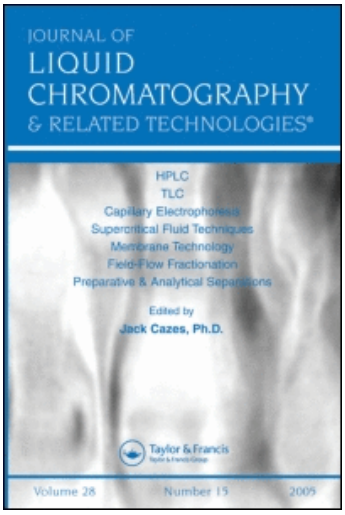

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DEVELOPMENT OF AN HPLC METHOD WITH UV DETECTION FOR THE PHARMACEUTICAL QUALITY CONTROL OF THE NOVEL MARINE ANTICANCER AGENT KAHALALIDE F

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ABSTRACT

Kahalalide F is a cyclic depsipeptide derived from the marine mollusc Elysia rufescens, an organism living in the seas near Hawaii. On the basis of its in vitro and in vivo selectivity, kahalalide F is currently developed as a potential anticancer agent against androgen independent prostate tumors. The development and validation of a reversed-phase high performance liquid chro-

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matography (RP-HPLC) method with ultra-violet (UV) detection for the quantification and purity determination of kahalalide F in raw drug substance and pharmaceutical dosage form is described. Linear calibration curves in the range of 0.5-12.5 µg/mL of kahalalide F with correlation coefficients > 0.999 were obtained. Within-run and between-run precisions were ≤ 3.0% and accuracy was within 100.4-103.2%. The assay proved selective, as determined by stress-testing, confirming its stability indicating capacity. Using liquid chromatography-mass spectrometry (LC-MS) analysis, kahalalide G, the hydrolyzed open-chain analog of kahalalide F, appeared upon heating and in acidic media. Furthermore, it was shown that kahalalide F remains its integrity in the freeze-dried pharmaceutical dosage form.

INTRODUCTION

Kahalalide F is a cyclic depsipeptide derived from the marine mollusc *Elysia rufescens*, an organism living in the seas near Hawaii (Figure 1).(1) Kahalalide F

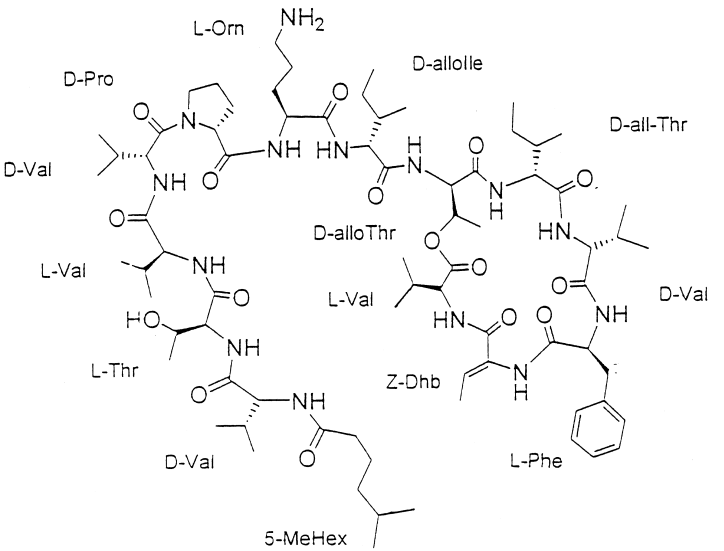

Figure 1. Chemical structure of kahalalide F ($C_{75}H_{124}N_{14}O_{16}$, Mw 1477; 5-MeHex: 5-methyl-hexane; Val: valine; Thr: threonine; Pro: proline; Orn: ornithine; Ile: isoleucine; Phe: phenylalanine).

displays both *in vitro* and *in vivo* activity in various solid tumor models, amongst which are breast, colon, non-small cell lung, and, in particular, prostate cancer. Preliminary data suggest the mechanism of action on the lysosome-level in the cell. On the basis of its selectivity, kahalalide F is now further developed as a potential anticancer agent against androgen independent prostate tumors.(1,2) For the forthcoming clinical trials, the drug is pharmaceutically formulated as a lyophilized solid for intravenous use containing 150 μg kahalalide F per dosage unit.

