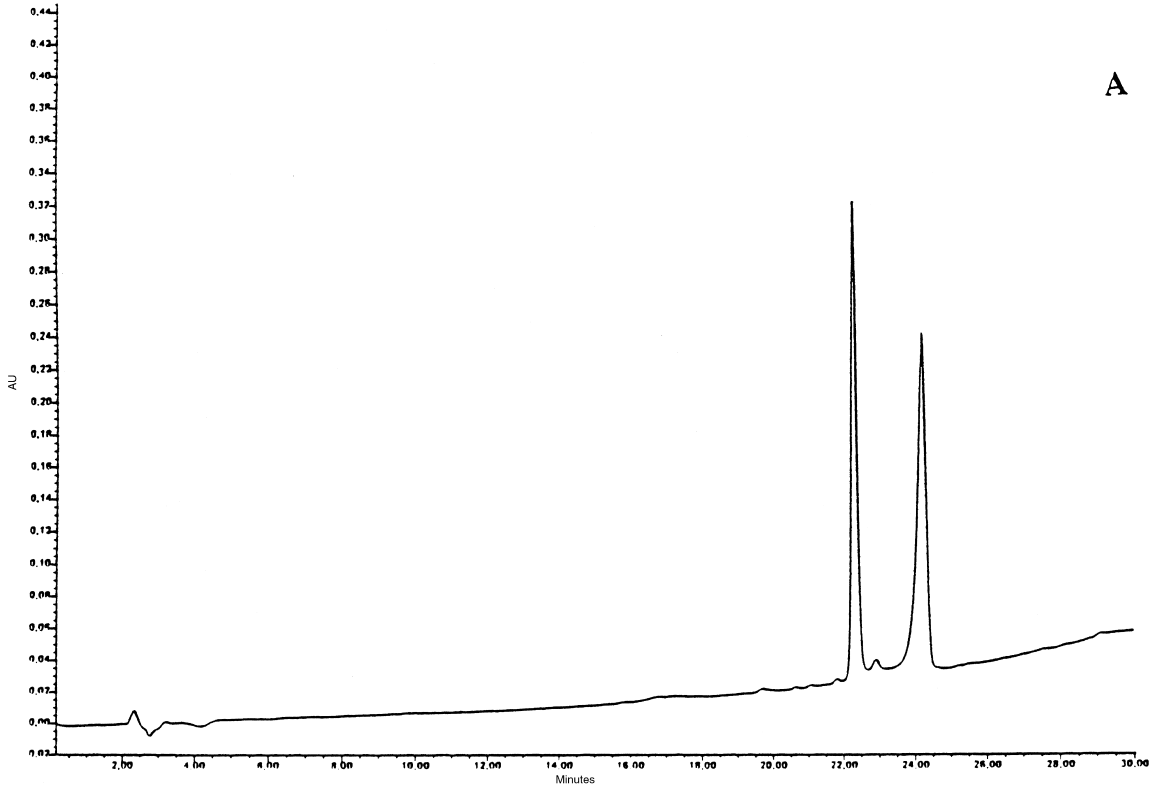
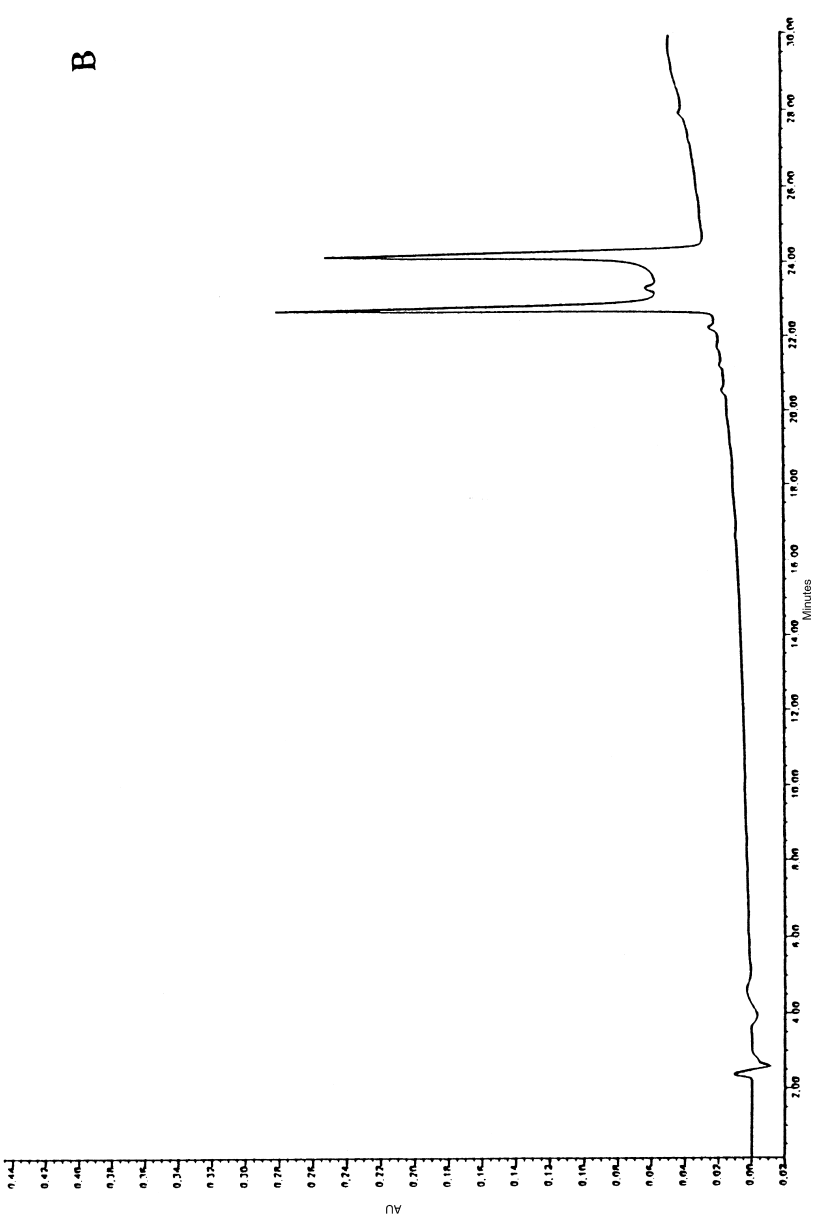


