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A rapid reversed-phase HPLC method for the simultaneous analysis of olanzapine and simvastatin in dual nanostructured lipid carriers

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In the present work, a rapid reversed phase high performance liquid chromatography (RP-HPLC) method was developed for the simultaneous determination of simvastatin, including the lactone prodrug (SV) and the respective active hydroxy acid form, simvastatin acid (SVA) and olanzapine (OL) in a formulation containing co-encapsulating-nanostructured lipid carriers (Combo-NLC). The desired chromatographic separation was achieved on a Phenomenex Luna Phenyl-Hexyl, 5 μm (150 × 3 mm) column at a temperature of 35 °C, under isocratic conditions using UV detection at 230 nm. The optimized mobile phase consisted of a mixture of ammonium acetate aqueous solution (0.02 M), methanol and acetonitrile (30 : 35 : 35, v/v/v) at a flow rate of 0.8 mL min^{-1}. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range 0.5–100 μg mL^{-1}, with determination coefficients, R^2, exceeding 0.9994 for all three compounds, SVA, SV and OL. The method was shown to be specific, without the interference of NLC components, precise at the intra-day and inter-day levels, as reflected by the relative standard deviation values, lower than 9.014%, accurate with bias not exceeding 15% and characterized by a recovery rate of 100 ± 8%. The limits of detection and quantification were, respectively, 0.07 and 0.22 μg mL^{-1} for OL, 0.12 and 0.36 μg mL^{-1} for SV and 0.09 and 0.27 μg mL^{-1} for SVA. The method was successfully applied for the determination of Combo-NLC entrapment efficiency.

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1 Introduction

Olanzapine, chemically thienobenzodiazepine described as 2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine[1] (Fig. 1a), is a second-generation antipsychotic drug used for the treatment of schizophrenia, bipolar mania and associated agitation.[2] Although it is more effective than other first-line second-generation drugs, olanzapine is associated with some adverse events, such as weight gain and development of dyslipidemia (it increases the levels of triglycerides and low-density lipoprotein cholesterol, LDL-C, and decreases high-density lipoprotein cholesterol, HDL-C). This calls for the need of a close monitoring of patients on olanzapine treatment so that, if these adverse events arise, the maintenance of the therapy may be evaluated.[3] In order to mitigate some of these unwanted effects, the treatment with statins, such as simvastatin, might be required.

Simvastatin, chemically butanoic acid, 2,2-dimethyl-1, 2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl)ethyl]-1-naphthalenyl ester, [1*S*-[1α,3α,7β,8β-(2*S**,4*S**),8*a*β]][1] (Fig. 1b), is the lactone prodrug that is hydrolyzed *in vivo*, in the liver and non-hepatic tissues, to the corresponding β-hydroxy acid (simvastatin acid, SVA, Fig. 1b).[4,5] The latter is a potent inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, involved in the cholesterol synthesis. This inhibition is mainly responsible for reducing LDL-C levels, but simvastatin has also been shown to reduce the levels of triglycerides and increase the levels of HDL-C. SV is also reported as effective in the reduction of the morbidity and mortality associated with coronary heart disease.[6] Moreover, it is known that the lactone form of statins would be easily converted to their corresponding hydroxy acids in water and plasma, and the conversion would be enhanced when they are in alkaline conditions.[5] This makes it necessary to quantify, in most systems, both forms.

Nanostructured lipid carriers (NLCs) for the co-encapsulation of OL and SV (referred to as Combo-NLC formulation) arise as a promising approach in a transdermal drug delivery system (TDDS). Moreover, the complementarity between the therapeutics and the advantage that clearly results in the improvement of treatment compliance, a reduction in the time of production and the challenge stemming from the simultaneous incorporation of drugs with different lipophilicity (log *P* values of 2.8 and 4.7 for OL and SV, respectively) into the same