The product is to be reconstituted with a mixture of the non-ionic surfactant polyoxyethylated castor oil (Cremophor EL), ethanol, and water (5/5/90% v/v/v, CEW), with further dilution in normal saline. Structural characterization studies of kahalalide F were conducted using mass spectrometry (MS), nuclear magnetic resonance (^{1}H NMR), and infra-red (IR) spectroscopy. Furthermore, the development and validation of a stability indicating analytical assay for the pharmaceutical quality control of both kahalalide F drug substance and final dosage form is described.

EXPERIMENTAL

Chemicals

Kahalalide F (Lot KHF-299) was obtained from Pharma Mar SA (Tres Cantos, Madrid, Spain). Kahalalide F lyophilized product for intravenous use containing 150 μg of active substance, 3 mg of anhydrous citric acid, 3 mg of polysorbate 80, and 150 mg of sucrose was manufactured in-house (Department of Pharmacy of the Slotervaart Hospital/The Netherlands Cancer Institute). All reagents were of analytical grade and used as received. Acetonitrile was purchased from Biosolve (Amsterdam, The Netherlands). Trifluoroacetic acid was obtained from Merck (Amsterdam, The Netherlands). Distilled water was used throughout the experiments.

Apparatus

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). The mass spectrum was obtained with a Finnigan LC-Q ion trap mass-spectrometer

(Thermoquest LC/MS Division, San Jose, CA, USA) equipped with a nanoflow electrospray source. LC-MS analyses were performed on a HP1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) connected to an API 2000 mass spectrometer (Sciex, Thornhill, ON, Canada) equipped with a turbo ion spray, and a Spectra 100 variable wavelength UV-VIS detector (Spectra-Physics, San Jose, CA, USA).

^{1}H NMR spectra were recorded with a Gemini 300 BB instrument (Varian Assoc., Palo Alto, CA USA) at 300.1 MHz. The IR spectrum was obtained with a PU9700 IR spectrometer (Philips, Eindhoven, The Netherlands).

MS, ^{1}H NMR, and IR Spectroscopy Conditions

MS: Kahalalide F was dissolved to a concentration of approximately 50 μM in methanol; collision induced dissociation (CID) spectra were recorded by mass-selecting the precursor ion with an isolation width of 2 daltons, followed by resonance RF excitation and detection.

^{1}H NMR: Kahalalide F (1 mg) was dissolved in deuterochloroform ($CDCl_3$). $CHCl_3$ at δ =7.26 ppm was used as the internal reference line.

IR: Samples were prepared by grinding approximately 1 mg of kahalalide F with approximately 200 mg of potassium bromide and annealing the powder into a disc using a hydraulic press. Spectra from 400-4000 cm^{-1} were recorded.

Chromatographic Conditions

Injections of 75 μL 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) and kept at a temperature of +80°C. The eluent was comprised of a linear gradient of acetonitrile and water containing 0.04% trifluoracetic acid from 35% up to 90% acetonitrile in 25 minutes. The flow rate was 0.6 mL/min and UV-detection was performed at 215 nm. A total run time of 30 minutes was employed.

LC-MS Conditions

LC-MS conditions were similar to those described under "Chromatographic conditions". 20 μL injections were performed, which were subsequently splitted to the mass spectrometer (1/20 part) and the UV-detector (19/20 part). 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 are given in Table 1.

Table 1. MS Settings

Parameter	Setting
Ion spray voltage (positive ion mode)	+5500 V
Declustering potential	+16 V
Focussing potential	+360 V
Entrance potential	−11.5 V
Ion Energy Q1	0.6 V
Collission cell entrance potential	+34.16 V
Collision energy	+51 V
Collision cell exit potential	+7.54 V
Ion Energy Q3	2.5 V
Deflector	−200 V
Channel Electron Multiplier	2300 V
Dwell time	mrm 150 ms; Q1 700 ms
Nebulizer gas (compressed air) pressure	35 psi
Curtain gas (N_2) pressure	20 psi
Turbo gas (N_2) pressure	45 psi
Temperature	350°C
Collision Activated Dissociation gas (N_2) pressure	3 psi
Kahalalide F (srm)	
Quad 1 selected mass (amu)	739.8
Quad 2 selected mass (amu)	212.21
Kahalalide F (Q1)	
Start mass (amu)	100
Stop mass (amu)	1800