Figure 3. Effect of column temperature on aplidine chromatography: (A) +10°C; (B) +25°C; (C) +40°C; (D) +80°C (concentration of 250 mg/mL aplidine in acetonitrile).



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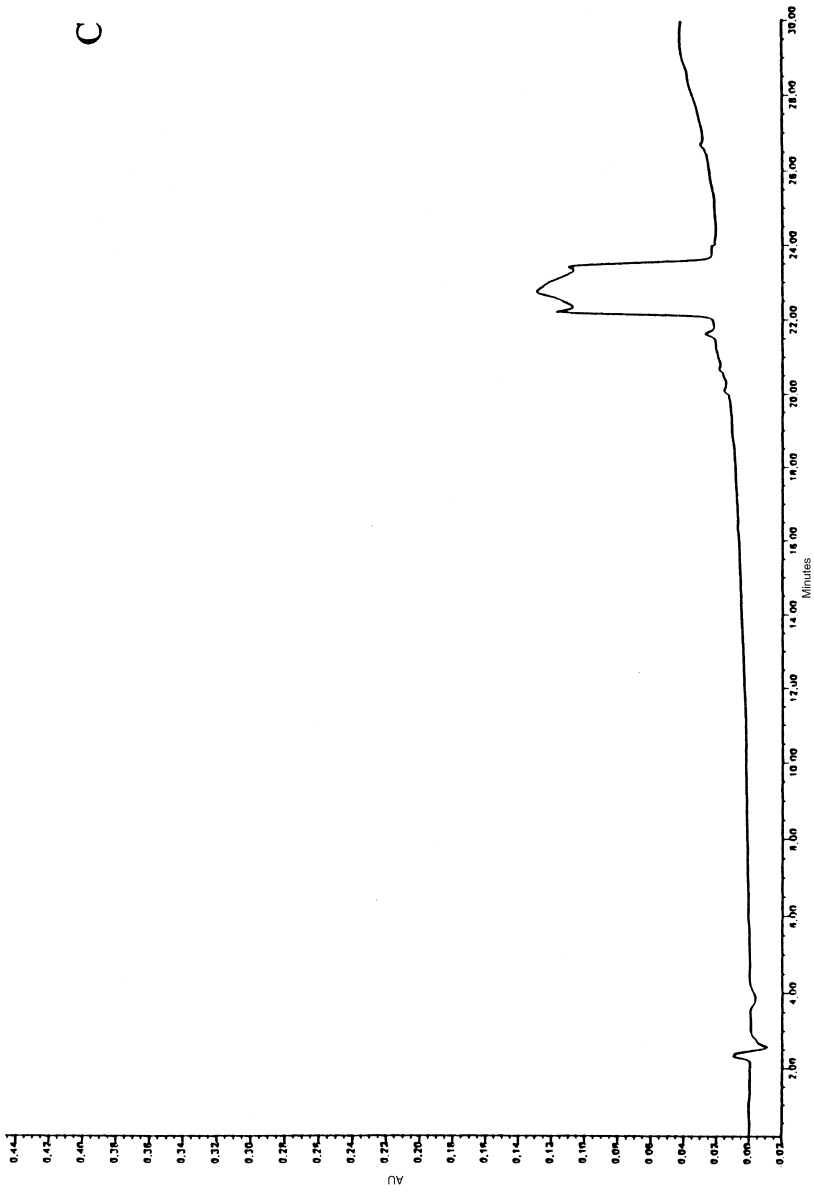