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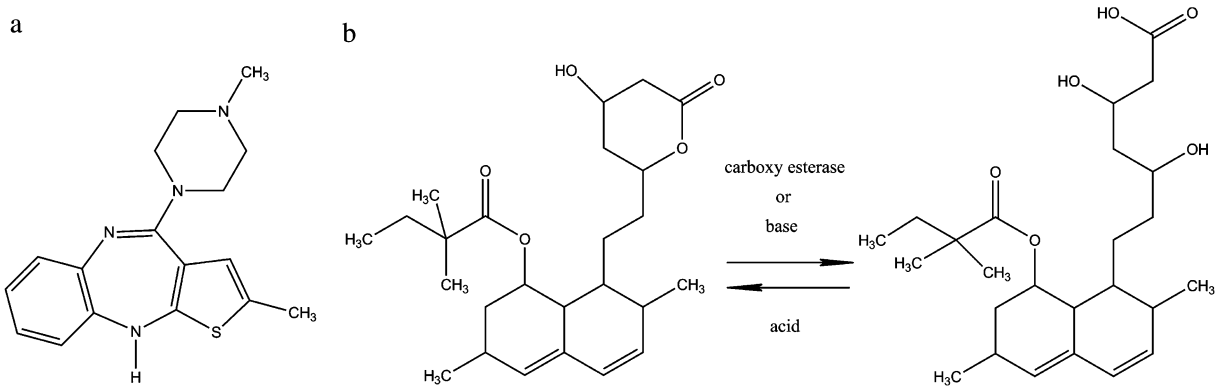

Fig. 1 Chemical structures of (a) olanzapine and (b) simvastatin (left), the prodrug as an inactive lactone form with simvastatin acid (right), the respective active β-hydroxy acid form.

colloidal carrier, are also appealing factors from a nanotechnological point of view.

In order to fully characterize the Combo-NLC formulation, a suitable and validated method is required for a critical assessment of pharmaceutical parameters such as drug content. Literature review reveals that HPLC methods have been reported for the quantitation of simvastatin[7] and olanzapine[8] separately, in combination with other drugs[9–11] and a few bioanalytical methods are also reported.[12–15] However, until now, there have been no published reports about the simultaneous quantitation of simvastatin and olanzapine by HPLC in a pharmaceutical formulation.

The purpose of the present study was to develop and validate a simple and time-saving RP-HPLC method with UV detection for the simultaneous determination of OL, SV and SVA. The validated method was applied to quantify both the content of SV/SVA and OL incorporated into the NLC after preparation.

2 Materials and methods

2.1 Materials

Simvastatin (99.4%) was kindly provided by Labesfal – Laboratórios Almiro, S.A. (Santiago de Besteiros, Portugal). Olanzapine (98.9%) was purchased from Zhejiang MYJOY Import & Export Co.,Ltd (Hangzhou, China). Glyceryl tripalmitate (tripalmitin, T8127) and polysorbate 80 (Tween® 80) were purchased from Sigma. Oleic acid was acquired from Fluka. Carbopol® Ultrez 10 NF was a gift from Lubrizol (Quimidroga, Barcelona, Spain). All other reagents and solvents were of analytical or HPLC grade.

2.2 Instrumentation and chromatographic conditions

The HPLC analysis of OL, SV and the active form SVA was carried out using a Shimadzu LC-2010C HT apparatus (Shimadzu Co., Kyoto, Japan) equipped with a quaternary pump, a vacuum degasser, an autosampler, an oven and a variable UV/visible dual wavelength detector. The column used for the analysis was a Luna Phenyl-Hexyl, Phenomenex® (Torrance, USA), with 5 μm particle size, 3 mm internal diameter and 150 mm length, supported with a SecurityGuard™ cartridge Phenomenex® (Torrance, USA), with 3.0 mm internal diameter, in an oven at a temperature of 35 °C. The results were acquired and processed using Shimadzu LC-solution version 1.12 software. Chromatographic analysis was conducted in isocratic mode. The mobile phase consisted of a mixture of ammonium acetate aqueous solution (0.02 M) : methanol : acetonitrile of 30 : 35 : 35 (v/v/v) at a constant flow rate of 0.8 mL min^{-1}. A run time of 7 min was established for separation of the three compounds. The detection was carried out at 230 nm. An injection volume of 10 μL was used for all standards and samples.