Validation Procedure

Linearity

From a 250 μg/mL stock solution of kahalalide F in an acetonitrile/water/trifluoroacetic acid mixture (35:65:0.04%, v/v/v, solvent), calibration curves at six concentration levels (0.5, 1.0, 2.5, 5.0, 10.0, and 12.5 μg/mL kahalalide F in solvent) were prepared and analyzed, in duplicate, in three separate runs. Least-squares regression analysis of concentration, weighted by [1/concentration], versus the area of the kahalalide F 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 were determined by assaying samples at three concentration levels (1.0, 5.0, and 10.0

μg/mL kahalalide F in eluent) in triplicate, in three separate analytical runs. Samples were prepared from an independently prepared stock solution of 250 μg/mL kahalalide F in solvent. Accuracy was measured as the percent deviation from the nominal concentration. The within-run and between-run precision were 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 kahalalide F stock solution to heat, acidic, and alkaline stress testing conditions. Solutions at a concentration of 100 μg/mL kahalalide F in water, 0.1 N hydrochloric acid, and 0.1 N sodium hydroxide were prepared, respectively, and subsequently stored at +50°C in the dark. Samples were analyzed for degradation of kahalalide F in time, using LC-MS. Kahalalide F 150 μg/vial lyophilized product was reconstituted with 3 mL of water and subsequently diluted five-fold with solvent.

RESULTS AND DISCUSSION

Structural Characterization

With MS analysis, the $[M+H]^+$-ion was found at *m/z* 1477, corresponding to the mass of the stipulated molecular structure of kahalalide F. Figure 2 shows the product ion spectrum of *m/z* 1477. Assignments to the major fragment ions are given in Table 2. As can be seen, fragmentation mainly occurs at the side-chain attached to the depsipeptide-ring of the kahalalide F molecule. As the presence of many identical amino-acids in the molecule resulted in highly overlapping signals, the ^{1}H NMR spectrum is largely inconclusive. As for MS, the IR spectrum was indicative of the structure of kahalalide F (Table 2).

HPLC-UV Method Development

On the basis of the structural resemblance with kahalalide F, the current assay of the marine compound aplidine, also a cyclic depsipeptide, was taken as a starting point in the development of a stability indicating analytical method. Modifications were made to optimize the system for the analysis of kahalalide F. As for aplidine, a gradient HPLC system was applied. With gradient elution, all the peaks will be eluted, either during the gradient or within a few minutes after

