ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 903–912

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Microbiological and densitometric TLC analyses for peptides in liposomes

M. Ricci *, L. Tuttobello, G. Luca, C. Rossi

Department of Chimica e Tecnologia del Farmaco, Via del Liceo, 1, Università degli Studi, 06123 Perugia, Italy

Received 17 September 2000; received in revised form 14 December 2000; accepted 30 December 2000

Abstract

Quantitative determination of Leucinostatins and/or of similar peptides, such as Peptaibols, is sometimes quite difficult to perform especially when they are entrapped in vectors, i.e. liposomes, whose components display UV absorbances that may obscure those of the active principle. Therefore, in these cases, it is useful to find alternative ways, especially when high pressure liquid chromatography (HPLC) is difficult to perform or needs long procedure times. In the present paper, the use of microbiological and densitometric methods for quantitative analysis of Leucinostatin A (Leu-A) are described and the results compared with those from HPLC analyses. The use of microbiological and densitometric assays, furnished results comparable with those obtained by HPLC. Of the two methods used, the microbiological procedure appeared to be less accurate and precise.

Keywords: Liposomes; Leucinostatin A; Peptides; Densitometric analyses; Microbiological analyses; HPLC

1. Introduction

Leucinostatins (Fig. 1) are a family of linear nonapeptides which concerned Italian, Japanese and American research groups because of their interesting antimycotic, antimicrobial (against gram positive bacteria) and phytotoxic properties [1–7]. In spite of the high biological activities, the clinical use of these peptides is severely limited by their low DL_{50}, ranging from 0.8 (i.v.) to 5.4 mg/Kg (oral). Therefore, attempts have been made to find suitable carriers, which could reduce potential side effects, modify the distribution profile of these molecules and, at the same time, to possibly solve the problems due to their poor solubility in water as well [8–11].

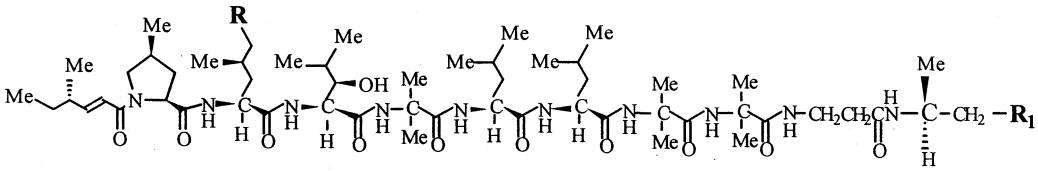
In order to study the efficiency of various leucinostatin formulations, it is necessary to estimate carefully the peptide liposome loading. Although reversed phase high pressure liquid chromatogra-

Abbreviations: Chol, cholesterol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; Leu-A, leucinostatin-A; MLVs, multilamellar vesicles; S.D., standard deviation; TEAP, triethylammoniumphosphate; TEM, transmission electron micrscope.

* Corresponding author. Tel.: +39-075-5855125; fax: +39-075-5855163.

E-mail address: riky@unipg.it (M. Ricci).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00365-X



Leucinostatin A	$R = CH(OH)CH_2COCH_2CH_3$	$R_1 = N(CH_3)_2$
Leucinostatin B	$R = CH(OH)CH_2COCH_2CH_3$	$R_1 = NHCH_3$
Leucinostatin C	$R = CH(OH)CH_2COCH_2CH_3$	$R_1 = NH_2$
Leucinostatin D	$R = H$	$R_1 = N(CH_3)_2$
Leucinostatin F	$R = H$	$R_1 = NHCH_3$
Leucinostatin H	$R = H$	$R_1 = NO(CH_3)_2$
Leucinostatin K	$R = CH(OH)CH_2COCH_2CH_3$	$R_1 = NO(CH_3)_2$

Fig. 1. Molecular structures of Leucinostatins.

phy (HPLC), is the method of choice for quantitative analysis of peptides, namely Leucinostatins and Peptaibols [16], it is sometimes time-consuming and difficult to perform, especially when high concentrations of other constituents, as sterols (data not published) are present in the formulation. Therefore, in such circumstances, alternative analytical methods are needed in order to estimate in an unambiguous way the amount of these peptides entrapped in liposomes.

