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Development and validation of a reversed-phase HPLC method for the simultaneous analysis of simvastatin and tocotrienols in combined dosage forms

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ABSTRACT

A RP-HPLC method for the simultaneous analysis of tocotrienol isoforms (TRF) and simvastatin (SIM) in SIM–TRF nanoparticles (NPs) was developed. Analytes were monitored by UV detection at 238 and 295 nm for SIM and TRF, respectively, using a gradient methanol/water elution. Calibration curves for TRF and SIM were linear over concentration range of 20–80 μg/mL and 1–10 μg/mL with correlation coefficients 0.9990 and 0.9991, respectively. The recovery of TRF and SIM from the NPs was in the range from 97.35 to 102.19% and from 92.71 to 104.35%, respectively. This developed method was successfully employed in quantifying both drugs in NPs for future use in cancer therapy.

1. Introduction

Statins, such as simvastatin (SIM, Fig. 1), represent a class of drugs that are commonly used for the management of hypercholesterolemia [1,2]. Chemically, SIM is 2,2-dimethyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-1naphthylenyl ester, [1S-[1α,3α,7β,8β(2S*,4S*),-8aβ]] butanoic acid. It is frequently prescribed for the treatment of hypercholesterolemia and was shown to significantly decrease the mortality associated with coronary heart disease [3,4]. SIM, however, is a pro-drug. After its oral administration, SIM is hydrolyzed in the liver to its active form, the β-hydroxy acid, which competitively inhibits 3-hydroxyl-3-methylgutarylcoenzyme A (HMG-CoA) reductase. HMG-CoA reductase is an enzyme involved in the conversion of HMG-CoA to mevalonate, which is the rate-limiting step in cholesterol biosynthesis [2].

Inhibition of the HMG-CoA reductase and subsequently blockade of mevalonate synthesis was also shown to decrease tumor growth *in vivo*. Therefore, statins were evaluated for their potential use in cancer therapy [5–8]. The antiproliferative and apoptotic activity of SIM against breast cancer cells, for examples, was demonstrated in recent studies [9–11]. Similarly, palm oil rich in tocotrienols, commonly referred to as tocotrienol-rich-fraction or TRF (Fig. 2), was shown to display potent antiproliferative and apoptotic activity against breast cancer cells, which was also attributed to HMG-CoA reductase down-regulation [12–15]. TRF is an oily mixture of tocopherols and tocotrienols, in which tocotrienols constitutes 70–80% of the blend [12]. Both tocopherols and tocotrienols have similar chemical structure characterized by a phytyl side chain attached to a chromane ring. The fundamental structural difference between the two groups is the phytyl chain, which is unsaturated in tocotrienols (Fig. 2). Isoforms of tocopherols and tocotrienols differ from each other by the degree of methylation of the chromane ring.

Due to their similar mechanism of activity, blends of statins and TRF were evaluated for their therapeutic effects. It was found that combined low-dose treatment of γ-tocotrienol or TRF and SIM demonstrates synergistic antiproliferative effects against mammary tumor cells. These findings strongly suggested that a SIM–TRF combination therapy could provide significant health benefits in the prevention and/or treatment of breast cancer and would avoid the myotoxic side effects caused by high-dose administration of SIM if taken alone [16].

Based on the observed synergistic effect, SIM–TRF nanoparticles (NPs) were manufactured for potential targeted therapy of breast cancer. This necessitated the development of a quantitative and sensitive HPLC method that could discriminate between SIM, TRF isoforms, and the excipients used in the preparation of the NPs. Several HPLC methods were reported for the analysis of SIM alone [17,18] or in combination with other drugs, such as ezetimibe [19,20]. Similarly, HPLC methods have been devel-

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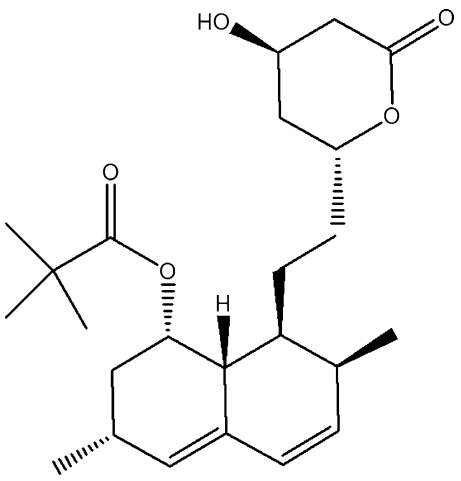


Fig. 1. Chemical structure of simvastatin (SIM).

oped and validated for the analysis of individual tocotrienols and tocopherols isoforms in human plasma [21]. None of the reported methods, however, addressed the analysis of composite TRF mixture of tocopherols and tocotrienols in the presence or absence of statins. The objective of this study was therefore to develop a simple, sensitive, and precise HPLC method for the simultaneous analysis of TRF and SIM for use as a QC tool in the development of SIM–TRF NPs. The HPLC method reported in this study was validated by assessing system suitability, specificity, limit of quantitation and detection, linearity, precision, accuracy, and recovery.