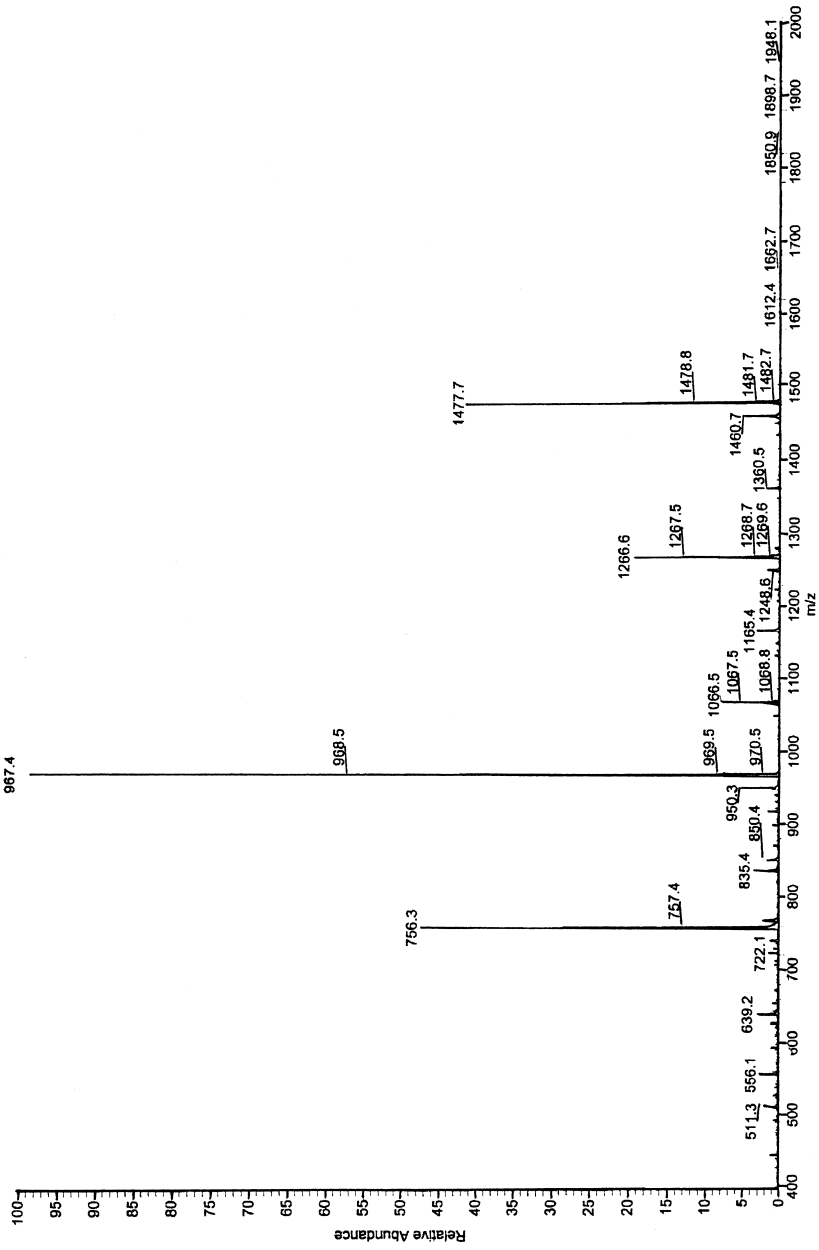

Figure 2. Mass spectrum of kahalalide F (MS-MS).

Table 2. Characterization of Kahalalide F Raw Drug Substance (Lot KHF-299)

Analytical Method	Results
Appearance	White amorphous powder
Mass spectrum	Molecular formula: $C_{75}H_{124}N_{14}O_{16}$; Protonated molecular ion $[M+H]^+$ *m/z* 1477; $[M+2H]^{2+}$ *m/z* 739; MS-MS (MS2): *m/z* 1266.6 $[M+H]^+$-211, *D*-Val-5-MeHex; *m/z* 1165.4 $[M+H]^+$-313, *L*-Thr-*D*-Val-5MeHex; *m/z* 1066.5 $[M+H]^+$-410, *L*-Val-*L*-Thr-*D*-Val-5-MeHex; *m/z* 967.4 $[M+H]^+$-511, *D*-Val-*L*-Val-*L*-Thr-*D*-Val-5-MeHex; *m/z* 756.3 $[M+H]^+$-721, *D*-Pro-*L*-Orn-*D*-Val-*L*-Val-*L*-Thr-*D*-Val-5-MeHex
IR spectrum	Characteristic absorption bands (approximately): 3300 cm^{-1}: N-H stretching (amide); 2900–3000 cm^{-1}: C-H stretching (aliphatic); 1650 cm^{-1}: N-C=O stretching (amide); 1520 cm^{-1}: C=C stretching (aromatic); 1400 cm^{-1}: C-H deformation; 1100–1200 cm^{-1}: C-O stretching

the gradient is complete. This is especially of interest during the development of a stability indicating assay capable of identifying impurities and degradation products, apart from the substance of interest, with a broad range of polarities. Using a gradient starting at an eluent composition of acetonitrile/water/trifluoroacetic acid 35:65:0.04%, v/v/v going up lineary to a composition of 90:10:0.04%, v/v/v kahalalide F eluted at a retention time of approximately 15 minutes.

The addition of trifluoroacetic acid to the eluent improved the shape of the kahalalide F peak. As determined with PDA detection, kahalalide F shows no absorption maxima and hardly any absorption at wavelengths above 280 nm. Absorption of the phenyl moiety appears negligible. UV-detection at a wavelength of 215 nm was selected for the analysis of kahalalide F. Using the initial gradient eluent composition of acetonitrile/water/trifluoroacetic acid (35:65:0.04%, v/v/v) as solvent medium, kahalalide F eluted as a single, sharp peak in the chromatogram. The peak identity was confirmed by on-line MS analysis (Figure 3). For the aplidine-analysis, a high column temperature of +80°C was necessary to make the *cis-trans* isomers of the molecule, which appear as two distinct peaks in the chromatogram at room temperature, elute as a single peak (3). Figure 4 gives the molecular structure of aplidine and the *cis-trans* isomerism at the pyruvoyl-proline amide bond.