Classical quantitative determination by UV spectroscopy of leucinostatins is difficult because of their absorbance peaks at 204 and 220 (sh) nm, which are often superimposed by other signals. Capillary zone electrophoresis (CZE) was also used as a sensitive, specific and rapid method to identify, with good resolution, mixtures of leucinostatins [12]. Nevertheless, this method failed the quantitative determination of these peptides in liposomes.

The aim of this paper was to develop easy and fast analytical methods, as alternatives to HPLC, to perform unambiguous titration of leucinostatins in liposomes with different compositions.

The present paper deals with the titration of Leu-A, taken as a model compound, following two ways namely microbiological and thin layer chromatography-ultra violet (TLC-UV) densitometric methods. The former was chosen for the high biological activity of the peptide [4], while the latter for its simplicity, low cost, high resolving power and short analysis times. Both methods have been proved to be valid and the results obtained were compared with those from HPLC as regard to their linearity, precision, accuracy and sensitivity.

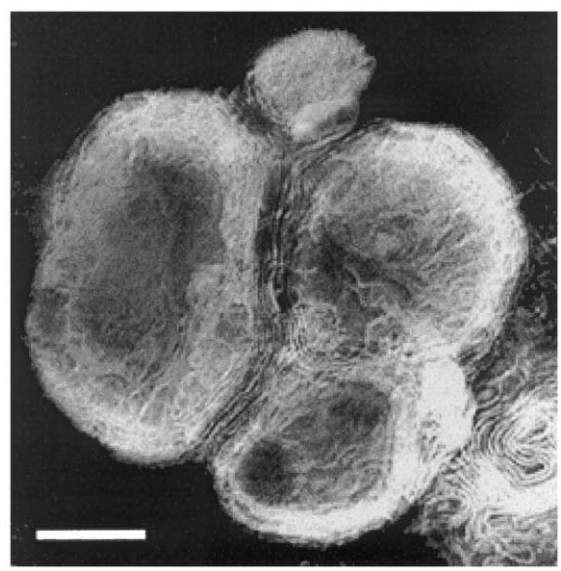


Fig. 2. Transmission electron micrograph (TEM) of Leu-A loaded DPCC ML versus bar corresponds to 2 μm.

Table 1
Statistics for HPLC calibration curve data

Concentration (μg/ml)	Peak area (mean value ± S.D., $n = 5$)	Confidence intervals (0.05 significance level)	%R.S.D.
6.12	21.32 ± 0.14	±0.20	0.7
25.16	79.88 ± 0.50	±0.71	0.6
99.96	292.28 ± 1.76	±2.50	0.6
299.88	904.35 ± 4.27	±6.07	0.5
340.00	1021.32 ± 4.10	±5.77	0.4

Table 2
Statistics for TLC-UV calibration curve data

Concentration (μg/ml)	Peak area (mean value ± S.D., $n = 5$)	Confidence intervals (0.05 significance level)	%R.S.D.
75	24 931.89 ± 249.99	±355.52	1.0
100	35 448.82 ± 427.84	±608.45	1.2
150	48 467.50 ± 459.67	±653.71	0.9
225	69 364.33 ± 837.34	±1190.80	1.2
300	92 928.15 ± 791.17	±1125.15	0.8

Table 3
Statistics for microbiological calibration curve data

Log [Conc (μg/ml)]	Halos area (mean value ± S.D., $n = 5$)	Confidence intervals (0.05 significance level)	%R.S.D.
1.096	13.52 ± 0.91	±1.29	6.7
1.390	15.37 ± 0.85	±1.21	5.5
1.700	17.35 ± 0.88	±1.25	5.1
2.000	18.37 ± 0.64	±0.91	3.5
2.300	20.50 ± 0.68	±0.96	3.3

2. Materials and methods

2.1. Chemicals and reagents

Leu-A·HCl was obtained from cultural broth benzene extract of *Paecilomyces marquandii* Massee (Hughes) and purified by extensive flash chromatographies on Silica Gel columns followed by crystallization with ethylacetate [13]. Cholesterol (Chol), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), distearoylphosphatidylcholine (DSPC), were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The triethylammonium phosphate (TEAP) buffer solution (pH 3, 1.0 M) used was provided by Fluka (Buchs, Switzerland). The purity of lipids was checked by TLC using Silica Gel plates as stationary phase from Merck, (Darmstadt, Germany) and $CHCl_3$–MeOH–H_2O (65:25:4 v/v/v) as mobile phase. Development of the chromatogram, performed with the Dragendorff's reagent, showed one spot at appropriate R_f [14]. All other reagents and solvents were of the highest purity available. The sterile nutrient agar medium was composed by Alimento Carneo Assimilabile Standardizzato (ACAS, Costantino & Co., Favria, Italy) (1% w/v), NaCl (0.5% w/v), Costantino Peptone (Costantino & Co., Favria, Italy) (1% w/v), and glucose (1% w/v).