2. Experimental

2.1. Chemicals and reagents

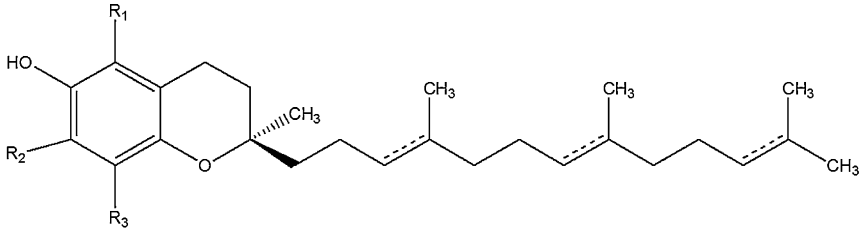
Simvastatin was purchased from Haorui Pharma-Chem Inc. (Edison, NJ); tocotrienol-rich-fraction of palm oil (TRF, which contains 20.2% α-tocopherol, 16.8% α-tocotrienol, 44.9% γ-tocotrienol, 14.8% δ-tocotrienol, and 3.2% of a non-vitamin E lipid soluble contaminants) was a gift from the Malaysian Palm Oil Board (Selangor, Malaysia); Compritol 888 ATO US/NF (glyceryl behenate, a mixture of ~15% mono-, 50% di-, and 35% triglycerides of behenic acid [C_{22}]) was provided by Gattefossé (Paramus, NJ), Lutrol® F 68 NF (poloxamer 188) was obtained from BASF (Florham Park, NJ); methanol HPLC grade, chloroform, (±)-α-tocopherol (used as internal standard, IS), and sodium hydroxide were purchased from Sigma–Aldrich Co. (St. Louis, MO); alcohol USP (95%) was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, Kentucky); water was obtained from NanoPure purification system. All chemicals were used as received without further modification.

2.2. Instrumentation and chromatographic conditions

The HPLC analysis was carried out by a SpectraSystem apparatus (Thermo Electron Corporation, San Jose, CA) equipped with a SCM1000 vacuum degasser, a P2000 pump, an AS1000-010 autosampler unit, and a UV3000-160 UV/Visible variable wavelength detector. A C_{18} (4.6 mm × 100 mm) Onyx® monolithic analytical column (Phenomenex®, Inc., Torrance, CA) was used for the analysis. The column was supported with an analytical guard cartridge system (Phenomenex® Inc., Torrance, CA). Data acquisition was performed using a chromatography software ChromQuest™ version 4.2 (Thermo Electron Corporation, San Jose, CA). The column was flushed with methanol at a flow rate of 3 mL/min for 5 min prior to the analysis of each set of three samples. For the separation and identification of TRF isoforms, an isocratic elution using 5% (v/v) water in methanol at a flow rate of 0.8 mL/min was used. The mobile phase was allowed to run for 5 min prior to each analysis. The detection of TRF was carried out at λ_{max} = 295 nm. For the simultaneous analysis of SIM and TRF, a gradient elution was initially carried out using a 15:85 (v/v) water/methanol system and a flow rate of 0.8 mL/min for 5 min. During this time, λ_{max} was set to 238 nm for the detection of SIM. Thereafter, λ_{max} was adjusted to 295 nm for the detection of TRF isoforms. The composition and flow rate of the mobile phase were also adjusted to a 5:95 (v/v) water/methanol blend and a flow rate of 1 mL/min. An injection volume of 20 μL was used in all experiments and the elution was carried out at controlled room temperature (20–25 °C). For method development and optimization, retention factor (k) was calculated using the following equation:

$$k = \frac{t_R - t_M}{t_M} \quad (1)$$

where t_R is the elution time for the most retained isoform of TRF, which corresponds to α-tocopherol; t_M is the elution time of the solvent front.



Compound	R_1	R_2	R_3	Phytyl chain
α-tocopherol	CH_3	CH_3	CH_3	Saturated
γ-tocopherol	H	CH_3	CH_3	Saturated
δ-tocopherol	H	H	CH_3	Saturated
α-tocotrienol	CH_3	CH_3	CH_3	Unsaturated
γ-tocotrienol	H	CH_3	CH_3	Unsaturated
δ-tocotrienol	H	H	CH_3	Unsaturated

Fig. 2. Generalized chemical structure of TRF isoforms.