2.3 Preparation of stock solutions, calibration standards and quality controls

Three methanolic stock solutions at 1 mg mL^{-1} of OL, SV and SVA were prepared. The OL and SV stock solutions were prepared by accurately weighing approximately 10 mg of OL and SV in 10 mL of methanol. Regarding SVA, since it is reported that lactones are unstable at alkaline pH,[5] it was obtained by alkaline hydrolysis of SV, according to ref. 16 and 17. Briefly, a SV methanolic solution of 2 mg mL^{-1} was firstly prepared. A certain volume of this solution was added to an equal volume of 0.04 M NaOH solution, heated at 60 °C for 45 min and kept overnight at room temperature. The mixture was subsequently neutralized with 1 M HCl, yielding a SVA solution with a concentration of *ca.* 1 mg mL^{-1}. The complete hydrolysis of SV was confirmed by the absence of the SV peak in HPLC. These results were also supported by LC-MS/MS, which presented a transition ion *m*/*z* at 459.5/343.3 for SVA in the positive-ion mode, comparable to what is reported in the literature.[18] Two working standard solutions containing OL, SV and SVA at concentrations of 100 and 10 μg mL^{-1} were prepared by further dilution of each stock solution with the mobile phase. Eight standard solutions (0.5, 1, 5, 10, 25, 50, 75 and 100 μg mL^{-1}) were obtained by appropriate dilution of the working standard solutions with the mobile phase. As quality control (QC), six replicates of 0.5, 1.5, 50 and 100 μg mL^{-1} standards containing the three compounds were considered. For the determination of the limit of detection (LD) and limit of quantitation (LQ) of the method, six standard solutions, between 0.1 and 1.25 μg mL^{-1},

were obtained from the 10 μg mL^{-1} working solution. All stock solutions were stored at −20 °C and working solutions were freshly prepared each day.

2.4 Method validation

The HPLC method was validated according to the US Food and Drug Administration (FDA) regulations,[19] including also some complementary aspects taken from the International Conference on Harmonization (ICH) guidelines.[20] The parameters considered for the validation included selectivity and specificity, linearity, accuracy, precision, recovery, limits of detection and quantitation, system suitability and stability.

2.4.1 System suitability. The system suitability parameters were determined by injecting six times the standard solution containing OL, SV and SVA at a concentration of 75 μg mL^{-1}. The acceptance limit was RSD ≤ 2% of the peak area and the retention time of the three compounds.[21] Other chromatographic parameters, such as capacity factor (k'), resolution (R) tailing factor (T) and theoretical plate number (N), were also analyzed.[22–25] The capacity factor is a measure of where the peak of interest is located with respect to the void volume, *i.e.*, corresponds to the elution time of the non-retained components. R is a measure of the degree of separation of two peaks. The tailing factor is a measure of the peak symmetry, and the theoretical plate number is a measure of the column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram.[26–28]

2.4.2 Limits of detection and quantification. The limits of detection and quantitation were determined based on a specific calibration curve obtained from six standard solutions (0.1, 0.25, 0.5, 0.75, 1 and 1.25 μg mL^{-1}) containing the three analytes at concentrations in the proximity of these limits values. LD and LQ were calculated according to LD = $3.3\sigma/S$ and LQ = $10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.[20]

2.4.3 Linearity. Calibration curves were constructed with eight standard solutions, containing the three compounds simultaneously, ranging from 0.5 to 100 μg mL^{-1}. Linearity was determined through the calculation of a regression line by the method of least squares, representing the peak area as a function of the standard concentration. Data collected were analyzed using the Analysis ToolPak of Microsoft Excel® (Microsoft Corp., Redmond, WA) with linear regression by the least squares method. The analysis of the response factors, that is, the peak area divided by the concentration of each standard was also considered.

2.4.4 Accuracy and precision. Precision indicates the closeness of agreement, *i.e.*, the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample and it was determined by repeatability (intra-day) and intermediate precision (inter-day) for three consecutive days. Four standard solutions (quality controls), 0.5, 1.5, 50 and 100 μg mL^{-1}, respectively, were prepared six times each and analyzed according to the proposed method (intra-day precision) for three consecutive days (inter-day precision). The relative standard deviation (RSD) determined at each concentration level should not exceed 15%, except for the lower limit of quantitation, where it should not exceed 20%.[19]

The accuracy of the method expresses the closeness of agreement between the true value and the value found. It was determined by measuring six replicates of the four quality controls and by calculating the percentage of bias for each compound according to the equation: %accuracy = (observed concentration/nominal concentration) × 100. The mean value should be within 15% of the actual value, except at the LQ, where it should not deviate by more than 20%.[19]

2.4.5 Specificity. The specificity of a method may be defined as the ability to accurately measure the analyte in the presence of all potential sample components.[20,23] In this method, the response of OL, SV and SVA, the major metabolite of SV degradation, was compared with the response of a solution containing only the analytes, and used as a measure of its specificity. The comparison was made both with the nanoparticle components and the respective supernatant, the latter containing excess of lipids and surfactants.