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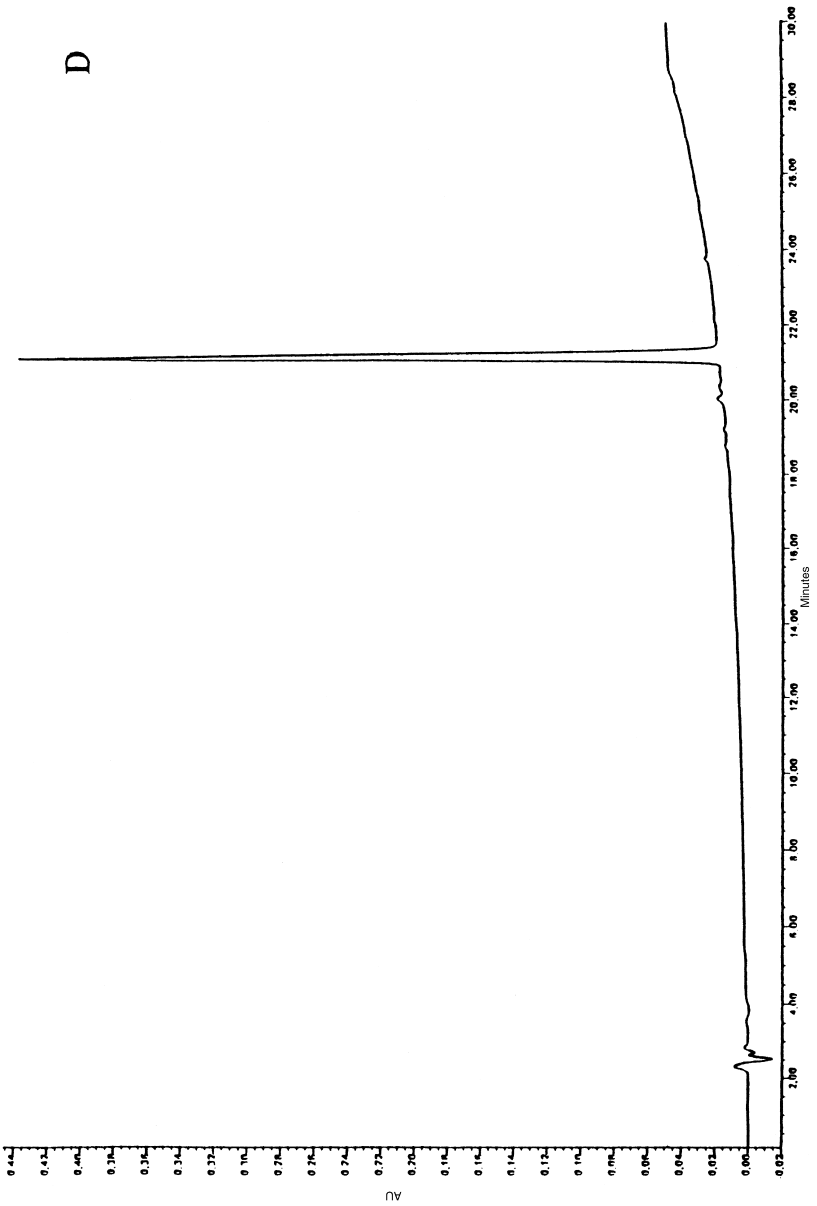


Figure 3. Continued.

