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GC determination of long chain fatty acids that compose D003 in 5-mg film-coated tablets

Ernesto Méndez Antolín, David Marrero Delange, Víctor González Canavaciolo *, Abilio Laguna Granja

National Center for Scientific Research, Center of Natural Products, PO Box 6414, Cubanacán, Playa, Havana City, Cuba

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Abstract

D003 is a new product which consists of a mixture of aliphatic fatty acids ($C_{24:0}$–$C_{36:0}$), that shows antiplatelet, antithrombotic and cholesterol-lowering effects in experimental models. A gas chromatographic (GC) method using a DB-5 wide-bore column and 1-nonadecanoic acid as internal standard was developed and validated in order to determine D003 in 5 mg film-coated tablets. The acids were analyzed as methyl esters derivatives, prepared using 5% aqueous HCl–methanol. Developed method was specific for the active principle, even when samples were subjected to stress conditions. Good linearity (correlation coefficient >0.99) and accuracy (total average recovery = 99.60%) were proven over a range 38–150% of the nominal concentration. Within-day and intermediate precisions at the nominal dose (100%) were $<1.5\%$. The method was suitable for quality control and stability studies of these tablets.

Keywords: D003; Very long chain fatty acids; GC method; Identification; Quantification; Validation

1. Introduction

D003 is a new product consisting of a mixture of 13 very long-chain primary fatty acids ($C_{24:0}$–$C_{36:0}$). This product is isolated and purified from sugar cane (*Saccharum officinarum* L.) wax [1], the composition of this mixture is highly reproducible from batch to batch at the industrial scale [2]. D003 has demonstrated good cholesterol-lowering [3,4] as well as antiplatelet and antithrombotic [5] effects in experimental models. Also, it was studied its acute and oral subchronic toxicity [6] where no drug-related toxicity has been observed after single or short term repeated administration of D003 to rats. D003 is under study in clinical trials Type II, using film-coated tablets containing 5 mg of it.

Gas chromatography (GC) shows to be the best technique for determining fatty acids; and with this aim, they are usually converted to the simplest convenient volatile derivatives, often methyl esters (FAMEs) or other esters [7,8]. However, to our knowledge, published works only deal about acids with less than 26 carbon atoms and contain limited

* Corresponding author. Fax: +53-733-6837.
E-mail address: dalmer@ip.etecsa.cu (V.G. Canavaciolo).

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information concerning the details of the quantitative determination.

In the present work was described the validation of a GC method for determining the fatty acids that compose D003. They were extracted from the 5-mg film-coated tablets and converted to methyl esters by an acid-catalyzed reaction. This method is used for quality control and stability studies of these tablets.

2. Experimental

2.1. Apparatus

The GC system (a) consisted of Shimadzu GC-14A with a flame ionization detector and Shimadzu C-R4A computerized data processor, (Shimadzu, Kyoto, Japan). The column used was a DB-5 Wide-bore fused-silica capillary column (30 m, 0.53 mm id, 1.5 μm D_f; J&W Scientific, Folsom, USA) set to the injection port intended for packed column (fitted with a 3.8 mm id silanized quartz glass liner) by means of a wide-bore adapter. Operated at a program from 250 to 320 °C at 5 °C min^{-1} and isothermal for 10 min at 320 °C while injector and detector temperatures were 300 and 320 °C, respectively. Carrier gas (H_2) flow, 11.4 ml min^{-1}. To form the flame, hydrogen gas flow, 40 ml min^{-1}, and air gas flow, 400 ml min^{-1}, were used.

GC system (b) used, only, in the intermediate precision study (Laboratory 2). Same chromatograph as (a) with a BPX-5 Wide-bore fused-silica capillary column (25 m, 0.53 mm id and 1.0 μm D_f, SGE, TX, USA). Operating at a program from 220 to 340 °C at 5 °C min^{-1} and isothermal for 10 min at 340 °C while injector and detector temperatures were 320 and 340 °C, respectively. Carrier gas (Ar) flow was 4.5 ml min^{-1}.

The GC/MS system (c) GC 8000 coupled to a MD800 Series (Fisons Instruments, Manchester, England) with a computerized data processor, with a capillary column SPB-5 (30 m, 0.25 mm id and 0.5 μm D_f; Supelco, Bellefonte, USA). Operating conditions: column programmed from 100 to 200 °C at 40 °C min^{-1}, from 200 to 320 °C at 10 °C min^{-1} and isothermal for 30 min at 320 °C. Helium carrier gas flow, 1 ml min^{-1}. Injector, ion source, and interface temperatures were 300, 250, and 250 °C, respectively. Ionization energy was 70 eV. The mass spectrum was continuously acquired from 40 to 600 m/z with a scan speed of 1 s per decade in full scan mode.

