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UV spectroscopy and reverse-phase HPLC as novel methods to determine Capreomycin of liposomal fomulations

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Abstract

Capreomycin (CS) is an antitubercular drug active against several *Mycobacterium* strains, in particular, against *M. Avium*. In spite of its activity, it is considered a second line drug because it can induce severe renal and hepatic damages when administered as free drug.

However, it is possible to employ drug delivery systems, such as liposomes, to reduce the toxicity of the peptide without loss of its biological activity. For this purpose, appropriately validated time and money saving analytical methods are needed for a careful capreomycin dosage.

In the present paper, UV spectroscopy and a reverse-phase HPLC (RP-HPLC) were investigated as alternative methods for capreomycin quantitative analysis. These techniques were validated against the USP XXVI microbiological turbidimetric assay and the normal-phase HPLC (NP-HPLC) method reported in the British Pharmacopoeia 2003. The results obtained showed that either UV spectrophotometry or RP-HPLC are techniques having higher accuracy and reproducibility with respect to the microbiological assay. Moreover, the RP-HPLC method provided improved performances if compared to NP-HPLC. In fact, RP-HPLC showed: (i) enhanced sensitivity and (ii) increased resolution. Thus we propose RP-HPLC and UV as valid alternative methods to the conventional procedures for capreomycin quantitative analysis.

Keywords: Liposomes; Capreomycin; RP-HPLC; NP-HPLC; Spectrophotometric analysis; Microbiological turbidimetric assay

1. Introduction

Capreomycin (CS) is an antitubercular drug produced by fermentation from *Streptomyces capreolus* and first described in the 1960s [1]. CS is characterized by a cyclic pentapeptide structure and it exists as a mixture of four active components, namely IA, IB, IIA, IIB, distributed as follows: 90% IA and IB forms and 10% IIA and IIB forms (Fig. 1). This peptide shows a high bacteriostatic activity both in vitro and in vivo against *Mycobacterium tuberculosis*, *M. Avium*, *M. bovis*, and *M. kansasii*. Recent studies demonstrated that only 10% of the 46 drug resistant strains of *M. Avium* isolated from Italian patients were resistant to CS [2].

In spite of its biological activity, CS is considered a second-line drug and it can be used in the treatment of tuberculosis when multi-resistance to conventional drugs, such as isoniazid and ethambutol, is developed [3]. In fact this peptide can induce progressive renal damages and severe abnormalities in liver functions. Rare cases of hypersensitivity reactions are also reported [4,5].

The current strategy for reducing the toxicity of available drugs, without losing their biological activity, is to entrap these molecules inside drug delivery systems able to slowly release the drug over long periods of time.

Consequently, a selection of suitable CS carriers was necessary. The attention was focused on liposomes as potential

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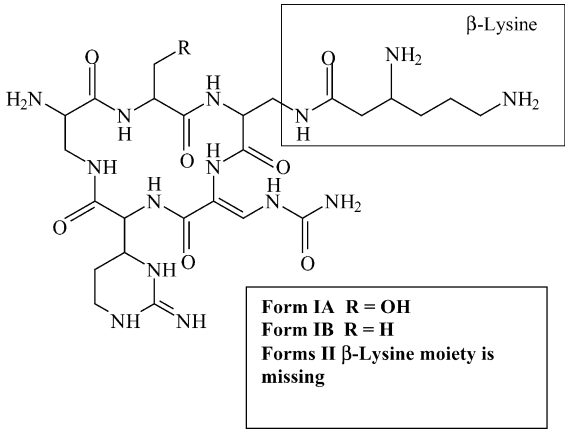

Fig. 1. Structure of CS: IA, IB forms R= OH, H; IIA, IIB forms: without β-lysine.

carriers because it was demonstrated that i.v. injection of CS loaded multilamellar vesicles (MLVs) increased the therapeutic index of this drug [6].

It is also known that liposomes can be directly employed in the treatment of lung infections through aerosolisation [7]. In particular, liposomes are employed in the treatment of intracellular infections as they are rapidly removed after administration by pulmonary macrophages in which microorganisms (in this case mycobacteria) are located and reproduce themselves [8].