2.3. Preparation of standard solutions

The stock solution of TRF was prepared by dissolving accurately weighed 10 mg in 10 mL of methanol to obtain a final concentration of 1.0 mg/mL. The prepared stock solution was stored at −10 to −20 °C in amber glass scintillation vial. From this stock solution, standards within a 20–80 μg/mL concentration range were freshly prepared prior to analysis. The stock solution of (±)-α-tocopherol, as the internal standard, was prepared by dissolving the IS in methanol at a final concentration of 1 mg/mL. The IS solution was stored under conditions similar to that for TRF. A constant concentration of 1 μg/mL of the IS [(±)-α-tocopherol] was used for all calibration points. Similarly, stock solution of SIM was prepared by dissolving accurately weighed 10 mg in 100 mL of methanol to obtain a final concentration 100 μg/mL. The prepared stock solution was stored at −10 to −20 °C in amber glass scintillation vial. From this stock solution, standards within a 1–10 μg/mL concentration range were freshly prepared prior to analysis. Triplicate 20-μL injections were made for each concentration of both compounds and were analyzed under the conditions described above. The mean peak area ratio of TRF/IS and SIM/IS of each standard solution were then plotted as a function of their corresponding concentration in order to construct the calibration curves and subsequently calculate the equations of the calibration lines.

2.4. Construction of calibration curves for individual TRF isoforms

TRF calibration standards were prepared at concentrations of 10, 20, 40, 60, 80, and 100 μg/mL from a standard solution of 100 μg/mL by appropriate dilution with methanol. The individual TRF isoforms (δ-tocotrienol, γ-tocotrienol, α-tocotrienol, and α-tocopherol) were identified by injecting onto the column a standard of known quantity of each isoform separately. Calibration curves were then constructed by plotting the peak ratio of each isoform/IS against TRF concentrations. JMP statistical software package (SAS Institute Inc., version 7.0) was subsequently used to perform linear regression analyses and test the fitted models. Correlation coefficients were calculated and the results of the statistical analyses were considered significant if their corresponding p-values were less than 0.05.

2.5. Preparation of SIM–TRF NPs

SIM–TRF NPs were manufactured using Lutrol® F68 (polyethylene–polypropylene block copolymer) as the primary surfactant and Compritol® 888 ATO (glycerol behenate) as the lipid core encapsulating TRF and SIM. Hot O/W microemulsion with a high-shear homogenization technique was used to manufacture the SIM–TRF NPs [22]. Briefly, Compritol® 888 ATO, SIM and TRF were dissolved in chloroform, which was allowed to evaporate under a stream of nitrogen. The remaining lipid residue (containing Compritol®, TRF and SIM) was melted at 80 °C. An aqueous surfactant solution containing 0.125% (w/v) of Lutrol® F68, preheated to 80 °C, was then added to the molten lipid blend. The final concentration of the lipid blend in the solution was 0.25% (w/v), containing 1 mM of SIM and TRF each. The dispersion of the lipid blend in the surfactant solution was homogenized at 20,000 rpm for 5 min using IKA® Ultra-Turrax T8 mixer (IKA® Works Inc., NC, USA). The formed hot microemulsion was then sonicated for 10 min using ultrasonic homogenizer (Biologics, Inc., VA, USA). SIM–TRF nanoparticle dispersions were subsequently formed by cooling the sonicated microemulsion overnight in the refrigerator. The mean particle size (±S.D.) of the SIM–TRF NPs was 253.7 nm (±0.45), which was measured by photon correlation spectroscopy (PCS) using a Nicomp™ 380 ZLS particle size analyzer (PSS Inc., Santa Barbara, CA). Prior to HPLC analysis, NP samples were prepared by diluting 100 μL of the SIM–TRF nanoparticle dispersion with 900 μL of methanol.