2.2. Chemicals

D003, batch 990702, was provided by CNIC (Havana, Cuba); all other chemicals were analytical reagent grade: Hydrochloric acid (37%), methanol, toluene, hydrochloric acid (0.1 M), hydrogen peroxide (30%), sodium hydroxide (0.1 M); (Merck, Darmstadt, Germany), and chloroform (Riedel-de-Haën, Seelze, Germany). 1-Nonadecanoic acid (99%, Sigma, St. Louis, MO, USA), 1 mg ml^{-1} in chloroform was used as internal standard.

The stock solution was prepared as follows: weighed 6.5 mg 1-tetracosanoic ($C_{24:0}$), 4.5 mg 1-pentacosanoic ($C_{25:0}$), 14 mg 1-hexacosanoic ($C_{26:0}$), 12 mg 1-heptacosanoic ($C_{27:0}$), 145.5 mg 1-octacosanoic ($C_{28:0}$), 8 mg 1-nonacosanoic ($C_{29:0}$), 80 mg 1-triacontanoic ($C_{30:0}$) and 5 mg 1-hentriacontanoic ($C_{31:0}$) acids; all >99% GC, (Sigma) into a 100 ml volumetric flask complete volume with chloroform and mix in order to give final concentrations of 0.06, 0.04, 0.14, 0.12, 1.45, 0.08, 0.8, and 0.05 mg ml^{-1}, respectively. This solution was found to be stable for 1 month, when stored at +4 °C.

The methylation solution (MSoln) was prepared with hydrochloric acid–methanol (5:95, v/v). This solution should be weekly prepared and stored at +4 °C.

2.3. Tablet formulation

The test procedure was applied to a commercial formulation of D003 in film-coated tablets, consisting of 5 mg D003, lactose, cornstarch, gelatin, microcrystalline cellulose, magnesium stearate, and sodium croscarmellose as excipients. Tablets were coated with a mixture of cellulose acetophthalate, polyethylene glycol 20 000, special talc for tablets, titanium dioxide, and FD&C green lake. Mass tablet was 125 ± 6.25 mg.

2.4. Test procedure

2.4.1. Tablets

An amount of powdered tablets equivalent to 5 mg of D003 was added to a 10 ml test tube with screw cap, 0.5 ml of the internal standard solution and 3 ml of chloroform were added, and the tube was heated at 80 °C for 15 min with occasional shaking. The extract was hot filtered to another test tube, and 1.0 ml of the filtrate was transferred to a 1.8 ml crimp vial. This volume was evaporated to dryness at 80 °C with the help of a nitrogen flow and 0.5 ml of the MSoln was added. The vial was sealed and heated at 80 °C for 90 min. The content of the vial was evaporated to dryness at 80 °C with nitrogen flow, 150 μl of toluene were added, the vial was sealed and heated at 80 °C for 3 min, one μl portions were examined by GC.

The mass (mg) of each acid was obtained by the internal standard method [9] according to the following equation:

$$\text{Mass of compound } i = \frac{\text{Area of compound } i \times \text{Mass of Internal standard} \times f_i^{\text{w}}}{\text{Area of Internal standard}}$$

Where, f_i^{w}, relative mass response factor for compound i.

In order to determine f_i^{w}, 0.5 ml of the stock solution and 0.25 ml of the internal standard solution were transferred to a 1.8 ml crimp vial, the content was evaporated to dryness at 80 °C with a gently nitrogen stream. Afterwards, 1 ml of MSoln was added and the mixture was heated at 80 °C for 90 min. Content was evaporated to dryness, 250 μl of toluene were added and the mixture was heated at 80 °C for 3 min. This procedure was performed in triplicate, and f_i^{w} was calculated as follows:

$$f_i^{\text{w}} = \frac{\text{Area of Internal standard} \times \text{Mass of compound } i}{\text{Area of compound } i \times \text{Mass of Internal standard}}$$

Commercial standards of $C_{32:0}$, $C_{33:0}$, $C_{34:0}$, $C_{35:0}$, and $C_{36:0}$ acids are not available, then the f_i^{w} of $C_{30:0}$ acid was used for $C_{32:0}$, $C_{34:0}$ and $C_{36:0}$ acids, and for $C_{33:0}$ and $C_{35:0}$ acids was used the f_i^{w} of $C_{31:0}$ acid. The total mass of D003 was considered as the summatory of the masses of all these fatty acids. This value was corrected by taking into account sample mass and tablet average mass.