From the literature, the occurrence of more than one conformation in proline-containing peptides is well known.(4) Like aplidine, kahalalide F contains a proline-moiety in the side-chain attached to the depsipeptide-ring. However, iso-

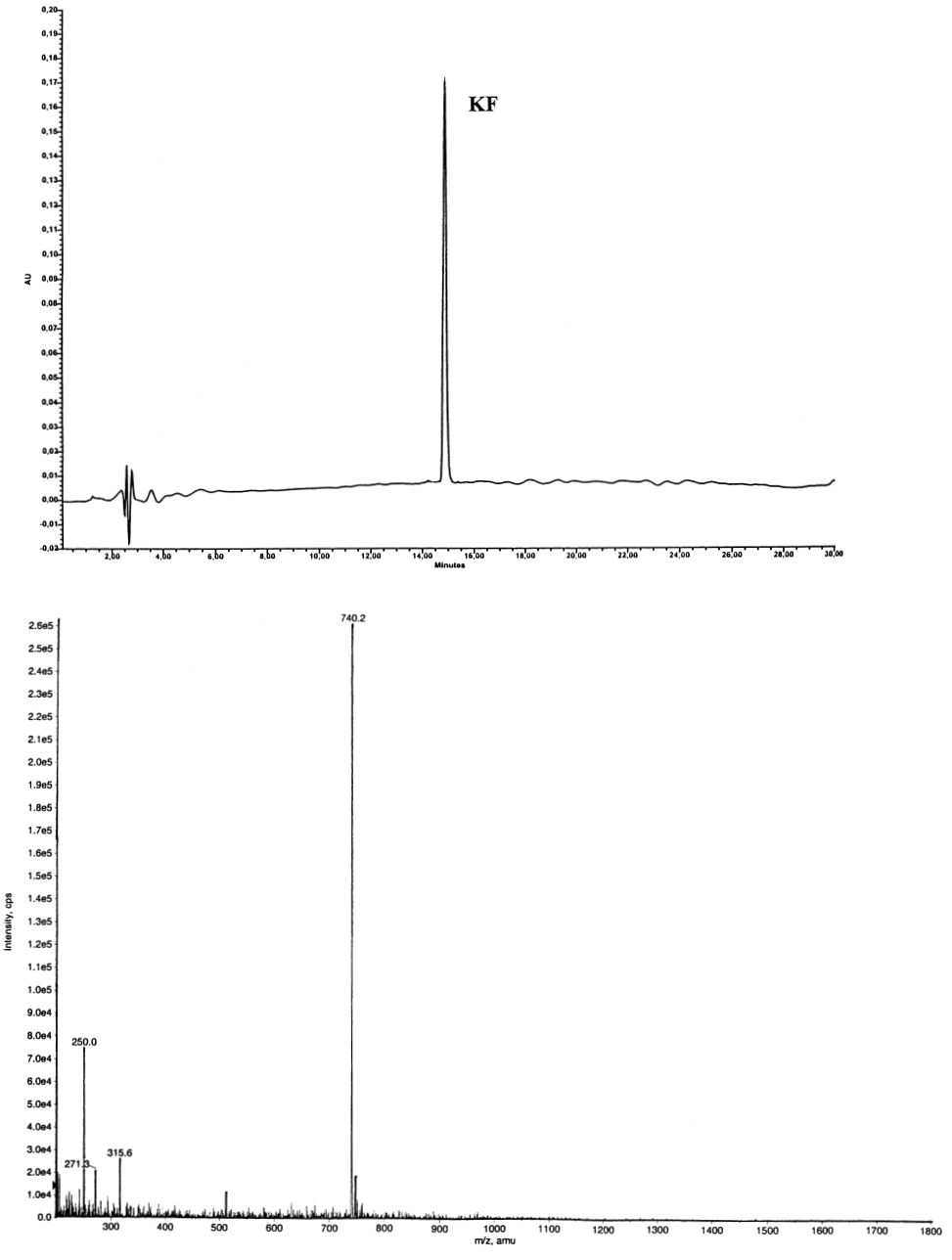

Figure 3. Representative chromatogram of kahalalide F (KF, 0.75 μg absolute on column) and its mass spectrum (on-line).