2.2. Preparation and characterization of liposomes

Multilamellar vesicles (MLVs) were prepared by the film method [15]. Chloroform solutions containing lipids and Leu-A (at right molar ratio) were poured into 50 ml round bottom flasks and placed on a Büchi T-51 rotating evaporator (Flawil, Switzerland). Rapid evaporation of the organic solvent, carried out under a stream of nitrogen over the gently warmed solutions, resulted in the deposition of thin films on the flask walls. Dry lipid films were maintained overnight under reduced pressure to remove traces of organic solvent. Finally, appropriate amounts of buffer solutions were added yielding 45 mM phospholipid concentrations. Films were hydrated by shaking in a Gallenkanp orbital incubator (Fisons Instruments, Crawley, UK) above 10°C (the gel-liquid-crystalline phase transition temperature T_m of the phospholipids) until homogeneous white milky suspensions formed (approximately 1 h). The MLV suspensions obtained were slowly cooled down to room temperature and stored under nitrogen at 4°C. Liposomes were morphologically characterized by means of Transmission Electron Microscopy (TEM) using a Philips EM 400T microscope (Eindhoven, NL). For negative staining, a drop of liposome suspension was floated on the surface of a 300-mesh carbon coated copper grid earlier treated with a polylysine solution. After a minute, the liposome suspension was drawn off the grid and replaced with a drop of negative stain (ammonium molybdate solution 1% w/v, pH 7.0 in distilled water).

2.3. HPLC analyses of Leu-A

Reversed phase-HPLC Leu-A analytical determination was performed by using a Hewlett Packard model HP 1050 chromatograph (W-7517 Waldbronn, Germany) and an endcapped Delta-Pack (Waters, Milford, MA, USA) reversed phase column (C18, 100 Å, 300 × 3.9 mm). Elution was performed in an isocratic manner using, as mobile phase, a mixture of acetonitrile–isopropanol–15 mM TEAP buffer solution (52.5:42:10.5 v/v/v). Leu-A was monitored with a spectrophotometer detector set at 220 nm.

Calibration curve for HPLC assays of Leu-A was performed with five solutions of concentration range 6.12–340 μg/ml (each sample in triplicate). The data reported in Fig. 5 showed a correlation coefficient > 0.990.

Leu-A retention time was 5.28 min. The Leu-A content in liposomes was determined by difference between Leu-A initial amount and free peptide in the supernatants. Loading was expressed as a percentage of the Leu-A initial amount.

The data reported (Table 1) are the average of five peak area values and the error was calculated as standard deviation ($\pm$ S.D.).

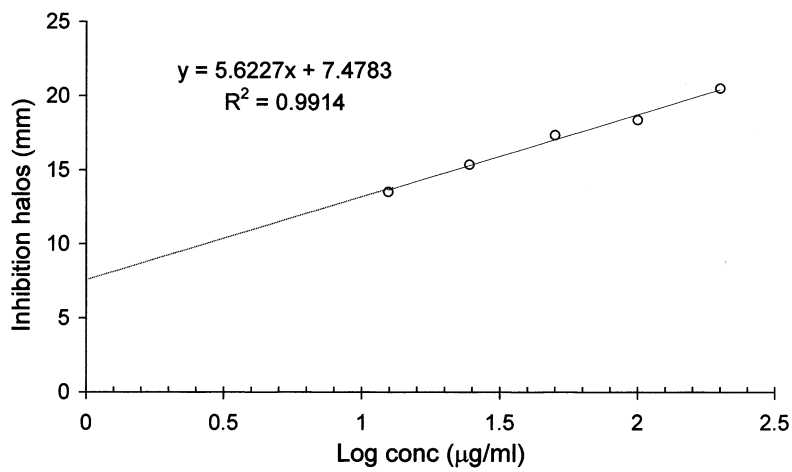


Fig. 3. Calibration curve of Leu-A standard solutions for microbiological analysis.