2.5. Validation of test procedure

2.5.1. Specificity

In order to determine the specificity of the chromatographic system was compared the chromatograms of placebo, internal standard solution, original tablets and that of tablets stressed under degradation conditions. Tablets were crushed to a fine powder and degradation conditions were obtained as follows: thermolysis (105 °C, 2 weeks); base and acid hydrolysis (sample suspended in 0.1 M of sodium hydroxide and hydrochloric acid, at 1 g in 10 ml, at 105 °C, 1 day); oxidation (sample suspended in 30% hydrogen peroxide, at 1 g in 10 ml, at 25 °C, 7 days); and photolysis (254 nm UV light, at 25 °C, 7 days). These tests were performed in neutral glass ampoules, which were flushed with nitrogen and sealed ($n = 3$). Peak purity was checked by gas chromatography/mass spectrometry analysis.

2.5.2. Linearity and accuracy

Linearity and accuracy were assessed over the range 37.8–151% of the nominal concentration ($n = 3$). The matrix (120 mg placebo) was spiked with 2, 3, 4, 5, 6, 7 and 8 ml of a working standard solution of D003 (0.945 mg ml^{-1} in chloroform). Samples were evaporated to dryness, and then the mixture was analyzed according to the analytical method. The regression lines ($y = a + bx$) were calculated by the method of least squares based

on the amount found (y) versus the amount added (x). Evaluation of the linearity of the total amount quantification was made according to the following parameters ($P = 0.05$):

- Correlation coefficient $\geqslant 0.99$.
- Relative standard deviation of response factor (R.S.D.$_f$) $< 5\%$, where response factor is defined as y/x.
- Relative standard deviation of slope (R.S.D.$_b$) $\leq 2\%$, with

$$\text{R.S.D.}_b(\%) = \frac{\text{S.D.}_b}{b} 100$$

where: S.D.$_i$ = standard deviation of the slope.

- $t_{exp}b < t_{tab}(0.05, n-2)$, with

$$t_{exp}b = \frac{|b|}{\text{S.D.}_b}$$

- The zero value should be included in the confidence intervals (CI) of the intercept:

$$\text{CI} = a \pm t \cdot \text{S.D.}_a$$

where: S.D.$_a$, standard deviation of the intercept.

Accuracy was assessed by a recovery study. Recoveries were calculated according to the following equation:

$$\text{Recovery } (\%) = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Average recovery was checked to 100% with the Student's t-test. The experimental value of t was calculated as follows:

$$t = \frac{|100 - \text{Recovery}|\sqrt{n}}{\text{R.S.D.}}$$

The null hypothesis (the recovery is closed to 100% and the method is accurate) was accepted for a significance level greater than 5%. To determine if the concentration factor affects the results, the Cochran test for $P = 0.05$ was used.

2.5.3. Precision

Repeatability and intermediate precisions were evaluated by assaying commercial tablets in two laboratories. Each operator followed the procedure under conditions of repeatability ($n = 10$). Fisher's (F) and Student's (t) tests for $P = 0.05$ were performed to determine significant differences between results. The R.S.D. values were evaluated by comparison with the Horwitz's criterion [10,11].

2.5.4. Ruggedness

Ruggedness was evaluated by an intralaboratory study, in which the influence of small changes in the operating conditions on the analytical result was measured. Seven operating conditions were studied, performing eight experiments ($n = 6$) as described by Youden [12]. The selected factors and levels are shown in Table 1. The results that were evaluated are: amount of D003 (mg), precision (S.D.), resolution between acids $C_{28:0}$ and $C_{30:0}$ ($Rs_{C28:0\text{-}C30:0}$) and relative retention for acid $C_{28:0}$ ($r_{C28:0,C19:0}$).