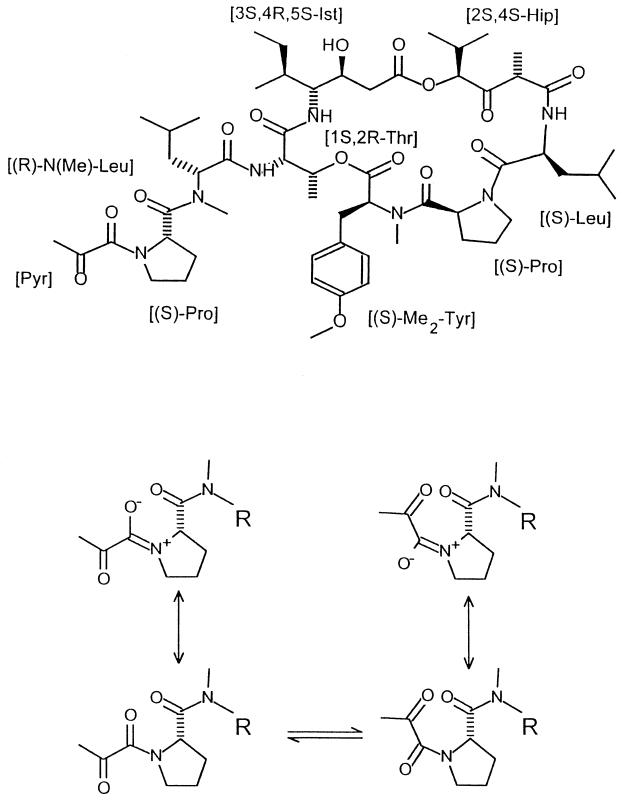

Figure 4. Structural formula of aplidine and cis-trans isomerism at the pyruvoyl-proline moieties.

merism was not noted for kahalalide F at +25°C. Apparently, in contrast to aplidine, any equilibrium reaction is too fast to elute the *cis* and *trans* isomers as distinct peaks and/or only a pyruvoyl group attached to proline can give this chromatographically detectable isomerism for these types of cyclic depsipeptides. However, as chromatography at +80°C resulted in some improvement of the peak symmetry and kahalalide F was stable on column under these conditions as determined by on-line MS, this temperature was selected for further development.

Validation

Linearity, accuracy, within-run, and between-run precision of the HPLC-UV method over a concentration range of 0.5-12.5 μg/mL was examined. This

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

Concentration (Nominal, μg/mL)	Concentration (Found, μg/mL)	RSD (%)	Accuracy (%)
0.5	0.5 ± 0.01	2.4	100.0
1.0	1.0 ± 0.03	3.0	99.3
2.5	2.5 ± 0.05	2.2	99.8
5.0	5.1 ± 0.07	1.3	102.3
10.0	10.0 ± 0.06	0.6	99.7
12.5	12.5 ± 0.13	1.0	99.5

range corresponds to 5-125% of the intended test concentration of 10 μg/mL for the pharmaceutical quality control of kahalalide F raw drug substance and final product. 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 ≤ 2.3%. The within-run and between-run precisions at concentration levels of 1.0, 5.0, and 10.0 μg/mL were ≤ 3.0% with accuracies between 100.4-103.2% (Table 4).

Selectivity of the assay was examined by accelerated stress testing. Upon heating of an aqueous solution of kahalalide F, degradation products at approximately 14 (degradation product 1) and 28 minutes (degradation product 2) merged in the chromatograms (Figure 5a). Using LC-MS, the doubly-charged ion for the peak at 14 minutes was detected at *m/z* 749, indicating the addition of water to the kahalalide F molecule. Likely, hydrolysis of the ester-bond between the *L*-Val and Thr-moieties in the depsipeptide chain occurs, resulting in the open-chain analog of kahalalide F, kahalalide G (Mw 1496) (5). No conclusive mass peak could be detected for degradation product 2.