2.4.6 Stability. The stability of OL, SV and SVA quality controls was assessed after a short-term storage at room temperature (~25 °C) for 12 h, after a long-term storage for 30 days at −20 °C, in order to simulate sample handling and after 24 h of storage in the autosampler for the autosampler stability. The effect of three freeze–thaw cycles on the stability of the analytes was also investigated. QC samples were stored at −20 °C for 24 h, thawed unassisted at room temperature and, when completely thawed, the samples were refrozen for 24 h under the same conditions until completion of the three cycles.[19]

2.4.7 Recovery. The recovery of OL, SV and SVA from the nanoparticle supernatant was determined by comparing the respective concentrations with those of standard solutions in the mobile phase at three concentration levels 1, 50 and 100 μg mL^{-1} by repeated analysis (n = 6).

2.5 Method applicability

2.5.1 Preparation of Combo-NLC dispersion. The NLCs were prepared by using a hot high pressure homogenization technique previously optimized.[29] Briefly, 80 mg of SV and OL were dissolved in 0.75 g of the lipid phase (tripalmitin : oleic acid, in a 50 : 50 weight ratio) melted at 80 °C and pre-emulsified in 30 mL of a 3% (w/v) polysorbate 80 solution at 80 °C for 2 min, using an Ultra-Turrax X1020 (Ystral GmbH, Dottingen, Germany) set to 25 000 rpm. The pre-emulsion was further subjected to hot high-pressure homogenization (HPH) at 1000 bar for 2.5 min using an Emulsiflex®-C3 (Avestin Inc., Ottawa, Canada). The dispersion thus obtained was cooled at 4 °C to form the Combo-NLC.

2.5.2 Determination of entrapment efficiency and drug loading. The entrapment efficiency of SV and OL in the NLC was determined indirectly by calculating the total amount of drug and subtracting that of the free drug in the aqueous phase of the nanoparticle dispersion. The separation of the two phases was carried out by ultrafiltration, using Amicon® Ultra-4 centrifugal

filter units (Merck Millipore, Darmstadt, Germany) with a 100 kDa molecular weight cut-off. The amount of free drug present in the aqueous phase was collected in the outer chamber of the centrifugal unit after separation, suitably diluted with the mobile phase, filtered by a 0.22 μm membrane and determined by HPLC. For the estimation of the total drug, a specific volume of NLC suspension was accurately taken, diluted with the mobile phase and heated at 60 °C for 15 min. The dispersion was further centrifuged for 10 min at 11 740 × *g* in a Minispin® (Eppendorf Ibérica S.L., Madrid, Spain), and the supernatant was filtered by a 0.22 μm membrane and analysed by HPLC. The entrapment efficiency (EE) and drug loading (DL) were calculated using

$$\%\mathrm{EE} = (W_{\text{total drug}} - W_{\text{free drug}})/W_{\text{total drug}}) \times 100 \quad (1)$$

$$\%\mathrm{DL} = (W_{\text{total drug}} - W_{\text{free drug}})/W_{\text{lipid}} \times 100 \quad (2)$$

where $W_{\text{total drug}}$ is the total amount of SV and OL determined in the whole system, $W_{\text{free drug}}$ is the amount of free drug determined in the aqueous phase after separation of the nanoparticles, and W_{lipid} is the weight of the lipid phase. As stated above, since statins exist, in general, in the lactone and hydroxy acid forms, the respective conversion was taken into consideration, and both forms were quantified. Thus, SV entrapment efficiency is a result of the sum of SV (after stoichiometric conversion into SVA) and SVA contributions.