Table 2. Solvent Effect on Ratio of *cis-trans* Rotamers of Aplidine (Determined at Room Temperature, +20-25°C)

Solvent	Ratio (*cis-trans*)
ACN	45:55
ACN-H_2O 75:25 v/v	48:52
ACN-H_2O 50:50 v/v	46:54
ACN-H_2O 35:65 v/v	51:49
ACN-H_2O:TFA 35:65:0.04 v/v/v	51:49

ACN = acetonitrile.

elution. Table 2 shows the relative insensitivity of the ratio of the *cis-trans* conformers to variations in the amounts of acetonitrile and water and the addition of trifluoroacetic acid. The column temperature, however, appeared of significant importance in the chromatographic behaviour of aplidine. As can be seen in Figure 3a, a sub-ambient column-temperature of +10°C results in two resolved peaks. Apparently, at low temperature, the on-column equilibrium reaction is very slow, allowing both isomers to elute as separate peaks. Increasing the column-temperature results in a gradual coalescence of the two conformers (coalescence point of approximately +40°C), with a sharp, single peak at +80°C (Figures 3c,d).

Validation

Linearity, accuracy, within-run, and between-run precision of the HPLC-UV method over a concentration range of 12.5-300 μg/mL was examined. The assay showed linearity as determined by the *F*-test for lack of fit (α=0.05). For all three calibration curves correlation coefficients > 0.999 were obtained by least-squares regression analysis. Table 3 gives the average back calculated concentrations for the different calibration concentration levels in six analytical runs. The deviation of the nominal concentration for all concentrations was less than 2%. The within-run and between-run precisions at concentration levels of 14.5, 144.9, and 241.5 μg/mL were less than 2.2% with accuracies between 100.6-101.4% (Table 4). The limit of detection (LOD), calculated as the absolute amount of aplidine injected resulting in a signal three times the baseline noise-level, was determined to be 0.2 ng.

Aplidine stock solutions subjected to heat, oxidation, acidic, and alkaline environments resulted in a significant decrease of the initial aplidine peak areas and the appearance of degradation peaks well separated from the parent peak (Figure 4). Stock solutions subjected to acid resulted in a similar profile as the

Table 3. Results of Back Calculated Aplidine Calibration Samples (n=6)

Concentration (Nominal, μg/mL)	Concentration (Found, μg/mL)	RSD (%)	Accuracy (%)
12.5	12.5 ± 0.25	2.0	100.4
25.0	24.8 ± 0.43	1.7	99.4
49.9	49.6 ± 0.53	1.1	99.4
99.8	100.1 ± 0.76	0.8	100.1
199.7	200.6 ± 2.27	1.1	101.1
299.5	300.0 ± 3.58	1.2	100.1

solutions subjected to an alkaline environment, with a complete disappearance of the aplidine peak after 30 minutes. The UV-spectra of the aplidine peak recorded during the forced degradation studies were identical to the initial spectrum, indicating that no degradation products co-eluted with aplidine and demonstrating the stability-indicating capability of the HPLC-UV method.

Impurity Profile of Aplidine Raw Drug Substance

Figure 5 shows a representative chromatogram of aplidine raw drug substance lot APL#397. LC-MS analysis was conducted for the structural elucidation of the impurities found in the samples. The recurrent impurities found for six lots of aplidine raw drug substance (Lots APL#397, APL#499, APL#599, APL#699, APL#799, and APL#899) are listed in Table 5. For each impurity, the retention time-intervals, as well as the relative retention time-intervals, with respect to the aplidine peak are given. Amounts of the respective impurities are expressed as percentages of the aplidine peak area. Also, the molecular masses as determined by LC-MS analysis are listed. The most prominent impurity eluting just before the aplidine peak at a relative retention time of approximately 0.97, was only first discovered with the gradient HPLC-UV method, confirming the specificity of the analytical method. Initial batches of aplidine raw substance

Table 4. Results of Aplidine Quality Control Samples (n=9)

Concentration (Nominal, μg/mL)	Within-Run Precision (%)	Between-Run Precision (%)	Accuracy (%)
14.5	2.2	2.2	101.4
144.9	0.9	0.9	101.3
241.5	1.0	0.5	100.6