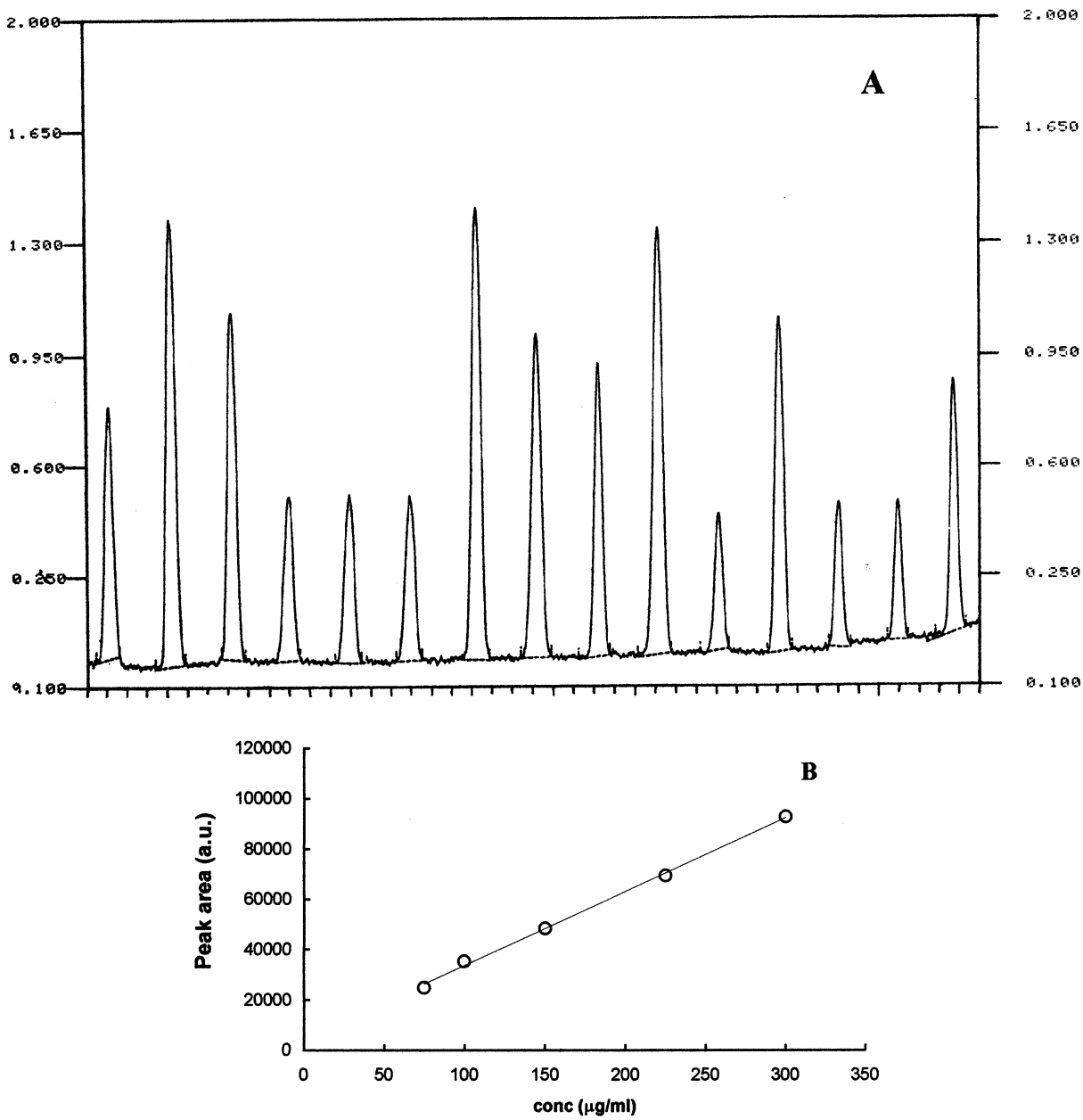


Fig. 4. Leu-A densitometric chromatogram (A) and related calibration curve (B).