3. Results and discussion

3.1. Specificity

Fig. 1 shows chromatograms of samples of D003 tablets, placebo, and internal standard. It can be observed that the chromatographic conditions give well-resolved peaks when samples were

Table 1
Factors and conditions investigated in ruggedness study

Factors	Examined conditions	
	Upper level (+)	Lower level (−)
(A) Temperature of injector (°C)	300	295
(B) Temperature of detector (°C)	320	315
(C) Flow rate (ml min^{-1})	11.4	9.4
(D) Temperature program (°C min^{-1})	6	5
(E) Initial oven temperature (°C)	250	240
(F) Injection volume (μl)	2	1
(G) Final oven temperature (°C)	320	315

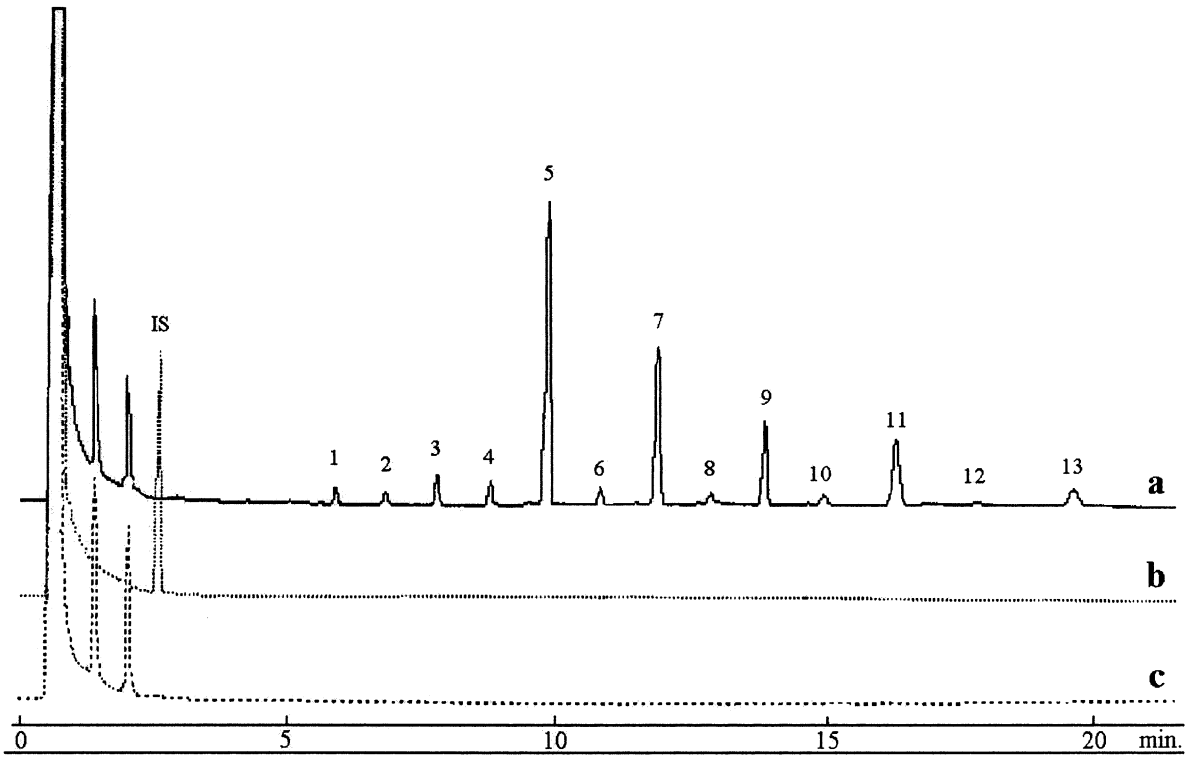

Fig. 1. GC profiles of (a) D003 tablets, (b) internal standard, and (c) placebo tablets, all of them after FAMEs preparation. Peaks correspond to derivatives of I.S (1-nonadecanoic acid), (1) 1-tetracosanoic acid, (2) 1-pentacosanoic acid, (3) 1-hexacosanoic acid, (4) 1-heptacosanoic acid, (5) 1-octacosanoic acid, (6) 1-nonacosanoic acid, (7) 1-triacontanoic acid, (8) 1-hentriacontanoic acid, (9) 1-dotriacontanoic acid, (10) 1-tritriacontanoic acid, (11) 1-tetratriacontanoic acid, (12) 1-pentatriacontanoic acid, and (13) 1-hexatriacontanoic acid.

analyzed. The specificity assay showed that no degradation occurs when the stress conditions were used.