3 Results and discussion

3.1 Method development and optimization

A high performance liquid chromatography method for the estimation of olanzapine, simvastatin and simvastatin acid in a nanoparticulate dosage form has been developed according to the principles of Good Laboratory Practices. Optimization trials were carried out using a Lichrospher C18, by testing different proportions of ammonium acetate aqueous solution (0.02 M) : methanol : acetonitrile. Since OL and SV are poorly water soluble and more soluble in organic solvents, a higher percentage of methanol and acetonitrile was considered to diminish the retention time. By changing the column to Phenyl-Hexyl, the retention times became even shorter and the peaks sharper. The optimized mobile phase was 30 : 35 : 35 (v/v/v) of ammonium acetate aqueous solution (0.02 M) : methanol : acetonitrile at a flow rate of 0.8 mL min^{-1}. Under these conditions, simvastatin acid, olanzapine and simvastatin eluted at 1.7, 2.0 and 5.5 min, respectively. The method was validated over the range 0.5–100 μg mL^{-1}.

3.2 Method validation

3.2.1 System suitability. The RSD of the peak area and retention time for OL, SV and SVA (Table 1 and Fig. 2) were lower than 2%, which indicates that the system is appropriate to simultaneously analyze the three compounds. The assessment of the column efficiency by the number of theoretical plates (N), the tailing factor (T), resolution (R_s) and capacity factor (k') shows that the peaks were symmetric and generally well resolved. Although a $R_s \geq 2$ is reported, if the peaks are not significantly different in height and possess nearly Gaussian shapes, the British Pharmacopoeia is less stringent, and recommends $R_s \geq 1.5$ (ref. 21) for their complete separation at the baseline level.

3.2.2 Limits of detection and quantitation. The estimated LDs for OL, SV and SVA were 0.07, 0.12 and 0.09 μg mL^{-1}, respectively. The LQs found for OL, SV and SVA were 0.22, 0.36 and 0.27 μg mL^{-1}, respectively.

3.2.3 Linearity. Linearity was evaluated over the concentration range 0.5–100 μg mL^{-1} for OL, SV and SVA, estimating the regression equation and the determination coefficient (R^2) obtained from the least squares method (Table 2). The coefficients of determination for the calibration curves of the three compounds were higher than 0.999, which is generally considered as evidence of an acceptable fit of the data to the regression line,[20,23] and indicating a good linearity over the concentration range proposed. Moreover, from the analysis of the response factors a slope close to zero was obtained (−0.0389, −0.0054 and −0.0377 for OL, SV and SVA, respectively) and a relative residual standard deviation of 3.29%, 3.37% and 4.31%, thus confirming the method as linear. This complies with recommendations *e.g.*, from ref. 30.

Table 1 System suitability test parameters

	OL (75 μg mL^{-1})		SV (75 μg mL^{-1})		SVA (75 μg mL^{-1})		
Chromatographic parameters	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area	Acceptance criteria
Mean ($n = 6$)	2.04	4877.5	5.66	2903.3	1.67	2835.0	—
S.D.	0.01	23.2	0.03	18.0	0.01	33.8	—
%RSD	0.41	2.04	0.59	0.62	0.71	1.19	≤2.0%[a]
Theoretical plates (N)	1661		4951		1340		>1000[a]
Capacity factor (k')	1.07		4.86		0.73		>2.0[b]
Tailing factor (T)	1.71		1.18		1.67		≤2.0[b]
Resolution (R_s)	1.76		1.98		1.86		>2.0[b] (>1.5[ac])

[a] Ref. 21 , [b] Ref. 26 , [c] Ref. 24.

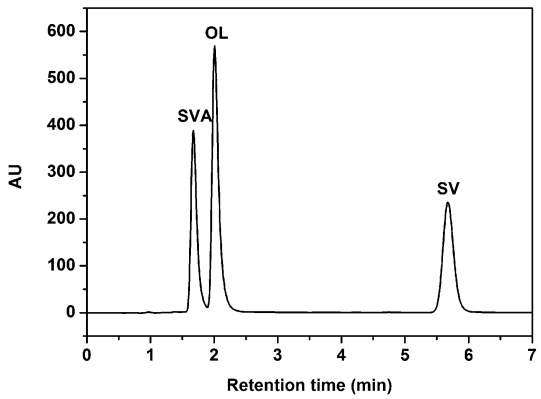

Fig. 2 Chromatogram of the standard 75 μg mL^{-1} solution of OL, SVA, and SV considered for the assessment of the system suitability.