2.6. Determination of SIM–TRF NPs entrapment efficiency

The entrapment efficiency of the SIM–TRF NPs was determined by measuring the concentration of the free unloaded compounds in the aqueous phase of the nanoparticle dispersion. Centrifugation was carried out using Centrisart filters (molecular weight cutoff 20,000 Da). Approximately 2.5 mL of the nanoparticle dispersion was placed in the outer chamber of the filter assembly. The assembly was then centrifuged at 5000 rpm (approximately 3000 × g) for 15 min at 5 °C using Eppendorf® 580R centrifuge (Hamburg, Germany). The NPs along with encapsulated compounds remained in the outer chamber whereas the aqueous dispersion medium containing the free unloaded compounds moved to the sample recovery chamber through the filter membrane. After separation, the amount of the free compounds in the dispersion medium was estimated by HPLC. The entrapment efficiency was subsequently calculated from the following equation:

$$\text{entrapment efficiency (\%)} = \frac{\text{total amount of drug} - \text{amount of free drug in the aqueous dispersion}}{\text{total amount of drug}} \times 100$$

2.7. Method validation for simultaneous TRF/SIM assay

2.7.1. System suitability

The system suitability was evaluated by six replicate analyses of a TRF/SIM mixture at a concentration of 60 μg/mL of TRF and 20 μg/mL of SIM. The acceptance limit was ±2% for the percent coefficient of variation (%CV) of the peak area and the retention time of TRF and SIM.

2.7.2. Detection and quantitation limits (sensitivity)

Limits of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio [23,24]. LOD is defined as the lowest concentration resulting in a peak area of three times the baseline noise. LOQ is defined as the lowest concentration that provides a signal-to-noise ratio higher than 10, with precision (%CV) and accuracy (%bias) within their acceptable range (10%).

2.7.3. Linearity (calibration curve)

The calibration curves were constructed with eight concentrations (simultaneously prepared) ranging from 1 to 10 μg/mL and from 20 to 80 μg/mL for SIM and TRF, respectively. Calibration curves were constructed by plotting the ratio of the mean peak area of either γ-tocotrienol or SIM to IS versus the concentration. The linearity was assessed by linear regression analysis, which was calculated by the least square method.

2.7.4. Accuracy and precision

Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days [23,24]. Three different concentrations of SIM and TRF were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). Every sample was injected in triplicate. The accuracy of the method, which is defined as the nearness of the true value and found value [23,24], was evaluated as %bias for TRF and SIM according to the following equation:

$$\%\text{accuracy} = \frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$$

Table 1
Regression characteristics of the TRF isoforms determined by the HPLC method.

Analyte	ANOVA				Average retention time (t_R) ± S.E.
	Average peak area[a] ± S.E.	r^2 [b]	p-value	Regression equation[c]	
δ-Tocotrienol	4.231 ± 0.2034	0.9338	<0.0001	$y = 1.631 + 0.1691x$	4.0533 ± 0.0622
γ-Tocotrienol	6.559 ± 0.0628	0.9990	<0.0001	$y = 1.535 + 0.4617x$	4.76 ± 0.0808
α-Tocotrienol	3.897 ± 0.4376	0.9278	<0.0001	$y = 0.7767 + 0.2357x$	5.4366 ± 0.0949
α-Tocopherol	19.833 ± 2.0204	0.2780	0.0245	$y = 12.0 + 0.0953x$	9.94 ± 0.1960

[a] Average peak area $\times 10^5$.
[b] R-squared.
[c] y: estimated peak area ($\times 10^5$) at x TRF concentration (μg/mL).

Table 2
System suitability study.

Parameters	TRF (60 μg/mL)		SIM (20 μg/mL)		Internal standard (1 μg/mL)	
	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area
Mean ($n = 6$)	10.47	24433.12	3.93	195714.67	12.95	7534.24
S.D.	0.06	92.94	0.027	883.52	0.037	22.45
%CV	0.58	0.38	0.69	0.45	0.29	0.30

2.7.5. Specificity

The specificity of an analytical method may be defined as the ability to detect the analyte peak in the presence of the analyte by-products, or other inactive components, such as dosage form excipients or impurities [23,24]. In this method, detection of TRF and SIM in the presence of de-esterified SIM, as the major by-product of SIM degradation, as well as nanoparticle lipids and surfactants excipients was used as a measure of its specificity. Forced degradation of SIM was carried out under basic hydrolytic conditions as described previously [25]. Briefly, SIM was dissolved in a 70% ethanol solution containing 0.1 M NaOH and then incubated at 50 °C for 1 h to convert SIM to its corresponding open-ring hydroxyl derivative. The de-esterified SIM and reference SIM solutions were then mixed into a blend containing 20 μg/mL of each component. The blend was injected into the column for HPLC analysis using water/methanol system (15:85, v/v) at a flow rate of 1 mL/min with a λ_{max} adjusted to 238 nm. All experiments were performed in triplicates.

2.7.6. Stability

The stability of the drug solution was determined by analyzing QC samples after a short-term storage at controlled room temperature (20–25 °C) for 12 h. The long-term stability was determined by analyzing samples stored at 4 °C for 30 days. The autosampler stability was determined by analyzing the samples after 24 h of storage in the autosampler.