In a previous paper, CS loaded liposome formulations were characterized under the chemical–physical point of view [9].

These studies outlined that liposomes can be promising carriers for CS delivery and, moreover, the distearoylphosphatidylcholine (DSPC) vesicles were found to have the best characteristics [9].

In this regard, careful analytical methods are needed for CS quantitation. Microbiological turbidimetric assays [10] and normal-phase HPLC (NP-HPLC) methods [11] are currently employed as official methods for CS analysis. In addition, electrophoresis techniques have been already developed for CS quantitation and identification [12].

The present paper was aimed at the development and the validation of UV spectrophotometry and reverse-phase HPLC (RP-HPLC) as alternative analytical methods to the official techniques already cited [10,11].

UV spectrophotometry was investigated because of its simplicity, low cost and short time of analysis, while RP-HPLC was chosen for its accuracy and sensitivity in the determination of low molecular weight proteins and peptides.

The study was carried out by preparing several CS containing liposome based formulations made of different phospholipid composition using dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG) and hydrogenated phosphatidylcholine (HCP).

The data from the RP-HPLC and UV analyses were compared in terms of linearity, precision, accuracy and repeatability with those from the official USP microbiological assay. Moreover, the RP-HPLC and NP-HPLC assays were compared as regard to their sensitivity and resolution to validate RP-HPLC as a new alternative method with respect to the BP official HPLC procedure.

2. Materials and methods

2.1. Chemicals and reagents

CS obtained from *S. capreolus*, DPPC, DPPG, DSPC, DSPG, HCP and ammonium hydrogensulfate 99.999% were purchased from Sigma Chemicals (Milan, Italy).

Water Plus for HPLC, methyl alcohol and acetonitrile for HPLC were obtained from Carlo Erba (Milan, Italy), heptafluorobutyric acid was provided by Fluka (Butchs, Switzerland). Chloroform and KH_2PO_4 salt were purchased from J.T. Baker (Milan, Italy). All other reagents and solvents were of the highest purity available.

Klebsiella Pneumoniae (ATCC No. 13883) and *Bacillus Subtilis* (ATCC No. 6633) for microbiological tests were obtained from Oxoid (Milan, Italy).

2.2. Liposome preparation

Large unilamellar vesicles (LUVs) were composed of DPPC–DPPG, DSPC–DSPG or HPC–DPPG (90:10, w/w). Blank liposomes were prepared by the thin layer evaporation (TLE) method. Briefly, lipids were dissolved in chloroform into 250 mL round bottom flasks. Then the organic solvent was evaporated under nitrogen stream and the dry lipid films were maintained under reduced pressure to remove traces of the solvent.

Films were hydrated by adding an appropriate amount of water to yield 10 mg/mL phospholipid concentration, while shaking in a Gallenkanp orbital incubator (Fisons Instruments, Crawley, UK) at a temperature 10 °C above the phospholipid gel–liquid crystalline phase transition temperature (T_m), until homogeneous milky suspensions were obtained. The MLV suspensions were extruded through a polycarbonate filter (pore size 0.1 μm) using an Emulsifer C5 (Avestin, Fausitano s.r.l. Milan, Italy) and the LUVs obtained were stored overnight at 4 °C.

CS containing liposomes were prepared by modification of the method by Ramaldes et al. [13]. Three millilitres of blank LUVs, prepared as described previously, were mixed with an equal volume of CS aqueous solution (1.6 mg/mL) and were shaken with a vortex mixer for 5 min. Then, each suspension was frozen using a dry ice–ethanol bath for 5 min and thawed in a water bath held at 50 °C for the same period of time. This cycle was repeated 10 times. Free CS and encapsulated were separated by ultracentrifugation (70,000 rpm, 2 h, 4 °C) using an Optima™ Ultracentrifuge with a TLA 100.4