Table 2 Results obtained from the regression analysis by the least squares method for OL, SV and SVA

Analyte	Mean R^2 ± S.E.	Mean slope ± S.E. ($n = 6$)	Mean intercept[a] ± S.E. ($n = 6$)
OL	0.9996 ± 0.0001	57.8 ± 1.7	3.9 ± 1.6
SV	0.9998 ± 0.0001	37.1 ± 0.9	−0.4 ± 2.1
SVA	0.9994 ± 0.0003	35.8 ± 0.4	3.1 ± 0.9

[a] Intercept is expressed in μg mL^{-1}.

3.2.4 Accuracy and precision. Accuracy and precision for the quality controls in the intra-day and inter-day run are shown in Table 3. All the data fulfill the acceptance criteria. The intra- and inter-day RSD values did not exceed 9.014%. The intra- and inter-day bias values were found in the interval −2.11 to 3.08%, −1.429 to 7.575%, and −4.15 to 1.91% for OL, SV and SVA, respectively. These data indicate that the developed method is accurate, reliable and reproducible, since neither RSD nor bias exceeded 15%, which is in agreement with acceptance recommendations.[19]

3.2.5 Specificity. The specificity of the method was analyzed both in the presence of components and the supernatant of the nanoparticles, containing excess of lipids and surfactants (see chromatograms, Fig. 3a and b, respectively). As shown, neither the nanoparticle content nor the supernatant exhibits peaks interfering with those of the analytes, thus indicating that the method is specific.

3.2.6 Stability. The stability data for OL, SV and SVA under conditions likely to be found during the analytical process and sample storage included short-term, long-term, autosampler and freeze–thaw stability analysis and are gathered in Table 4. According to the results, it can be inferred that the analytes are stable under the studied conditions, since the % mean concentration found was within the acceptance limit (90–110%).[9]

3.2.7 Recovery. The %recovery of OL, SV and SVA from the NLC supernatant was comprised between 93.32 and 102.55, 94.02 and 97.60, and 91.71 and 97.95, respectively (Table 5). This indicates that the developed method is adequate to simultaneously quantify the three compounds.

3.3 Method applicability

The method developed in this work was used to determine the content of OL and SV in NLC with a mean particle size of 150 nm and a zeta potential of *ca.* −36 mV. To calculate the entrapment efficiency of Combo-NLC, nanoparticles were submitted to ultrafiltration–centrifugation and the amount of free drug was determined indirectly in the filtrate, as described in Section 2.5.2. A total percentage of 77 ± 2 for OL and 96 ± 4 for SV (corresponding to the joint contribution of SV and SVA) was obtained from the nanoparticle dispersion. This yielded an EE of 99.72 ± 0.05% for SV and 97.0 ± 0.5% for OL, which corresponded to a drug loading of 10.637 ± 0.005% for SV and 10.34 ± 0.05% for OL,[29] thus making these carriers suitable for co-encapsulation of drugs with a different polarity.

Table 3 Intra-day and inter-day precision and accuracy results for OL, SV and SVA ($n = 6$)

	Intraday ($n = 6$)			Interday ($n = 18$)		
Nominal concentration (μg mL^{-1})	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias
OL (0.5)	0.49 ± 0.01	5.85	−2.11	0.51 ± 0.03	6.81	1.12
OL (1.5)	1.50 ± 0.07	4.56	0.09	1.55 ± 0.12	7.95	3.08
OL (50)	49.35 ± 0.22	0.45	−1.31	49.4 ± 0.1	0.73	−1.23
OL (100)	99.8 ± 1.5	1.47	−0.24	100.4 ± 1.7	1.67	0.37
SV (0.5)	0.52 ± 0.02	3.66	4.54	0.51 ± 0.04	8.49	1.29
SV (1.5)	1.60 ± 0.12	7.33	6.69	1.58 ± 0.11	6.63	5.41
SV (50)	49.29 ± 0.16	0.32	−1.43	49.4 ± 0.8	1.70	−1.19
SV (100)	107.6 ± 0.8	0.76	7.58	104 ± 3	3.08	3.80
SVA (0.5)	0.51 ± 0.02	4.45	1.14	0.49 ± 0.04	9.01	−1.68
SVA (1.5)	1.53 ± 0.07	4.58	1.91	1.49 ± 0.08	5.57	−0.88
SVA (50)	49.6 ± 0.4	0.73	−0.78	49.0 ± 0.6	1.25	−1.93
SVA (100)	99.9 ± 0.9	0.89	−0.11	96 ± 3	3.22	−4.15