3.2. Linearity and accuracy

The calculated regression line for total amount of acids was $y = (1.01 \pm 0.04)x - (0.04 \pm 0.18)$, with CI calculated at $P = 0.05$. Correlation coefficient (r), R.S.D.$_f$ and R.S.D.$_b$ (0.9994, 1.50 and 1.71%, respectively) fulfilled the acceptance criteria. Taking into account the small content of some acids in the mixture, the Student's t-test for the slope was, also, applied to the individual regression lines. In all cases (Table 2) the $t_{exp}b$ was $> t_{tab}$ (2.093), therefore, there is a probability $> 95\%$ that all slopes were different from zero. The confidence intervals of all the intercepts included the zero value for each acid and for the total of them; thus, all the lines passed through the origin. The procedure could be considered linear and did not present bias in the concentration range studied.

Recoveries from spiked placebos were between 98.0 and 101.1% of the expected amounts with R.S.D.s $< 2\%$ (Table 3). These high recoveries were possible because of the addition of the internal standard at the beginning of the extraction. The average recovery and 100% value were not significantly different, neither for each concentration nor for the total average recovery, according to the t_{exp} values which were lower than t_{tab} for $P = 0.05$ (4.303 and 2.085, respectively); consequently, the method is accurate. The G_{exp} (0.3606) was $< G_{tab}$ (0.5612), therefore, the concentration factor did not affect the variability of results.

Table 2
Linearity of GC determinations of D003 in film-coated tablets

Acids	$b \pm t_{tab} \times$ S.D.$_b$	$t_{exp}b$	$a \pm t_{tab} \times$ S.D.$_a$	Correlation coeficient (r)
$C_{24:0}$	0.016±0.002	20.236	0.004±0.009	0.9948
$C_{25:0}$	0.013±0.001	20.240	0.003±0.007	0.9948
$C_{26:0}$	0.035±0.004	18.860	0.012±0.020	0.9938
$C_{27:0}$	0.032±0.004	18.163	0.011±0.019	0.9933
$C_{28:0}$	0.373±0.011	69.749	0.010±0.057	0.9996
$C_{29:0}$	0.023±0.003	17.368	0.008±0.014	0.9927
$C_{30:0}$	0.205±0.008	51.194	0.001±0.042	0.9992
$C_{31:0}$	0.015±0.002	15.030	0.004±0.011	0.9904
$C_{32:0}$	0.105 ±0.005	48.128	0.004±0.023	0.9991
$C_{33:0}$	0.016±0.002	17.730	0.000±0.010	0.9935
$C_{34:0}$	0.124±0.005	48.559	0.005±0.027	0.9991
$C_{35:0}$	0.008±0.001	18.497	0.002±0.005	0.9937
$C_{36:0}$	0.043±0.002	44.896	0.000±0.010	0.9990
Total	1.007±0.036	58.419	0.044±0.183	0.9994

a, intercept; b, slope.

3.3. Precision

Good results were obtained in the precision study between two laboratories (Table 4), within-day R.S.D. values of quantification of the total amount were lower than the Horwitz's criterion (2.8%) [10] for both series of analysis; these results prove that the method is repeatable. Considering the results of both series of analysis (Table 5), the R.S.D. fulfilled the Horwitz's criterion too. No significant differences were found in the precision or the means, obtained from two laboratories, demonstrated through the experimental F and t values (1.19 and 0.669, respectively), which were lower than the critical values (3.18 and 2.101, respectively) for $P = 0.05$. The confidence limit was (5.00±0.03) mg, therefore, the real value will lie within the range 4.97–5.03 mg for $P = 0.05$.

3.4. Ruggedness

In the ruggedness study were calculated the means of results for each experiment (Table 6), the effects on observed results and the limit values of these effects, as expressed by S.D. $\sqrt{2}$ (Table 7). The only parameter that was significantly affected was the $Rs_{C28:0-C30:0}$ when the volume of injection was 2 μl, it could be due to a column overload [13].