Table 1
Statistics for UV calibration curve data

Concentration (μg/mL)	Absorbance (mean value ± S.D., $n = 3$)	Confidence intervals (0.05 significance level)	%R.S.D.
5	0.105 ± 0.001	±0.001	1.1
10	0.211 ± 0.006	±0.007	2.9
15	0.348 ± 0.004	±0.005	1.2
20	0.443 ± 0.008	±0.009	1.8
25	0.57 ± 0.01	±0.01	1.7
30	0.668 ± 0.002	±0.003	0.4
35	0.780 ± 0.002	±0.002	0.3

Table 2
Statistics for RP-HPLC calibration curve data of CS IA + IB

Concentration (μg/mL)	Area (mAU s) (mean value ± S.D., $n = 5$)	Confidence intervals (0.05 significance level)	%R.S.D.	Rs[a]
5	501 ± 6	±8	1.19	4.7
10	953 ± 28	±31	2.91	5.4
15	1903 ± 7	±9	0.35	4.7
20	2936 ± 13	±18	0.45	4.7
25	3540 ± 30	±42	0.85	4.3
30	3827 ± 8	±12	0.22	4.4
35	4645 ± 20	±28	0.44	4.3

[a] Resolution factor calculated according to BP.

rotor (Beckman, Palo Alto, CA, USA). Supernatant volumes were filtered through a syringe filter (pore size 0.22 μm) and the final volume was adjusted to 5 mL.

Free CS concentration was determined in the supernatants by means of spectrophotometric, HPLC and microbiological methods.

2.3. Liposome characterization

Liposomes were morphologically characterized by means of transmission electron microscopy (TEM) using a Philips EM 400T microscope (Eindhoven, NL). Samples were prepared according to the conventional method [14]. A drop of liposome suspension was floated on the surface of a 200 mesh formvar coated copper grid earlier treated with a poly-lysine solution. After 3 min the liposome suspension was drawn off and replaced with a drop of negative stain (phosphotungstic acid 2%, w/v and trehalose 1%, w/v, pH 6.5 in distilled water).

Dimensional distribution analysis was performed using a Nicomp 370 (PSS Inc., Santa Barbara, USA) autocorrelator equipped with Coherent Innova 70-3 (Laser Innovation, Moorpark, CA, USA) argon ion laser.

2.4. CS spectrophotometric determination

CS analytical determination was performed by using a spectrophotometer V-520 (Jasco, Tokyo, Japan) set at 268 nm.

The calibration curve for UV assays was carried out with seven standard solutions in the concentration range 5–35 μg/mL (Table 1). The data reported are the average of three measurements ($n = 3$) and the confidence interval was also determined at 0.05 significance level.

The CS content in liposomes was expressed as the difference between peptide initial amount and free CS in the supernatants, whereas the loading was determined as percentage of the CS initial amount.

2.5. CS reverse-phase HPLC analysis

RP-HPLC analysis was carried out by means of a Hewlett Packard HP 1050 Series chromatograph (Hewlett Packard, Germany) and a reverse-phase column (C18, 3 μm, 150 mm × 2 mm) (Phenomenex, USA). CS was monitored with a spectrophotometer HP 1050 Series detector (Hewlett Packard, Germany) set at 268 nm.

Elution was performed in an isocratic manner (flow rate 0.2 mL/min) with a mixture of acetonitrile–KH_2PO_4 buffer solution (pH 2.3; 0.2 M) with 0.3% of heptafluorobutyric acid (10:90, v/v).

Column, mobile phase and samples were equilibrated at 25 °C prior to each measurement.

The calibration curve for CS RP-HPLC analyses was drawn with seven solutions in the concentration range of 5–35 μg/mL (Table 2). The data were reported as the sum of the CS IA and IB peak areas. These data are the average of five measurements ($n = 5$) and the confidence interval was also determined at 0.05 significance level. The peptide content in liposomes was calculated by difference between the initial amount and the free peptide in the supernatants while the loading was expressed as percentage of the CS initial amount.

2.6. Normal-phase HPLC analysis

NP-HPLC analysis was performed according to the procedure proposed in the British Pharmacopoeia (BP) 2003 [10] by using a Hewlett Packard HP 1050 Series chromatograph (Hewlett Packard, Germany) equipped with a normal phase column (CN, 5 μm, 150 mm × 4.6 mm) (Waters-Spherisorb, Milan, Italy). CS was monitored with a spectrophotometer HP 1050 Series detector (Hewlett Packard, Germany) set at 268 nm.