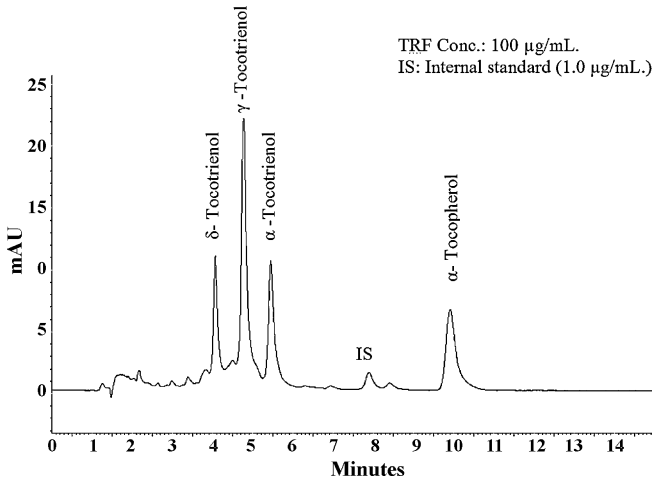


Fig. 3. TRF chromatogram at 0.8 mL/min isocratic elution using water/methanol at a ratio of 5:95 (v/v). λ_{max} was adjusted to 295 nm.

2.7.7. Recovery

The absolute recovery was computed from the peak area of TRF/SIM methanolic standard solutions to those containing TRF/SIM in the NPs at four different concentrations.

2.8. Statistical analysis

Data collected in this study were analyzed using JMP statistical software package by one-way analysis of variance (ANOVA). Univariate linear regression analysis using least square method was applied to test the fitted model. Correlation coefficient was calculated and the results of the statistical analysis were considered significant if their corresponding p-values were less than 0.05.

3. Results and discussion

3.1. Method development and optimization

The chromatographic conditions were optimized for the simultaneous determination of TRF and SIM within a short analysis time (<15 min) and an acceptable peak resolution ($R_s > 2$). To accomplish these objectives, the chromatographic column was first chosen based on peak shapes and resolution. In preliminary experiments, the sample retention time increased with an increase in column length. In order to avoid long run-times, a C_8 column was initially used. This, however, resulted in peak overlap between SIM and γ-tocotrienol, which is a component of TRF, with a consequent peak shape inconsistency during TRF elution. Therefore, a C_{18} monolithic column (5 μm, 100 mm × 4.6 mm i.d.), which permits the use of high flow rate with consequent low increase in back-pressure, was subsequently used to better resolve γ-tocotrienol from SIM peak, reduce elution time, and obtain sharp peaks for individual TRF isoforms.

With regard to the mobile phase, an HPLC method for SIM was described in the USP, in which phosphoric acid and acetonitrile (40:60, v/v) was used as the mobile phase and C_{18} column as the stationary phase (USP 28 monograph). The use of salts in the mobile phase, however, can affect the lifespan of the column [26]. In order to avoid this drawback, a mobile phase containing various ratios of water and methanol was initially used. Water, however, produced a high retention factor for TRF isoforms. At 25% water, retention factor

Table 3
Results of regression analysis of TRF and SIM determined by HPLC.

Analyte	Mean $r^2 \pm$ S.E.	Mean slope ± S.E. ($n = 6$)	Mean intercept[a] ± S.E. ($n = 6$)
SIM	0.9991 ± 0.4052	1.6346 ± 0.0132	3.0005 ± 0.8539
γ-Tocotrienol	0.9990 ± 0.1197	0.4617 ± 0.0035	1.535 ± 0.2155

[a] Intercept is expressed in μg/mL $\times 10^5$.

(calculated from Eq. (1)) for the most retained peak (α-tocopherol) was $k = 12$ (i.e. $t_M = 26$). In addition, TRF isoforms could not be distinguished due to peak overlaps. This is presumably due to the high content of water in the mobile phase, which produced tailing. SIM and TRF are poorly soluble in water whereas they are freely soluble in organic solvents, such as methanol and acetonitrile. Therefore, an increase in the percentage of methanol in the mobile phase from 75 to 85% by volume decreased the retention factor, i.e. k, by approximately 50% ($k = 6.3$; $t_M = 14.6$). Furthermore, peak resolution between TRF isoforms was in the acceptable range ($R_s > 2$). A further increase in methanol (95%), however, resulted in very fast elution with poor SIM resolution from the solvent front ($R_s < 2$). This was observed when an isocratic elution using 5% water in methanol was carried out at a flow rate of 0.8 mL/min. This was resolved by using a gradient elution as outlined in Section 2. The internal standard [(±)-α-tocopherol] was used to eliminate the interferences due to the impurities in TRF (constitutes 3.2% of TRF).