Table 3
Accuracy of GC determination of D003 in film-coated tablets

Amount added (mg)	Amount found (mg)			Mean recovery±S.D. (%)	R.S.D. (%)	t_{exp}
	1	2	3			
1.89	1.84	1.90	1.88	99.12±1.619	1.63	0.935
2.84	2.82	2.79	2.74	98.01±1.424	1.45	2.377
3.78	3.76	3.75	3.81	99.82±0.847	0.85	0.367
4.72	4.81	4.76	4.75	101.13±0.681	0.67	2.921
5.67	5.62	5.52	5.60	98.41±0.935	0.83	2.899
6.61	6.64	6.69	6.62	100.60±0.546	0.54	1.924
7.56	7.55	7.48	7.67	100.09±1.274	1.27	0.123
Total				99.60±1.427	1.43	1.291

Table 4
Results of repeatability study between two laboratories ($n = 10$)

Acids	Laboratory 1		Laboratory 2	
	Mean (mg) ± S.D.	R.S.D. (%)	Mean (mg) ± S.D.	R.S.D. (%)
$C_{24:0}$	0.076 ± 0.002	2.08	0.078 ± 0.003	3.26
$C_{25:0}$	0.065 ± 0.003	3.99	0.065 ± 0.008	11.66
$C_{26:0}$	0.158 ± 0.003	1.79	0.159 ± 0.003	1.80
$C_{27:0}$	0.143 ± 0.004	2.95	0.148 ± 0.004	2.53
$C_{28:0}$	1.884 ± 0.031	1.65	1.868 ± 0.028	1.48
$C_{29:0}$	0.102 ± 0.005	5.28	0.104 ± 0.003	3.03
$C_{30:0}$	1.034 ± 0.017	1.67	1.022 ± 0.011	1.08
$C_{31:0}$	0.069 ± 0.002	3.23	0.068 ± 0.003	4.53
$C_{32:0}$	0.524 ± 0.009	1.71	0.519 ± 0.008	1.62
$C_{33:0}$	0.083 ± 0.009	10.39	0.073 ± 0.003	3.66
$C_{34:0}$	0.620 ± 0.011	1.74	0.619 ± 0.011	1.76
$C_{35:0}$	0.036 ± 0.001	2.79	0.036 ± 0.004	12.25
$C_{36:0}$	0.220 ± 0.004	2.03	0.227 ± 0.006	2.80
Total	5.013 ± 0.075	1.49	4.991 ± 0.068	1.37

Table 5
Results of intermediate precision ($n = 20$)

Acids	Mean (mg) $\pm t \times$ S.D./$n^{1/2}$	R.S.D. (%)
C24:0	0.077 ± 0.001	3.26
C25:0	0.065 ± 0.003	8.50
C26:0	0.158 ± 0.001	1.78
C27:0	0.145 ± 0.002	3.13
C28:0	1.876 ± 0.014	1.59
C29:0	0.103 ± 0.002	4.32
C30:0	1.028 ± 0.007	1.55
C31:0	0.069 ± 0.001	3.87
C32:0	0.522 ± 0.004	1.70
C33:0	0.078 ± 0.004	10.18
C34:0	0.620 ± 0.005	1.68
C35:0	0.038 ± 0.003	17.74
C36:0	0.223 ± 0.003	2.92
Total	5.002 ± 0.033	1.41

Taking into account this result, the injection volume must be carefully controlled.

4. Conclusions

Other reported methods for the determination of fatty acid only deal with compounds containing less than 26 carbon atoms or have limited information concerning the quantitative aspects of the analysis. The present validated method, instead, allows an accurate and precise determination of

Table 6
Means of results in ruggedness study ($n = 6$)

Experiment	D003 (mg)	S.D.	$r_{C28:0,\ C19:0}$	$Rs_{C28:0-C30:0}$
1	4.92	0.01	3.99	10.16
2	4.99	0.01	3.43	8.31
3	4.96	0.02	3.76	8.12
4	4.92	0.02	3.17	9.52
5	4.92	0.02	3.52	9.15
6	5.00	0.02	3.42	7.68
7	4.94	0.02	3.80	8.15
8	4.91	0.02	3.59	8.63

Table 7
Effects of operational changes on results of ruggedness study

Factor	D003 (mg)	S.D.	$r_{C28:0-C19:0}$	$Rs_{C28:0-C30:0}$
A	0.01	0.01	0.08	0.62
B	0.03	0.01	0.01	0.20
C	0.01	0.00	0.36	0.37
D	0.01	0.01	0.24	0.20
E	0.01	0.00	0.21	0.14
F	0.05	0.00	0.04	1.28
G	0.00	0.00	0.02	0.03
S.D.$\sqrt{2}$	0.06	0.03	0.37	1.17

the fatty acids from C_{24} to C_{36} carbon atoms and fulfills the acceptance criteria for the analysis of a pharmaceutical form; thus it can be used for the quality control and stability studies of tablets containing 5 mg D003.

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