Table 4
Linear regression curves for HPLC, TLC-UV and Microbiological methods for Leu-A assay[a]

Analytical methods	Regression curves				C
	a	S_a	b	S_b	
HPLC	3.00	0.01	0.63	0.25	0.9999
TLC-UV	294.20	2.67	4213.90	84.25	0.9978
Microbiological	5.62	0.22	7.48	1.17	0.9914

[a] *a*, slope, S_a, standard deviation of the slope, *b*, intercept, S_b, standard deviation of the intercept, *c*, fit correlation coefficients.

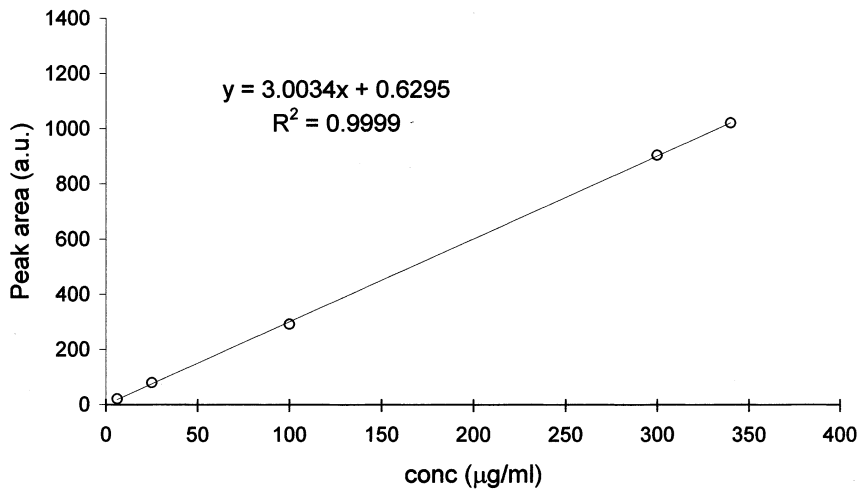


Fig. 5. Calibration curve of Leu-A standard solution for HPLC.

2.4. Microbiological method

Micrococcus luteus ISS 9341, from the collection of Istituto Superiore di Sanità (Rome, Italy), was reconstituted with sterile nutrient broth (36 h, 32°C) and then collected by centrifugation (2500 rpm, 15 min). Supernatants were discarded and the pellet suspended in sterile physiological saline. The final bacterial concentration was fitted colorimetrically (580 nm). A suitable volume of *M. luteus* suspension was added to the medium (900 ml, pH 7.2) to obtain a final concentration of 10^6 cells/ml). The homogeneous mixture was layered (8 mm height) on anodized aluminum plates (30 × 40 cm) and left to harden (1 h, 4°C). Leu-A standard curve was determined (halos (Y) vs. log of concentration (X)) using five scalar Leu-A concentrations (200, 100, 50, 25, 12.5 μg/ml), obtained from dilutions of an initial 1 mg/ml Leu-A water stock solution. In order to assay these standard samples, 15 small holes (8 × 8 mm) were punched in the agar plate and filled with 0.2 ml of each sample. After 24 h incubation (32 ± 1°C), the mean diameters (mm) of inhibition halos of the microorganism growth were measured.

Leu-A-liposome loading was determined by calculating the difference between the total amount of Leu-A used and the free Leu-A found in supernatants collected after ultracentrifugation and diluted with physiological solution to a final volume (10 ml).

Ultracentrifugation was performed (three times) with a Beckman Optima™ Series TL (Palo Alto, CA, USA) and a TLA 100.4 rotor (85 000 × *g* for 2 h at 4°C) was used. Aliquots of the recombined supernatants (0.2 ml) were put into the cavities punched in the agar plates as earlier described. The level of peptide binding was expressed as the percentage of molecule associated with the carrier with respect to the initial amount of Leu-A that was earlier dissolved in the preparation medium.

The data reported (Table 2) are the average of five measurements and the error was calculated as standard deviation (± S.D.).

2.5. Densitometric determination of Leu-A

Leu-A analytical determination by TLC densitometric analysis was performed using a Camag TLC Scanner II densitometer (Muttenz, Switzerland). Liposomal dispersions were ultracentrifuged (three times) and the recombined supernatant volumes were brought up to 10 ml. Aliquots (1 ml) were lyophilized and redissolved with a mixture (1 ml) of CH_3COOEt–MeOH (90:10 v/v).