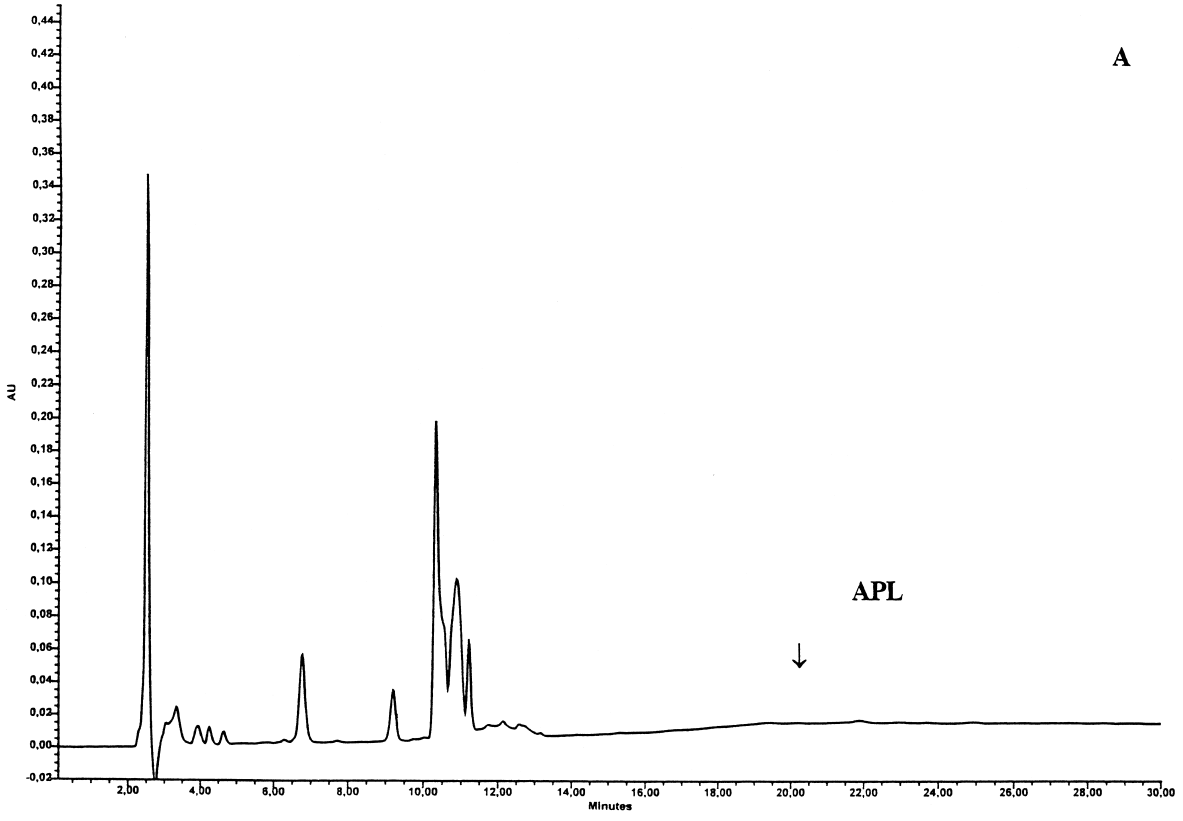
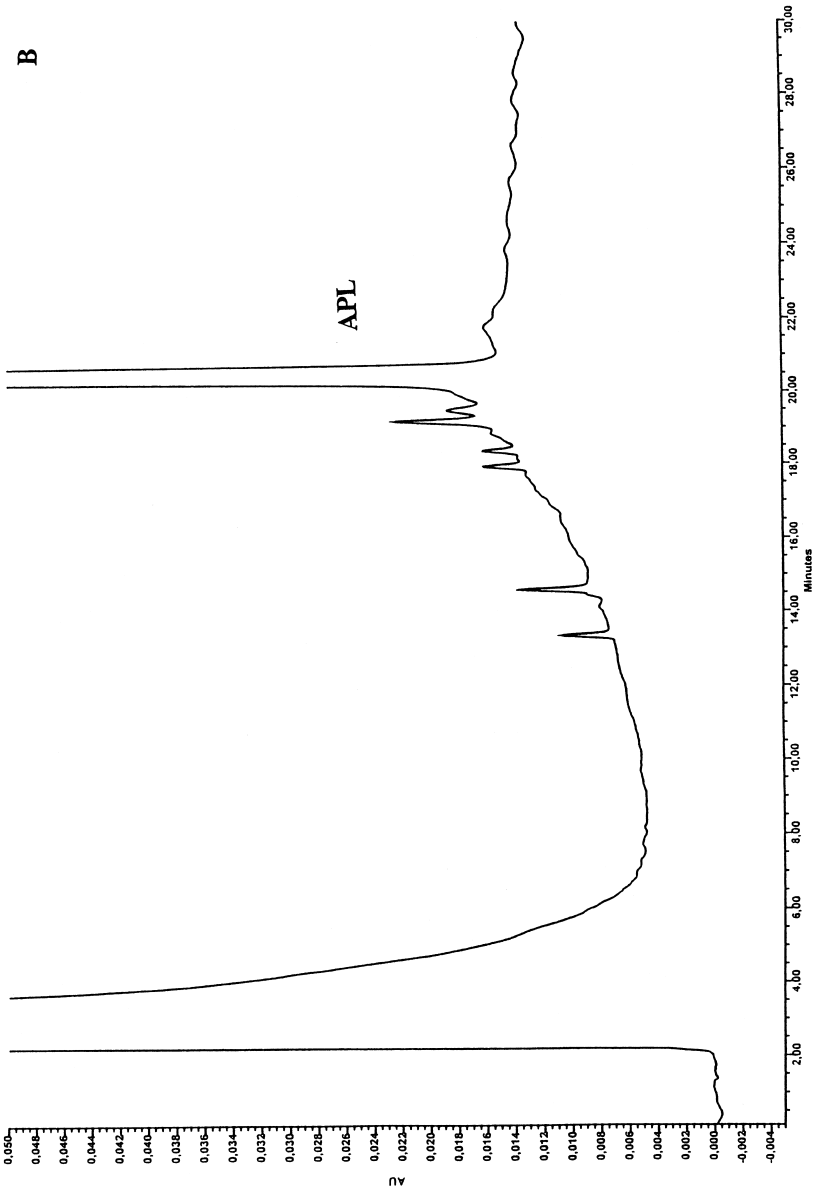