The solution of kahalalide F in 0.1 N hydrochloric acid showed the gradual appearance of degradation products at retention times of approximately 8 (degra-

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

Concentration (Nominal, μg/mL)	Within-Run Precision (%)	Between-Run Precision (%)	Accuracy (%)
1.0	1.4	3.0	103.2
5.0	1.2	1.5	103.1
10.0	0.8	1.0	100.4

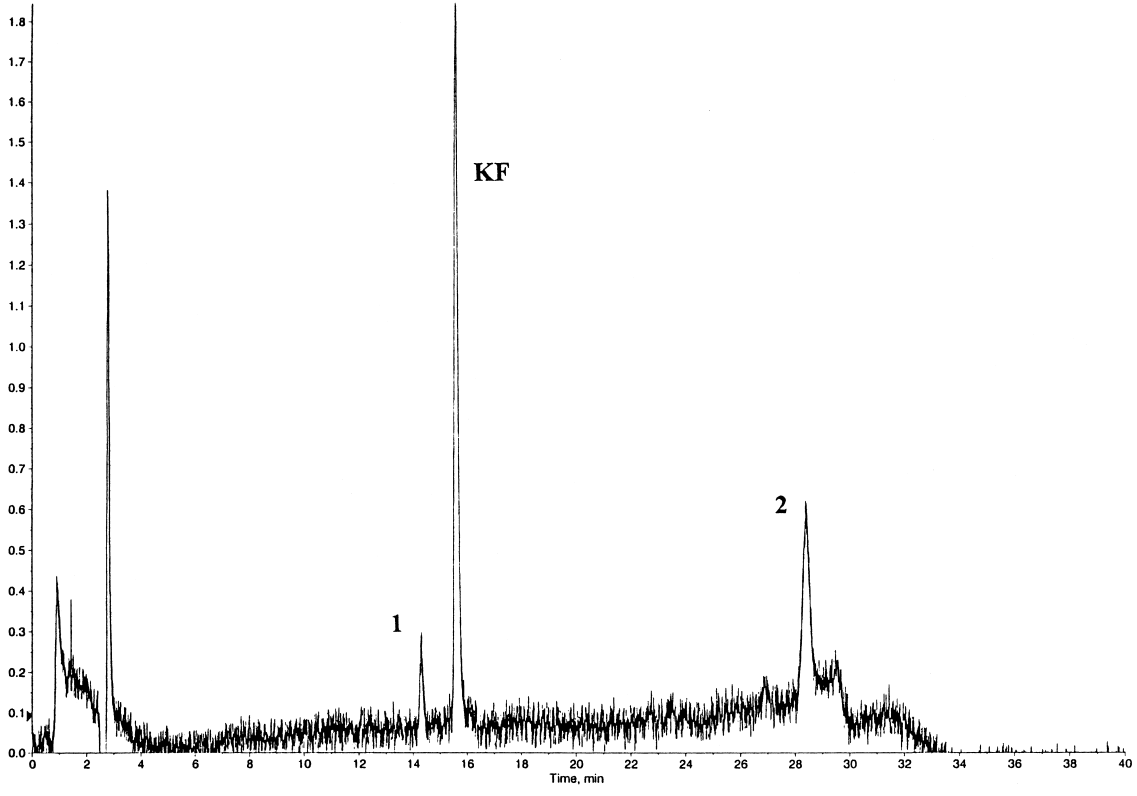

Figure 5. HPLC -chromatograms (UV-detection) of 20 µL injections of 100 µg/mL kahalalide F (KF) solutions after 24 hours at +50°C in the dark in, (a) water; (b) 0.1 N hydrochloric acid (the two peaks between 2-3 minutes originate from hydrochloric acid solution).