3.2. Development of an HPLC method for the separation of TRF isoforms

A single, isocratic and selective reversed-phase liquid chromatographic method was developed for the separation of TRF isoforms. TRF methanolic solutions (10 and 100 μg/mL) were injected under the chromatographic conditions outlined in Section 2. The selectivity of the RP-HPLC method is illustrated in Fig. 3, which shown satisfactory peak separation. Table 1 lists the results of the statistical analysis and the average peak area and retention times for the TRF isoforms. Retention time (t_R) ranged from 4.05 min for δ-tocotrienol to 9.94 min for α-tocopherol, which indicates a rapid analysis of TRF isoforms within a 12-min run-time. The p-values for the four isoforms in TRF were <0.05, which suggests significant linear model fit. The correlation coefficient (r) value was in the range from 0.278 for α-tocopherol to 0.999 for γ-tocotrienol. Therefore, γ-tocotrienol was used as an indicative tracer during the simultaneous analysis of TRF and SIM, as discussed in the subsequent sections. It is worth noting that the retention time of the synthetic (±)-α-tocopherol, which was used as the internal standard, was approximately 7.89 min as opposed to the 9.94 min observed with the natural α-tocopherol. This difference in retention time between the two compounds was verified by spiking the column with a pure α-tocopherol, which was previously extracted from natural TRF.

3.3. Method validation of the TRF/SIM simultaneous assay

3.3.1. System suitability

This step was accomplished under the conditions stated in Section 2. The %CV of peak area and retention time for TRF, SIM, and IS were within 2%. This indicates the suitability of the system to analyze TRF and SIM simultaneously (Table 2). To evaluate the column efficiency, the number of theoretical plates for TRF and SIM were 4872 and 14,696, respectively. The retention factor was 4.82 and 1.0 for TRF and SIM, respectively.

3.3.2. Detection and quantitation limits (sensitivity)

The LOD for TRF and SIM was found to be 7.5 and 0.5 μg/mL, respectively. The LOQ was 20 and 1 μg/mL for TRF and SIM, respectively.

3.3.3. Linearity (calibration curve)

The linearity of the calibration curves for TRF and SIM was calculated and constructed by least square regression method as illustrated previously. Table 3 lists the outcome of the statistical analysis. The correlation coefficient (r^2) for the standard calibration curves for TRF and SIM were 0.999 and 0.9991, respectively. This indicates linearity of the peak area ratio of TRF or SIM to IS in the range of 20–80 μg/mL and 1–10 μg/mL, respectively.

Table 4
Intra-day and inter-day precision and accuracy results of TRF and SIM ($n = 6$).

Nominal concentration (μg/mL)	Day 1			Day 2			Day 3		
	Mean[a]	%CV	%Bias	Mean[a]	%CV	%Bias	Mean[a]	%CV	%Bias
TRF (20)	20.535	2.990	2.675	20.251	3.091	1.258	20.293	1.694	1.467
TRF (40)	40.7733	1.488	1.933	40.643	2.234	1.608	40.175	2.016	0.438
TRF (60)	61.436	1.302	2.394	61.27	1.937	2.117	61.603	1.366	2.672
TRF (80)	80.72667	0.888	0.908	80.893	1.753	1.117	80.726	1.723	0.908
SIM (1.0)	0.991	3.574	-0.833	1.003	2.795	0.333	1.015	2.972	1.5
SIM (2.0)	2.071	3.668	3.583	2.046	1.793	2.333	2.036	2.701	1.833
SIM (6.0)	5.983	1.101	-0.278	5.995	0.0582	-0.083	6.001	1.103	0.028
SIM (10.0)	10.101	1.551	1.017	10.103	1.968	1.033	10.068	1.742	0.683

[a] Mean found concentration (μg/mL).

Table 5
Short-term, long-term, and auto-sampler stability for TRF and SIM combined solutions ($n = 6$).

Nominal concentration (μg/mL)	Short-term stability			Long-term stability			Auto-sampler stability		
	%Mean[a]	S.D.	%CV	%Mean[a]	S.D.	%CV	%Mean[a]	S.D.	%CV
TRF (20)	107.675	1.296	6.020	108.508	0.711	3.276	103.133	1.015	4.921
TRF (40)	104.017	0.757	1.818	101.933	1.739	4.265	102.937	2.338	5.679
TRF (60)	100.45	1.64	2.721	100.172	2.001	3.329	103.227	2.066	3.335
TRF (80)	100.908	2.418	2.995	101.741	3.430	4.214	103.154	3.343	4.051
SIM (1.0)	101.0	0.036	3.597	105.0	0.092	8.832	106.166	0.089	8.433
SIM (2.0)	101.583	0.048	2.379	101.583	0.048	2.379	108.5	0.213	9.853
SIM (6.0)	97.694	0.524	8.944	97.027	0.506	8.698	102.916	0.297	4.821
SIM (10.0)	101.933	0.549	5.393	102.933	0.517	5.027	102.783	00.359	3.501

[a] Expressed as percentage of nominal concentration.