Elution was carried out in a isocratic manner (flow rate 1.5 mL/min) with a mixture of methanol–$(NH_4)HSO_4$ solution (0.05, w/v) (45:55, v/v).

Calibration curve for CS NP-HPLC analysis was drawn by using six solutions in the concentration range 10–35 μg/mL and the data were reported as the sum of the CS IA and IB peak areas (Table 3). The data are the average of five measurements ($n = 5$) and the confidence interval was also determined at 0.05 significance level. The peptide content in liposomes was calculated by difference between the initial amount and the free peptide in the supernatants and the loading was expressed as percentage of the CS initial amount.

Table 3
Statistics for NP-HPLC calibration curve data

Concentration (μg/mL)	Area (mAU s) (mean value ± S.D., $n = 5$)	Confidence intervals (0.05 significance level)	%R.S.D.	Rs[a]
10	141 ± 6	±6	3.97	1.9
15	218 ± 5	±5	2.09	1.9
20	277 ± 4	±5	1.34	2.0
25	421 ± 5	±6	1.18	2.0
30	485 ± 9	±11	1.92	2.0
35	601 ± 1	±2	0.19	2.0

[a] Resolution factor calculated according to BP.

2.7. Preliminary microbiological analysis

In order to determine the best method for the CS microbiological assay, initially an agar diffusion method proposed by Le Conte et al. [6] was tested. *Bacillus Subtilis* ATCC 6633 [6] and *Klebsiella Pneumoniae* ATTC 13883 were used as bacterial indicator organisms in AM5 Agar Medium. The AM5 Agar Medium was composed by Agar Noble 20 g, meat extract 3 g, glucose 4 g, meat peptone 5 g, K_2HPO_4 1 g, NaCl 10 g, distilled water 1000 mL (pH = 7.2).

2.8. Microbiological method

The test was performed accordingly to the turbidimetric method described in the US Pharmacopoeia (USP) XXVI [10]. Briefly, *Klebsiella Pneumoniae* ATCC 13883 was allowed to grow in 250 mL of agar medium 1 contained in a Roux bottle at 36–37 °C for 24 h. At the end of this period, the surface growth was collected in 50 mL of sterile saline and part of the stock suspension obtained was diluted with sterile saline until its transmittance at 580 nm was 25% referred to a blank saline solution. 50 μL/mL of this suspension was used to infect 100 mL of medium 3, (inoculum). Both sterile medium 1, for incubation, and medium 3, for inoculum, used in the assay were prepared accordingly to USP XXVI.

The CS calibration curve was carried out using seven solutions prepared by diluting a CS stock solution (1 mg/mL) to obtain concentrations in the range of 2–8 μg/mL (Table 4).

One millilitre of each dose (standard dilutions or samples) and 9 mL of inoculum were added to the test tubes that were incubated at 37 °C for a proper period of time. This procedure was performed in triplicate. After incubation, 0.5 mL of diluted formaldehyde were added to each test tube and the transmittance was read at 530 nm. Control tubes consisted of inoculum without antibiotic.

Table 4
Statistics for microbiological calibration curve data

Concentration (μg/mL)	Transmittance % (mean value ± S.D., $n = 3$)	Confidence intervals (0.05 significance level)	%R.S.D.
2	36.5 ± 0.5	±0.6	1.4
3	40.7 ± 1.0	±1.1	2.4
4	49.6 ± 1.3	±1.5	2.6
5	61.1 ± 0.8	±0.9	1.3
6	70.7 ± 0.7	±1.0	1.0
7	81.0 ± 0.3	±0.4	0.4
8	87.8 ± 0.8	±1.1	0.9

The data were reported as the average of three measurements ($n = 3$) and the confidence interval was also determined at 0.05 significance level.

The CS content in liposomes was determined as the difference between the total CS amount and the free peptide found in the supernatant.