Figure 4. Degradation of aplidine (APL) in alkaline conditions for 30 minutes (**a**) and oxidative conditions for 24 hours (**b**).



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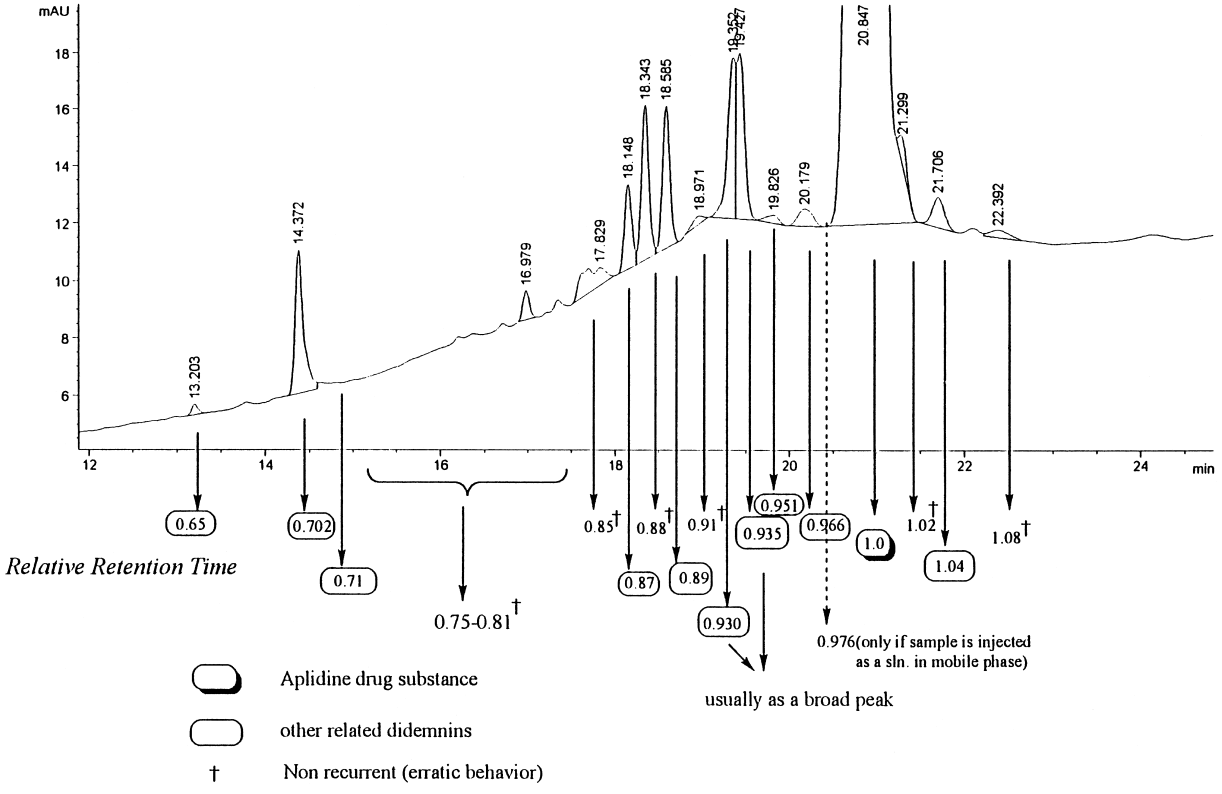

Figure 5. Representative chromatogram of aplidine raw drug substance, Lot APL#397 (impurity at a relative retention time of 0.71 was not present in this lot).