Five samples, (20 μl, in triplicate) including a Leu-A reference solution, were seeded (using the alternating deposition technique) on pre-coated Silica Gel F254 HPTLC (Merck, Darmstadt F.R.G.) plates (10 × 20 cm) earlier washed with a mixture of MeOH–CH_3COOEt–NH_4OH conc. (60:38:2 v/v/v) and a calibration curve was performed. Samples, in five repetitions each, for the determination of Leu-A in the different liposome systems were prepared as reported above and elutions were performed using the same mixture of solvents and stopped at 7 cm from seeding points. The R_f value of Leu-A was 0.5. Titrations were carried out by setting the densitometer at λ_{225} nm wavelength.

Leu-A in liposomes was calculated by difference between total and free Leu-A in the supernatants. Loading was expressed as a percentage of the Leu-A initial amount.

The data are reported (Table 3) and are the average of five measurements and the error was calculated as standard deviation (± S.D.).

3. Results and discussion

The low-shear 'shaking' technique used gave rise to large size and polydisperse MLV liposomal population. No appreciable instability problems that could generate titration errors were observed as confirmed by negative stain electron microscopy that showed no vesicle aggregation or fusion phenomena (Fig. 2) for more than 7 days.

The loading capacity of liposome suspensions, detected by means of microbiological and densitometric methods, were compared with those from HPLC.

Among various microbiological methods, the multi-well technique resulted the best in Leu-A testing. Differently, the cellulose disc technique, although less entangled, showed an incomplete release of the peptide from the discs, due to the strong adsorption of the molecule on the cellulose matrix. On the contrary, the multi-well method gave rise to better results in a wide range of peptide concentrations, as shown by the calibration curve of standards (Fig. 3) discussed at the end of the section.

Densitometric analyses were also performed (Fig. 4A–B). This procedure, based on classical TLC analysis, showed some difficulties in the choice of a suitable solvent with a proper volatility, which could ensure peptide seeding uniformity and repeatability. The use of a mixture of CH_3COOEt–MeOH (90:10 v/v) solved these problems and, at the same time, produced on deposition spots as smallest as possible. The eluant mixture, which produced the best spots with no tails and the highest resolution, was MeOH/CH_3COOEt/NH_4OH conc. (60:38:2 v/v/v). The ratio used seemed to be very critical for the success of the analyses, in fact, variations in percentage of the NH_4OH concentration in the mixture, could compromise the analytical results. An additional problem was to determine the optimal distance between the front and the seeding point in the TLC plate (7 cm). This was of particular importance to achieve the best Leu-A separation from all other components and to avoid any diffusion process of spots, which could generate broadening and, hence, a high percentage of error. As earlier specified, standard Leu-A solutions

have been used for the calibration curve (Fig. 4A–B). Five different Leu-A concentrations, varying from 75 to 300 μg/ml, were seeded (20 μl each in triplicate) using a volumetric micropipette. The above quantities were chosen for the following reasons: (1) lower quantities were not producing detectable spots, (2) higher quantity could produce broad spots not easily readable by the instrumentation, (3) to avoid large margin of error, (4) to achieve very good separation of spots. After elution, quantitative analyses were conducted directly on the plate setting the densitometer at λ_{220} nm. This particular wavelength corresponds to the maximum absorbance observed for the peptide.

Linearity was evaluated by performing calibration curves for HPLC, microbiological and densitometric methods. Using the least square regression method: slopes (a) and intercepts (b), their respective standard errors (S_a) and (S_b) and the correlation coefficients (c) were calculated as reported (Table 4). All correlation coefficients for the linear fit resulted very satisfactory (>0.990). The three methods were evaluated in terms of precision, accuracy and repeatability and, in particular, HPLC was taken as reference method to be compared with the densitometric and microbiological procedures. Every experiment was performed on five samples and the relative mean values are reported (Table 5). The TLC-UV method showed a noticeably higher precision and accuracy compared with the microbiological one. The TLC-UV method looked consistent with HPLC results and it is testified by the densitometric/HPLC ratio.

On the contrary, the microbiological method showed increased values of microbiological/HPLC and, hence, a lower accuracy compared with HPLC.

Differences in repeatability between microbiological and densitometric methods were also highlighted by the values of mean%R.S.D. (4.9) in the former and in the latter (1.0). The considerable difference in value demonstrated the higher precision of TLC-UV over the microbiological method. The mean%R.S.D. value of 0.5 for HPLC justified by itself its choice as a reference method.