2.9. Preliminary CS stability test

CS was investigated for its stability at the process temperatures. Briefly, two CS aqueous solutions were incubated for 1 h at 55 and 75 °C, then aliquots of these solutions were submitted to USP microbiological analysis in order to establish possible activity losses due to heat stress. The experiments were performed according to the procedure reported in USP XXVI [10] and described in the Section 2.8.

3. Results and discussion

CS loaded liposomes were prepared using the method described in the experimental section. Ten percent DPPG and DSPG were added to avoid vesicle aggregation or fusion phenomena in the liposomal dispersions. DSPG and DPPG stabilizing effects were confirmed by negative stain electron microscopy (Fig. 2). Dimensional analysis demonstrated that the liposome population was homogeneous with a mean diameter of 155.8 nm (Fig. 3).

LUV loading capacity was calculated as the difference between the starting CS amount and the free peptide found in the supernatants. Batches of supernatants were investigated by means of RP-HPLC and UV spectrophotometric methods

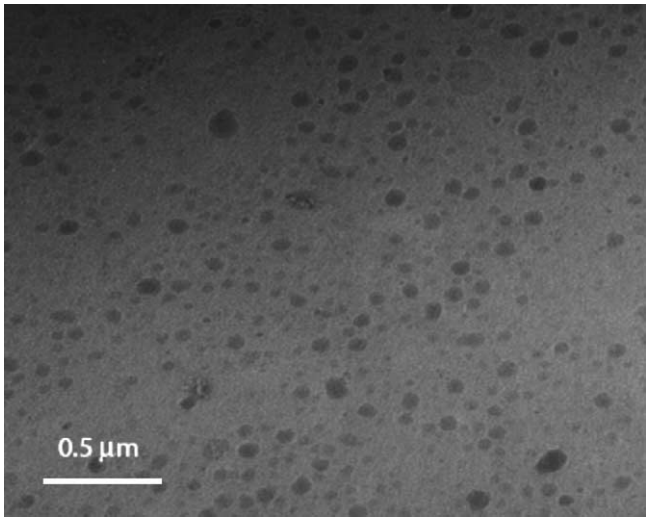

Fig. 2. Transmission electron micrograph (TEM) of CS loaded DPPC LUVs. Magnification 36,000×. The bar corresponds to 0.5 μm.

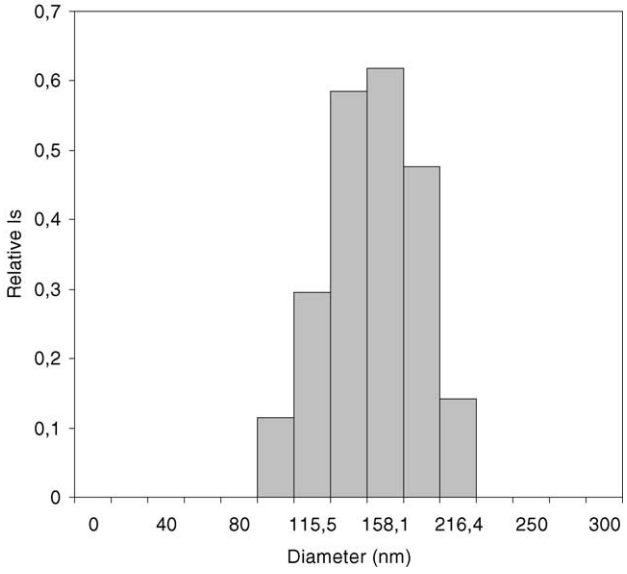

Fig. 3. Dimensional distribution analysis of CS loaded liposomes.

and the results obtained were compared with those from the USP microbiological assay (Table 5).

A preliminary work was performed in order to investigate different microbiological techniques and to establish the most suitable method for a concentration range of 5–35 μg/mL. The well method proposed in literature [6] and the turbidimetric method described in the USP XXVI [10] were tested.