Table 5. Impurity Profile of Aplidine Raw Drug Substance (Derived from Chromatographic Profiles of Lots APL#397, APL#499, APL#599, APL#699, APL#799, and APL#899)

Impurity	Retention Time Interval (min)	Relative Retention Time (interval)	Area % (interval)	Molecular Mass (MH^+)
1. Didemnin A	13.3–13.9	0.65 (0.63–0.66)	0.03–0.1	943
2. Unknown (degradation product)	14.2–14.9	0.70 (0.68–0.71)	0.09–0.35	—
3. Trifluoroacetyldidemnin A	14.9–15.8	0.71 (0.70–0.73)	0–0.1	1038
4. Unknown	17.7–17.9	0.85 (0.85–0.86)	0.08–0.5	1127
5. Unknown (degradation product)	18.0–18.4	0.87 (0.86–0.89)	0.02–0.09	1142
6. Unknown (degradation product)	18.4–18.9	0.89 (0.89–0.91)	0.03–0.14	—
7. Noraplidine		0.93[a]	0.27–0.36	1096
8a. Unknown, 8b. unknown (degradation products)	19.3–19.9	0.94 (0.93–0.95)	0.3-0.5 (total area 8a, 8b)	8a. 1110, 8b. 1142
9a. Noraplidine, 9b. unknown	19.5–19.9	0.95 (0.95–0.96)	0.1-0.2 (total area 9a, 9b)	9a. 1096, 9b. 1142
10. Acetyldidemnin A	20.1–20.6	0.97 (0.96–0.98)	0.11-1.97	986
11. Aplidine	20.7–20.6	1.0	> 98	1110
12. Unknown	21.3–21.4	1.02 (1.02–1.03)	0.13	1143
13a. Unknown, 13b. Unknown	21.4–22.4	1.04 (1.04–1.05)	0.02–0.1	13a. 1124, 13b. 1146

[a]Usually hidden under peak at relative retention time of 0.94 (8a,b).

(Lots APL#297 and APL#397) showed levels of around 2% of this specific impurity. After confirmation of its molecular mass (MH^+ 986) and co-elution of isolated and synthesized material with the impurity present in aplidine raw drug substance, the substance could be identified as acetyl-didemnin A.

Acetyl-didemnin A results from the reaction between unreacted didemnin A and O-acetylisourea originating from ethyl actetate and unreacted carbodiimide during the work-up step. In the manufacture of later batches of aplidine raw substance, the amounts of acetyl-didemnin A could be reduced to levels below 0.2% by complete quenching of the reaction mixture with 0.1 N hydrochloric acid before extraction with methylene chloride. Low levels of didemnin A (≤ 0.1%, MH^+ 943) were detected in a relative retention time range of 0.63-0.66 for all lots of hemisynthetic aplidine. The impurity at a relative retention time of 0.71 (MH^+ 1038), present in the lots examined at levels in the range of 0-0.1%, was assigned to trifluoroacetyl didemnin A (0% in Lot#APL397, Figure 5). The formation of this analytical method-related impurity can be explained in a similar manner to the formation of acetyldidemnin A, by reaction of trifluoroacetic acid present in the mobile phase, activated by unreacted carbodiimide, and didemnin A.

Due to the presence of the impurity nordidemnin A in the didemnin A starting material, noraplidine is formed during the synthesis of aplidine (Figure 6). In the lots examined, however, this impurity (MH^+ 1096) often co-elutes with two unknown impurities (MH^+ 1110 and 1142, respectively) appearing in a relative retention time-range of 0.93-0.95, thereby, hampering the determination of the relative amount of the compound. Peaks at relative retention times of approximately 0.70, 0.87, 0.89, and 0.94 refer, most probably, to degradation products as they increase with time upon standing. Thus far, these compounds have not been structurally identified. It was found, however, that the peaks at relative retention times of 0.70 and 0.89 only appeared after a significant increase in the peak area of the products at relative retention times of 0.87 and 0.94, suggesting a sec-

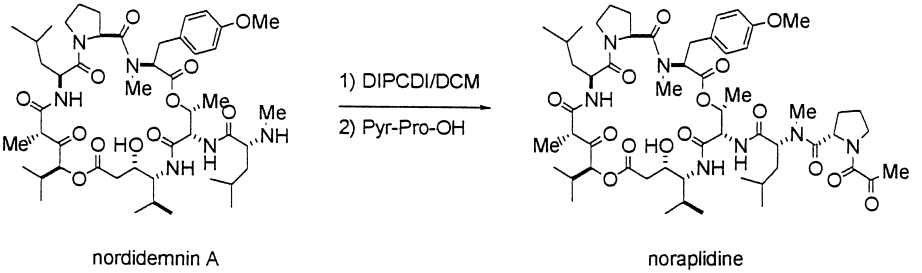

Figure 6. Formation of noraplidine from nordidemnin A.