The microbiological method resulted to be less precise and accurate, looking at the relevant differences in terms of repeatability and from HPLC. In fact, linearity showed a higher indetermination in the Y values compared with HPLC; data and confidence intervals are reported (Table 3). The high intercept value testified the low linearity of the system (curves expressed as halos vs. log C) due to two important factors: (1) the behavior of the biologic system that doesn't react according to a linear model, (2) the operator variable that produces great fluctuations in the determination of inhibition areas. The second factor doesn't affect the precision and accuracy of HPLC and TLC-UV and its contribution to the error in the microbiological method is definitely important. On the other hand, the microbiological represents a cheap and simple method, which provides an intrinsic enhanced specificity in comparison to HPLC and TLC-UV.

4. Conclusion

When quantitative analyses of compounds by HPLC are not easy to perform and/or require long experimental times, the microbiological and densitometric methods may represent alternative procedures. In fact, both of them showed, more or less, adequate linearity and repeatability, although the microbiological assay resulted less accurate and precise compared with TLC-UV densitometric method, and provided acceptable results if compared with those from HPLC. Moreover both procedures proved to be sensitive and rapid, allowing easy titration of compounds especially when they are mixed with others having similar UV absorption and, hence, are particularly difficult to test. Furthermore, antimicrobial activity of certain drugs, such as Leucinostatins, as discriminant factor in the microbiological test, makes this method highly specific. Therefore, these methods are proposed as alternative fast ways to detect Leu-A and/or similar peptides in liposomal dispersions.

Table 5
Precision, repeatability and accuracy of HPLC, HPTLC and Microbiological methods for Leu-A content determination in MVLs systems

Lipid Composition	Microbiological ($\pm$S.D.)	%R.S.D.[a]	Densitometric ($\pm$S.D.)	%R.S.D.	HPLC ($\pm$S.D.)	%R.S.D.	Δ Microbiological/HPLC	Δ Densitometric/HPLC
DSPC	39.29 ($\pm$0.95)	2.42	40.29 ($\pm$0.63)	1.56	40.50 ($\pm$0.33)	0.81	−3.08%	−0.52%
DSPC:Chol (10:2.5)	48.65 ($\pm$1.40)	2.88	48.10 ($\pm$0.22)	0.46	48.30 ($\pm$0.12)	0.25	+0.74	−0.41
DSPC:DPPG (10:1)	22.18 ($\pm$1.82)	8.21	21.60 ($\pm$0.12)	0.56	21.50 ($\pm$0.08)	0.37	+3.06%	+0.46%
DSPC:Chol:DPPG (10:2.5:1)	58.06 ($\pm$1.43)	2.46	56.90 ($\pm$0.33)	0.58	56.40 ($\pm$0.14)	0.25	+2.86%	+0.88
DPPC	30.64 ($\pm$1.60)	5.22	31.70 ($\pm$0.35)	1.10	31.50 ($\pm$0.09)	0.29	−2.80	+0.63
DPPC:Chol (10:2.5)	24.21 ($\pm$1.06)	4.38	23.50 ($\pm$0.42)	1.79	23.60 ($\pm$0.08)	0.34	+2.52%	−0.42%
DPPC DPPG (10:1)	26.54 ($\pm$1.74)	6.56	27.30 ($\pm$0.22)	0.81	27.50 ($\pm$0.28)	1.02	−3.62%	−0.73%
DPPC:Chol:DPPG (10:2.5:1)	23.99 ($\pm$1.66)	6.92	24.60 ($\pm$0.15)	0.61	24.80 ($\pm$0.15)	0.60	−3.38%	−0.81%
DMPC	35.44 ($\pm$1.53)	4.32	34.10 ($\pm$0.14)	0.41	34.20 ($\pm$0.15)	0.44	+3.50%	−0.29%
DMPC:Chol (10:2.5)	10.89 ($\pm$1.05)	9.64	10.70 ($\pm$0.25)	2.34	10.60 ($\pm$0.09)	0.85	+2.66%	+0.93%
DMPC:DMPG (10:1)	46.58 ($\pm$0.98)	2.10	48.50 ($\pm$0.47)	0.97	48.30 ($\pm$0.12)	0.27	−3.69%	+0.41%
DMPC:Chol:DMPG (10:2.5:1)	31.65 ($\pm$1.24)	3.92	30.70 ($\pm$0.14)	0.46	30.50 ($\pm$0.11)	0.36	+3.63%	+0.65%

[a] %Relative Standard deviation.