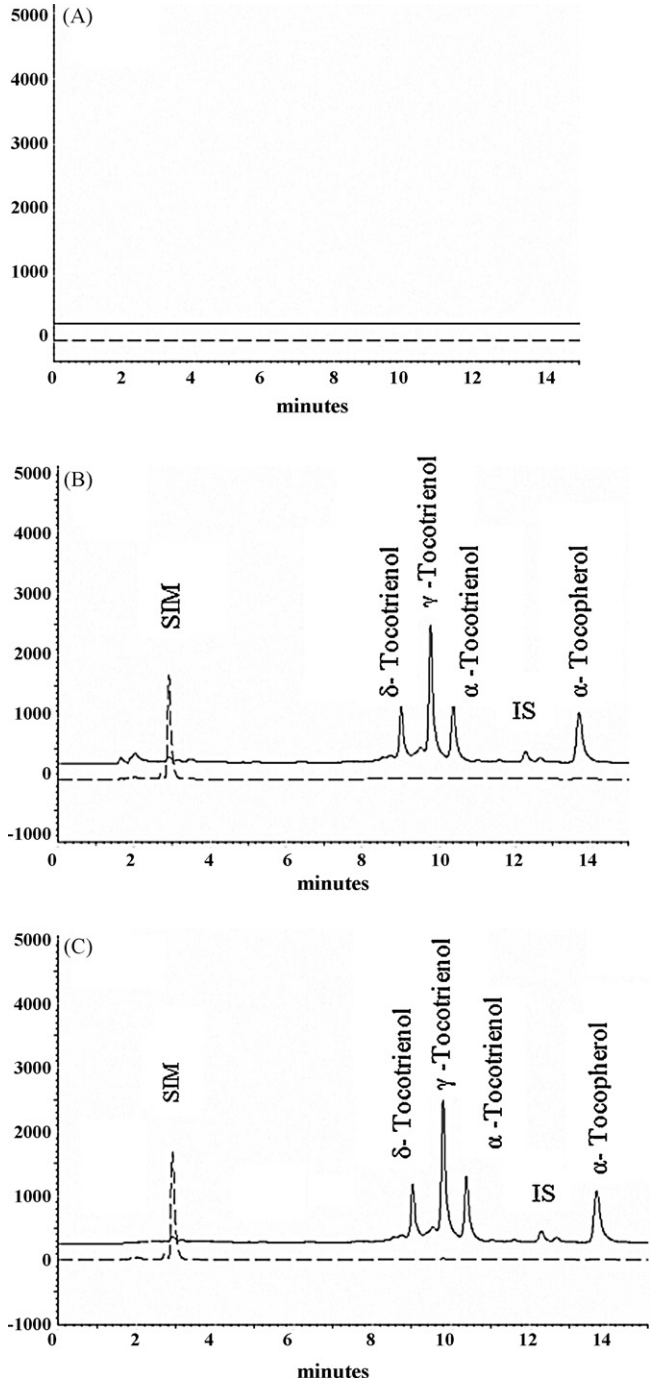


Fig. 4. The chromatograms of nanoparticle excipients (A), nanoparticles (before filtration) containing TRF (20 μg/mL) and SIM (20 μg/mL) (B), and standard solutions of TRF (20 μg/mL) and SIM (20 μg/mL).

3.3.4. Accuracy and precision

Accuracy and precision for the QC samples during the intra- and inter-day run are given in Table 4. As shown, the data were within the acceptance criteria (i.e. 5%). One-day intra-day accuracy (expressed as %bias) ranged from 0.908 to 2.675 and from −0.833 to 1.017 for TRF and SIM, respectively. The calculated intra-day precision (expressed as %CV) after 1 day ranged from 0.888 to 2.990 and from 1.101 to 3.668 for TRF and SIM, respectively.