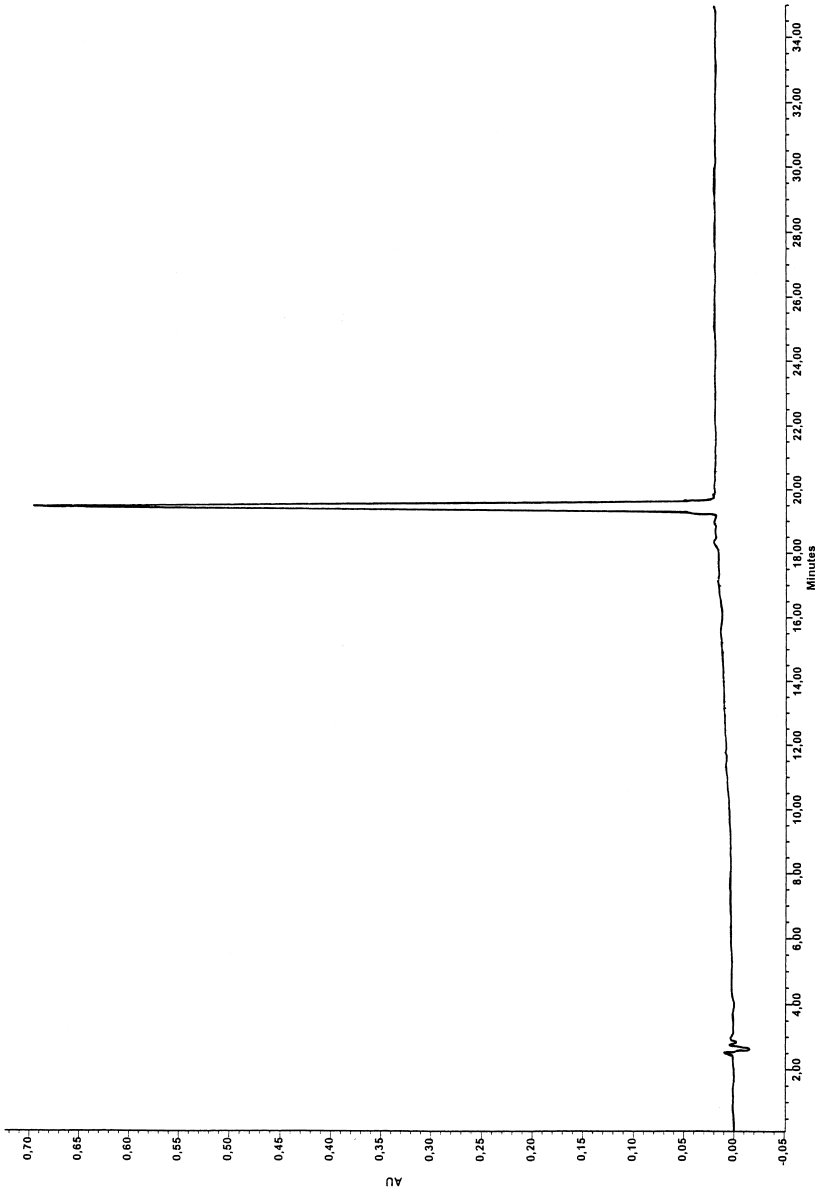

Figure 7. Representative chromatogram of aplidine 500 µg/vial lyophilized powder for intravenous use (Lot 99J11).

ondary reaction and resulting in the formation of four products. Furthermore, upon degradation, a significant increase in the peak attributed to didemnin A was found. Figure 7 shows a representative chromatogram of aplidine 500 μg/vial lyophilized powder for intravenous use, Lot 99J11, manufactured using hemisynthetic aplidine raw drug substance. No significant differences in the chromatographic profiles for both aplidine final product and aplidine raw drug substance could be found, indicating that the manufacturing process does not affect the integrity of aplidine.

CONCLUSION

In conclusion, the HPLC-UV method presented provides a specific and reproducible method for the quantification of aplidine and determination of impurities and degradation products in aplidine raw drug substance and the pharmaceutical product. Using the HPLC-UV system, an impurity profile of hemisynthetic aplidine was drawn up. Furthermore, a number of impurities were structurally elucidated by LC-MS analysis. Current research is now focused on the impurity profiling of synthetic material, which will be used in the manufacturing of aplidine pharmaceutical product in the forthcoming clinical studies.

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