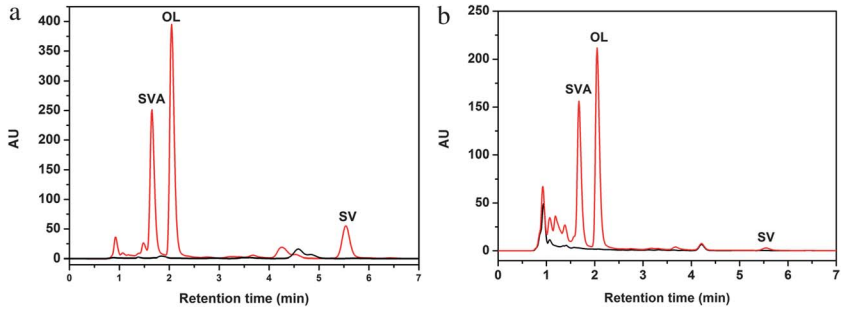

Fig. 3 (a) Chromatograms of nanoparticle components (black) and total Combo-NLC (red). (b) Chromatograms of nanoparticles (black) and Combo-NLC (red) supernatants.

Table 4 Short-term, long-term, autosampler and freeze–thaw stability (values in percentage of nominal concentration) of OL, SV and SVA quality controls ($n = 6$)

	Nominal concentration (μg mL^{-1})											
	Olanazapine				Simvastatin				Simvastatin acid			
Stability conditions	0.5	1.5	50	100	0.5	1.5	50	100	0.5	1.5	50	100
Short-term stability												
%Mean[a]	104.12	104.21	102.5	100.0	104.43	105.36	108.0	112.0	101.12	102.46	106.0	105.3
SD	0.05	0.14	2.3	3	0.04	0.17	2	3	0.05	0.08	3	1.9
%RSD	9.98	8.74	4.56	3.45	7.95	10.99	4.52	2.65	9.77	5.19	5.15	1.80
Long-term stability												
%Mean[a]	96.22	94.415	97.8	103.0	96.16	99.12	99.5	105.0	98.54	99.42	98.4	98.0
SD	0.05	0.023	2.2	8	0.03	0.07	1.9	8	0.05	0.03	1.9	7
%RSD	9.45	1.61	4.43	7.78	6.73	4.97	3.87	8.03	9.04	1.89	3.77	6.92
Autosampler stability												
%Mean[a]	98.17	102.545	102.6	105.0	109.44	98.84	98.1	103.8	105.92	102.68	98.3	94.6
SD	0.05	0.017	0.6	1.8	0.05	0.11	0.7	1.7	0.06	0.06	1.1	1.1
%RSD	9.67	1.08	1.17	1.71	9.50	7.46	1.40	1.65	10.55	4.12	2.32	1.16
Freeze–thaw stability												
%Mean[a]	98.11	104.70	95.7	96.0	108.12	104.36	95.3	104.0	97.93	102.17	95.6	95.7
SD	0.04	0.06	0.5	8	0.05	0.03	0.7	3	0.03	0.04	0.5	1.1
%RSD	8.50	4.05	0.95	8.29	9.32	2.07	1.51	2.49	5.64	2.59	1.01	1.11

[a] Expressed as percentage of nominal concentration.

Table 5 Percentage of recovery of OL, SV and SVA from the nanoparticle supernatant ($n = 6$)

	Nominal concentration (μg mL^{-1})								
	Olanzapine			Simvastatin			Simvastatin acid		
	1	50	100	1	50	100	1	50	100
%Recovery	102.7	92.1	92.5	97.3	94. 9	92.5	91.6	93.8	92.4

4 Conclusion

A specific, linear, accurate, reliable and reproducible new method for the simultaneous quantitation of OL, SV and SVA, the active form of SV, was developed and fully validated over the range 0.5–100 μg mL^{-1}. The method was successfully applied to measure the drug content in Combo-NLC formulation after preparation. The optimized Combo-NLC dispersion with a mean particle size of *ca.* 150 nm and a zeta potential of −36 mV renders an entrapment efficiency in excess of 97% for both drugs, which indicates that these nanoparticles are efficient carriers for co-encapsulation.

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