The well method was performed by using an agar diffusion technique. According to this procedure, several CS solutions were placed in wells made on agar plates together with *Bacillus Subtilis* as suggested by Le Conte et al. [6]. Unfortunately, inhibition halos were observed only above 160 μg/mL. The same method was repeated employing *Klebsiella Pneumoniae*, but also in this case no inhibition halos were observed in the concentration range 5–35 μg/mL (data not shown). In light of these results, an alternative disc diffusion test was carried out either by using sterile discs of filter paper wetted with different doses of CS against the same bacteria strains above mentioned. In these experiments too, halos were observed only at high CS doses. In turn, the USP turbidimetric method, performed using *Klebsiella Pneumoniae*, gave rise to better linear responses as shown by the calibration curve and a satisfactory linearity was confirmed in the concentration range 2–8 μg/mL with a correlation coefficient >0.99. Out of this range no significant transmittance variations were registered. Moreover, this range was close enough to the range required for UV and HPLC analysis (5–35 μg/mL).

The turbidimetric method was also employed to perform further experiments in order to test CS stability during the liposome preparation process. The microbiological analysis showed no modifications of CS activity, even for the samples incubated at 75 °C (data not shown).

A reverse-phase HPLC procedure was developed. RP-HPLC was chosen for its high accuracy and sensitivity that make this method suitable for the analytical determination of low molecular weight proteins and peptides.

In order to optimize this potentially innovative method for CS detection, the attention was focused on the variables that influence elution, such as eluant composition, elution manner, temperature and flow rate. These parameters were opportunately modified to assess the best conditions to obtain symmetrical peaks with high resolution and good reproducibility.

Optimal resolution and peak symmetry were achieved using a mixture of acetonitrile/PBS buffer solution (pH = 2.3) 10:90 (v/v) ratio with 0.3% heptafluorobutyric acid as mobile phase.

CS IA and IB retention times were 13.2 ± 0.6 and 16.8 ± 0.9 min, respectively (Fig. 4).

Reproducibility improved by equilibrating the column, the mobile phase and the sample at 25 °C, as heptafluorobutyric acid–CS ion pair performance is very sensitive to temperature variations.

UV spectrophotometric analyses were performed reading CS absorbance at 268 nm.

The RP-HPLC and UV methods were validated in terms of linearity, precision, accuracy and reproducibility. Linearity was evaluated by comparing UV, RP-HPLC and microbiological calibration curves. Concentration ranges employed both in HPLC and microbiological analysis were selected in agreement with the UV method, for which the linearity range was 5–35 μg/mL (Table 1). Then, according to the least square regression method, slopes (*a*), intercepts (*b*), their respective standard deviations (S_a and S_b) and correlation coefficients

Table 5
Precision, reproducibility and accuracy of Microbiological, UV and RP-HPLC methods for CS determination in LUV

Lipid composition	Microbiological[a] (±S.D.)	%R.S.D.[b]	UV[a] (±S.D.)	%R.S.D.	RP-HPLC[a] (±S.D.)	%R.S.D.	ΔUV/Micro (%)[c]	ΔHPLC/Micro (%)[c]
DPPC:DPPG (90:10, w/w)	800 ± 48	6.07	800 ± 28	3.42	830 ± 8	0.96	−0.73	+0.96
DSPC:DSPG (90:10, w/w)	700 ± 42	5.98	680 ± 4	0.58	690 ± 6	0.87	−2.56	−1.99
HPC:DPPG (90:10, w/w)	800 ± 48	5.93	800 ± 18	2.30	790 ± 8	1.01	−3.21	−1.97

[a] Micrograms of capreomycin per millilitre of supernatant.
[b] %Relative standard deviation.
[c] Comparison between the UV and HPLC methods and the microbiological reference method calculated as follows: ΔUV/Micro = (UV − Micro)/Micro; ΔHPLC/Micro = (HPLC − Micro)/Micro. All the terms of the equations refer to the capreomycin concentration calculated in the supernatants.

Table 6
Linear regression curves for UV, microbiological and HPLC methods for CS analysis[a]

Analytical methods	Regression curves				
	a	S_a	*b*	S_b	*R*
UV	0.0226	0.0001	−0.0048	0.0045	0.9992
Microbiological	9.1307	0.1650	15.4080	0.8274	0.9954
RP-HPLC (CS IA + IB)	138.7700	0.3960	−216.0000	10.7268	0.9908
NP-HPLC (CS IA + IB)	18.5500	0.0353	−60.3890	3.0879	0.9936

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

were determined as reported (Table 6). All correlation coefficients were >0.990.