3.3.5. Specificity

In order to confirm the specificity of the method for TRF and SIM in the presence of nanoparticle excipients, three methanolic solutions of the dissolved SIM–TRF NPs were injected into the HPLC. As discussed above, the specificity of an HPLC method is the ability to detect the analytes under research in the presence of other ingredients, such as the lipids and surfactants in the NPs. Peak identification was performed under the experimental conditions stated previously. Fig. 4 shows representative chromatograms of the SIM/TRF methanolic solution as well as those for the NPs in the presence and absence of the SIM–TRF blend. As shown, the signals detected during the analysis correspond to the TRF and SIM only. Nanoparticle excipients did not exhibit any peaks, and therefore no interferences were detected as indicated by the absence of signals in the chromatograms.

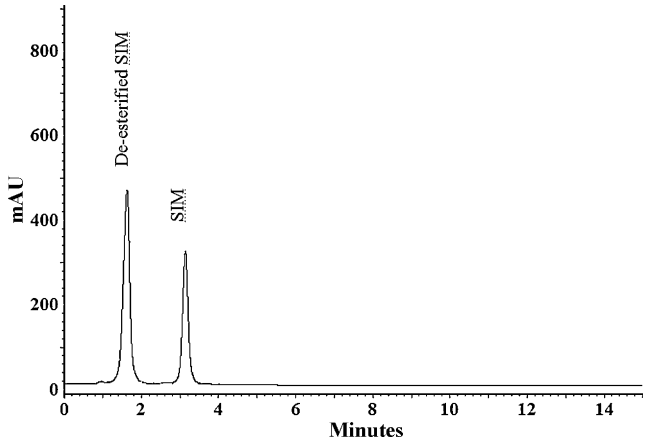


Fig. 5. The chromatograms of SIM and its by-product (de-esterified form with an open lactone ring), each existing at a concentration of 20 μg/mL. The mobile phase consisted of water/methanol at 15:85 (v/v) ratio. The flow rate was set at 0.8 mL/min and λ_{max} was adjusted to 238 nm.

Similarly, the specificity of the method in the presence SIM by-products was demonstrated by analyzing samples containing the reference and de-esterified SIM solutions as described in Section 2. Opening of the lactone ring to yield a hydroxy-derivative was reported as the only by-product of SIM degradation [25,27–30]. The chromatogram of SIM and its de-esterified form are shown in Fig. 5. SIM exhibited a retention time (t_R) of 3.00 ± 0.63 min whereas its corresponding degradation product had a t_R of 1.72 ± 0.33 min. No other interfering signals were observed during the elution of SIM even when λ_{max} was adjusted to 295 nm for the quantification of TRF.

3.3.6. Stability

The data for short-term, long-term, and the autosampler stability of the TRF and SIM solutions are given in Table 5. As shown, the %mean found concentration was within the acceptable limit (90–110%).

Table 6
Absolute recovery of TRF and SIM from SIM–TRF nanoparticles (n = 6).

Nominal concentration (μg/mL)	Mean[a]		
	Standard methanolic solution	Methanolic solution of nanoparticles	% Recovery
TRF (20)	19.67	20.1	102.186
TRF (40)	39.50	37.67	95.367
TRF (60)	61.98	60.34	97.353
TRF (80)	78.90	76.98	97.566
SIM (1.0)	0.96	0.89	92.708
SIM (2.0)	1.08	1.07	99.074
SIM (6.0)	6.07	5.95	98.023
SIM (10.0)	9.87	10.3	104.356

[a] Mean found concentration.

3.3.7. Recovery and application of the HPLC method in SIM–TRF nanoparticle analysis

Entrapment efficiency and absolute recovery were measured in order to demonstrate the applicability of the HPLC method for the simultaneous determination of SIM and TRF in NPs. To calculate the entrapment efficiency of SIM and TRF in the NPs, the free unloaded compounds were separated from the SIM–TRF NPs using Centrisart filter assemblies as described in Section 2. The concentration of the free unloaded SIM and TRF in the filtrate, as measured by the HPLC method, was 0.011 and 0.025 mmol%, respectively, which corresponds to an entrapment efficiency of 98.95% (±0.62) for SIM and 97.5% (±0.35) for TRF. The absolute recovery was computed by comparing the peak area of the TRF/SIM methanolic standard solutions to those containing SIM–TRF NPs at different concentrations. The results of this experiment are given in Table 6. The % recovery of SIM and TRF from the NPs was in the range from 92.71 to 104.35 and 97.35 to 102.19, respectively. This indicates the suitability of the developed method in simultaneously quantifying the concentration of both drugs in the NPs.

4. Conclusion

The RP-HPLC method developed in this study was shown to be rapid, sensitive, and accurate in simultaneously detecting and quantifying TRF isoforms and SIM either alone or in the presence of nanoparticle excipients, such as lipids and surfactants. This method has the advantage of being specific for both drugs without the need for additional sample preparation, such as the extraction of the active constituents.

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