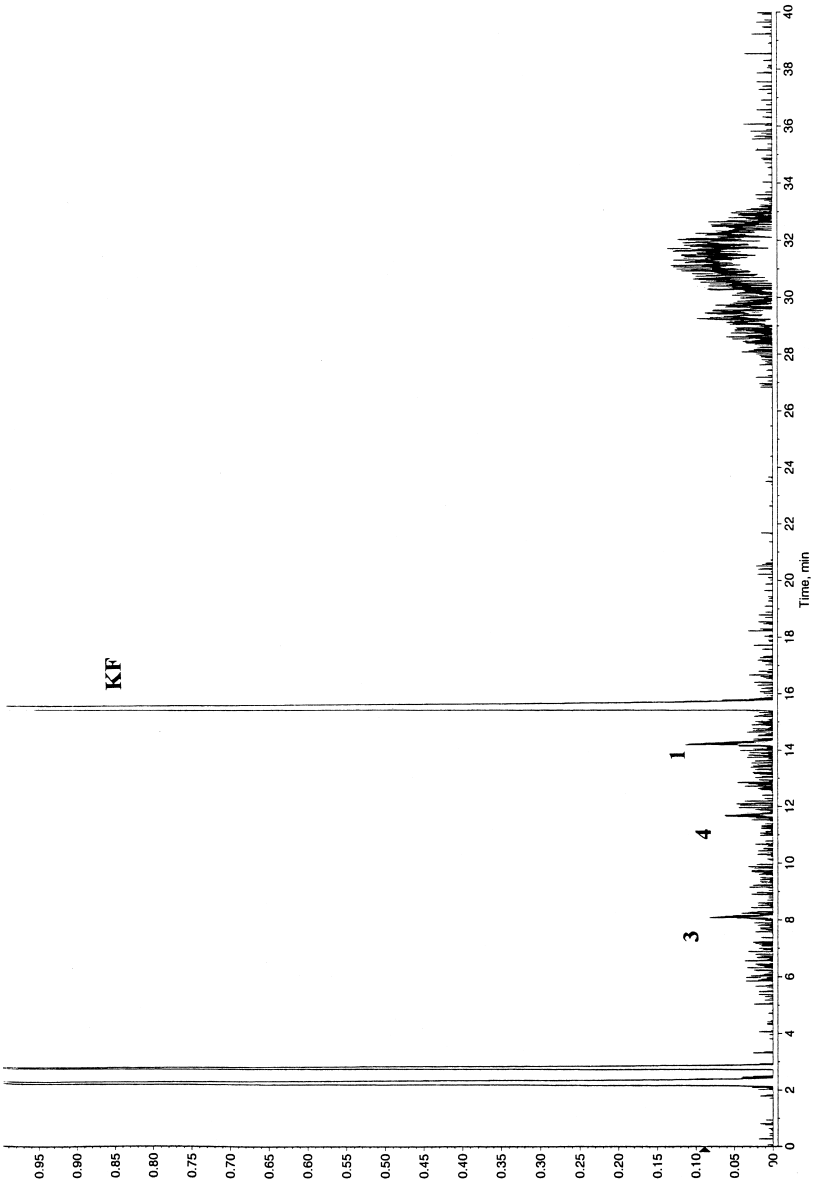

Figure 5. Continued.

dation product 3), 11.6 (degradation product 4), and 14 minutes (degradation product 1) (Figure 5b). Using LC-MS analysis, doubly-charged ions at *m/z* 690, 740, and 749 could be identified for these products, respectively. Degradation product 1 seems to originate from the hydrolysis of kahalalide F into kahalalide G, similar as described for the heated aqueous solution. Degradation product 3 (doubly-charged ion at *m/z* 690) might result from degradation of either kahalalide F or kahalalide G. Degradation product 4 with a doubly-charged ion at *m/z* 740, identical for kahalalide F, might result from epimerization in the parent molecule. Complete destruction of kahalalide F occurred in 0.1 N sodium hydroxide and no assignments could be given to the signals obtained in the mass spectrum. During all degradation studies the UV spectrum, as determined by PDA analysis and MS profile of the kahalalide F peak, did not change, excluding coelution of degradation products.

From these results, it can be concluded that the HPLC-UV system has good stability indicating capability. From *in vitro* assessments it was found that kahalalide G shows no cytotoxic activity. As kahalalide F is formulated in a relatively acidic medium (pH 2.8, 5 mM citric acid), in which hydrolysis of kahalalide F could occur, it was investigated to see if the substance remained intact during the manufacturing process and in the final pharmaceutical product. LC-MS analysis performed on reconstituted lyophilized product resulted in a single peak at a retention time of 15 minutes and a $[M+2H]^{2+}$-ion at *m/z* 740, confirming the integrity of kahalide F upon formulation. The chromatographic purity of kahalalide F raw drug substance Lot KHF-299 was determined at 98.85%.

CONCLUSION

In conclusion, a stability indicating HPLC-UV method was developed for the pharmaceutical quality control of kahalalide F raw drug substance and final product. The method was found to be linear in the range of 0.5-12.5 μg/mL, precise, accurate, and reproducible. The assay also proved selective, as determined by stress-testing, confirming its stability indicating capacity. Using LC-MS analysis, kahalalide G, the hydrolyzed open-chain analog of kahalalide F appeared upon heating and in acidic media. Furthermore, it was shown that kahalalide F retains its integrity in the final dosage form.

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