In order to evaluate precision, accuracy and reproducibility, every experiment was carried out on three samples and each one was replicated three times and the mean values were calculated (Table 5). UV and RP-HPLC methods showed a higher precision compared to the USP microbiological assay and, moreover, RP-HPLC revealed an enhanced accuracy with respect to UV, as testified by its narrower confidence interval. Differences in reproducibility between UV and RP-HPLC methods were pointed out by calculating the mean %R.S.D. that was 2.10 and 0.95, respectively.

These values were much lower if compared to the microbiological R.S.D. (5.99) as the behaviour of biological systems is usually characterized by a quite large variability. On the contrary, this variability was drastically reduced in the UV and HPLC methods (Table 5).

In addition, RP-HPLC was also compared and validated with respect to the NP-HPLC method described in the BP [10]. The NP-HPLC employed a normal-phase column modified with chemically-bonded cyano groups and a mixture of methanol/ammonium hydrogen sulphate solution in 45:55 (v/v) ratio as mobile phase. The limit of detection (LD) and quantitation (LQ) were 0.45 and 1.51 μg/mL, respectively for the normal phase and 0.20 and 0.68 μg/mL for the reverse-phase method. The limits were obtained considering the lowest peak which was the IA form.

New supernatant batches were prepared accordingly with the procedure previously reported in the Section 2.2 and they were investigated by means of both RP-HPLC and NP-HPLC (Table 7). In spite of the good linearity shown by NP-HPLC and confirmed by a $R > 0.990$, the new RP-HPLC method produced better performances in terms of peak resolution and sensitivity.

In fact, the chromatogram obtained according to the official BP procedure showed that CS IA and IB retention times were respectively 2.60 ± 0.01 and 3.10 ± 0.01 min and the two peaks were not completely separated and resolved (Fig. 5).

Differences in resolution between NP-HPLC and RP-HPLC were outlined by calculating the mean resolution factor

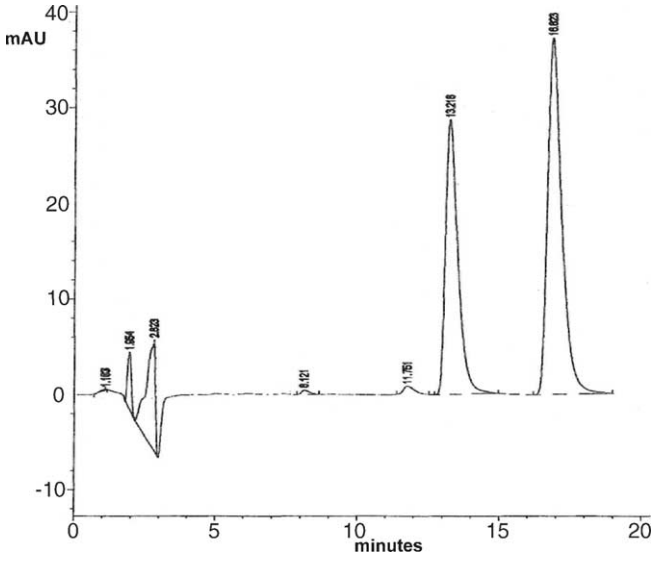

Fig. 4. CS chromatogram obtained by RP-HPLC (injected concentration 15 μg/mL). The peak at 13.218 min is related to the form IA and at 18.623 min to the form IB.