Acknowledgements

Authors wish to thank CNR (Italian National Research Council) and MURST (Italian Ministry for the University) for financial support.

References

[1] C.G. Casinovi, L. Tuttobello, C. Rossi, Z. Benciari, Determinazione della struttura dei peptidi fitotossici ed antibiotici prodotti in colture sommerse di *Paecilomyces marquandii*, Phytopathol. Medit. 22 (1983) 103–106.

[2] K. Fukushima, T. Arai, Y. Mori, M. Tsuboi, M. Suzuki, Studies on peptide antibiotics, Leucinostatins. I. Separation, physico-chemical properties and biological activities of Leucinostatins A and B, J. Antibiot. 36 (1983) 1606–1612.

[3] K. Fukushima, T. Arai, Y. Mori, M. Tsuboi, M. Suzuki, Studies on peptide antibiotics, Leucinostatins. II. Separation, physico-chemical properties and biological activities of Leucinostatins A and B, J. Antibiot. 36 (1983) 1613–1627.

[4] C.G. Casinovi, C. Rossi, L. Tuttobello, M. Ricci, The structure of Leucinostatin C. A minor peptide from *Paecilomyces marquandii*, Eur. J. Med. Chem. Chim. Ther. 21 (1986) 527–528.

[5] C. Rossi, C.G. Casinovi, M. Ricci, L. Tuttobello, L. Radics, Leucinostatin D, a novel peptide antibiotic from *Paecilomyces marquandii*, J. Antibiot. 40 (1987) 130–133.

[6] P. Csermely, L. Radics, C. Rossi, M. Szamel, M. Ricci, K. Mihaly, J. Somogyi, The nonapeptide leucinostatin A acts as a weak ionophore and as an immunosuppressant on lymphocytes, Biochim. Biophys. Acta 1221 (1994) 125–132.

[7] G.A. Strobel, R. Torczynsky, A. Bollon, *Acremonium* sp.: a leucinostatin A producing endophyte of European yew (*Taxus baccata*), Plant Sci. 128 (1997) 97–108.

[8] F.C. Szoka, Liposomal drug delivery: corrent status and future prospects, in: J. Wilschut, D. Hoekstra (Eds.), Membrane Fusion, Marcel Dekker, New York, 1991, pp. 845–890.

[9] A. Sherma, R.M. Straubinger, Novel taxol formulations: preparation and characterization of taxol-containing liposomes, Pharm. Res. 11 (1994) 889–896.

[10] M. Fresta, M. Ricci, C. Rossi, P.M. Furneri, G. Puglisi, Antimicrobial nonapeptide Leucinostatin A: dependent effects on physical properties of phospholipid model membranes, J. Colloid Interf. Sci. 226 (2000) 222–230.

[11] M. Ricci, P. Sassi, C. Nastruzzi, C. Rossi, Liposome-based formulations for the antibiotic nonapeptide Leucinostatin A: FTIR spectroscopic characterization and in vivo toxicological study. AAPS Pharm. Sci. Tech. Vol 1, Issue 1, article 2 2000.

[12] M.G. Quaglia, S. Fanali, A. Nardi, M. Ricci, C. Rossi, Separation of leucinostatins by capillary zone electrophoresis, J. Chromatogr. 593 (1992) 259–263.

[13] G. Vertuani, M. Boggian, A. Scatturin, M. Ricci, B. Meli Balbocchino, L. Tuttobello, C. Rossi, Structure activity studies on chemically modified homologues of the antibiotic phytotoxic Leucinostatin A, J. Antibiot. 48 (1995) 254–260.

[14] New R.R.C., Characterization of liposomes in Liposomes, a practical approach (R.R.C. New Ed.) IRL Press at Oxford University Press, 1989, 105–162.

[15] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1965) 238–252.

[16] T. Fujita, W. Shun-ichi, A. Iida, T. Nishimura, M. Kanai, N. Toyama, Fungal Metabolites. XIII. Isolation and structural elucidation of new peptaibols, Trichodecenins-I and II, from *Trichoderma viride*, Chem. Pharm. Bull. 42 (3) (1994) 489–494 References herein reported.