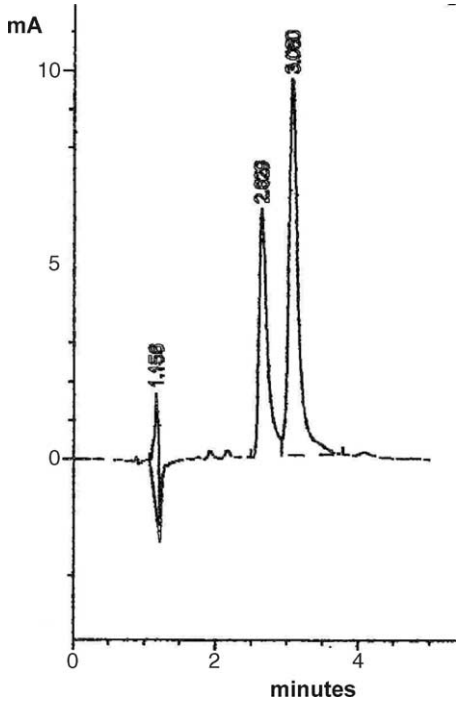

Fig. 5. CS chromatogram obtained by NP-HPLC (injected concentration 15 μg/mL). The peak at 2.629 min is related to the form IA and at 3.060 min to the form IB.

Table 7
Precision, reproducibility and resolution of NP-HPLC and RP-HPLC methods for CS determination in LUV

Lipid composition	RP-HPLC[a] (±S.D.)	%R.S.D.[b]	Resolution factor (Rs)[c]	NP-HPLC[a] (±S.D.)	%R.S.D.	Resolution factor (Rs)	Δ HPLC/NP-HPLC (%)[d]
DPPC:DPPG (90:10, w/w)	690 ± 4	0.58	4.4	640 ± 2	0.31	1.9	+8.24
DSPC:DSPG (90:10, w/w)	720 ± 4	0.56	4.4	640 ± 4	0.62	1.9	+11.19
HPC:DPPG (90:10, w/w)	730 ± 2	0.27	4.5	600 ± 2	0.33	1.8	+22.21

[a] Micrograms of capreomycin per millilitre of supernatant.
[b] %Relative standard deviation.
[c] Resolution factor calculated according to the British Pharmacopoeia. $Rs = 1.18(t_{r2} - t_{r1})/(W_{h1} + W_{h2})$. Expression to calculate resolution factor according to the British Pharmacopoeia. t_{r1}, t_{r2}, retention times of two chemical species, W_{h1}, $W_{h2,}$ width of the peaks at half height.
[d] Comparison between the RP-HPLC and NP-HPLC methods calculated as follows: ΔHPLC/NP-HPLC = (RP-HPLC − NP-HPLC)/NP-HPLC. All the terms of the equation refer to the capreomycin concentration calculated in the supernatants.

(Rs) [10], as reported in Eq. (1):

$$Rs = \frac{1.18(t_{r2} - t_{r1})}{W_{h1} + W_{h2}} \quad (1)$$

where t_{r1}, t_{r2}, are the retention times of two chemical species and W_{h1}, $W_{h2,}$ the widths of the peaks at half height.

The resolution values resulted respectively 1.87 and 4.43 for NP-HPLC and RP-HPLC.

The lower NP-HPLC resolution consequently implied a lower precision in the CS quantitative determination, as shown by the ΔHPLC/NP-HPLC ratio (Table 7).

Moreover NP-HPLC was unsuitable for investigating samples with concentration ≤5 μg/mL because either the adverse signal-to-noise ratio, as shown by the larger LD and LQ values with respect to RP-HPLC, or its lower resolution caused a larger variability and a lower reproducibility. For these reasons, a narrower concentration range was employed for the calibration curve (Table 3).

On the contrary, RP-HPLC was able to analyze samples characterized by peptide concentration ≤5 μg/mL as reported in Table 2, confirming its sensitivity in the quantitative dosage of low molecular weight peptides.

4. Conclusion

In light of the results obtained, UV and RP-HPLC methods can represent a very good alternative procedures to the USP microbiological and BP NP-HPLC methods for CS quantitative analysis. In fact, both of methods showed a good linearity, precision and a higher reproducibility in comparison with the microbiological assay. Moreover, although UV spectrophotometry resulted less accurate if compared to RP-HPLC, spectrophotometric analyses are easier, cheaper and they do not require the long experimental time needed for HPLC experiments. Furthermore, RP-HPLC produced better results in terms of sensitivity and resolution with respect to NP-HPLC as the BP procedure did not allow the perfect and complete separation of the signals. On these basis, UV and RP-HPLC are proposed as alternative valid and effective procedures for careful CS quantitation. In addition, RP-HPLC is proved to be a new performing HPLC method for CS quantitative analysis.

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