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Il Farmaco 58 (2003) 521–525

IL FARMACO

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Gas chromatographic method for determining very long chain fatty acids that compose D003 in aqueous suspensions from 20 to 200 mg/ml, used in pharmacological and toxicological studies

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Received 21 September 2002; accepted 31 December 2002

Abstract

D003 is a natural mixture of fatty acids ($C_{24:0}$ to $C_{36:0}$), which shows antiplatelet, antithrombotic, and cholesterol-lowering effects in experimental models. A specific gas chromatographic method, using a BPX-5 wide-bore column and 1-nonadecanoic acid as internal standard, was developed and validated to determine the content of D003 in 20–200 mg/ml aqueous suspensions, which are used in pharmacological and toxicological studies. Fatty acids were extracted with chloroform and converted to methyl esters derivatives using 5% aqueous HCl–methanol. The method was linear for suspensions ranging from 10 to 250 mg/ml (correlation coefficient = 0.9998) and showed a good accuracy, with average recoveries (98.5–101.28%) no significantly different from 100%, according to the Student *t*-test ($P = 0.05$). The RSDs were $< 2.2\%$, indicating that the method has a good repeatability. The intermediate precision was good too, with RSDs between 1.20 and 2.10%. This method is suitable for quality control of these suspensions.

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Keywords: D003; Suspensions; Fatty acids; GC determination; Validation

1. Introduction

D003 is a new product consisting of a mixture of 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], and its composition is highly reproducible from batch to batch [2]. D003 has demonstrated good cholesterol-lowering effects, determined both in vivo and in vitro models [3–5], as well as antiplatelet and antithrombotic [6] effects in experimental models. No drug-related toxicity has been observed after single or short term repeated administration of D003 to rats in acute and oral sub chronic toxicity studies [7]. This product does not show evidences of cytotoxic or genotoxic activity on both somatic or germ cells in rodents [8]. Aqueous suspensions of D003, containing acacia gum as vehicle, were used in all these studies because of the very low solubility of D003 in water and aqueous solutions [2]. It was necessary then to develop a method for determining the real concentration of D003 in these suspensions, which allows knowing the exact quantity of D003 administered to the animals.

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) [9,10]. However, to our knowledge, published works only deal about acids with less than 26 carbon atoms and contain limited information concerning the details of the quantitative determination. In the present work it is described the validation of a GC method for determining acids from $C_{24:0}$ to $C_{36:0}$ in D003 suspensions ranging from 20 to 200 mg/ml.

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doi:10.1016/S0014-827X(03)00072-7

2. Experimental

2.1. Instruments

The gas chromatographic system consisted of a GC-14B equipment with a flame ionization detector coupled to a C-R4A computerized data processor (Shimadzu, Kyoto, Japan). It was used a BPX-5 wide-bore fused-silica capillary column (25 m, 0.53 mm id, 1.0 μm D_f; SGE, USA). Oven was programmed from 220 to 340 °C at 5 °C/min and was maintained isothermal for 10 min at 340 °C, while injector and detector temperatures were 320 and 340 °C, respectively. Carrier gas (H_2) flow was 11.13 ml/min. To form the flame, hydrogen gas flow, 40 ml/min, and air gas flow, 400 ml/min, were used.

2.2. Chemicals

D003 (batch 990702) was provided by CNIC (Havana, Cuba); all other chemicals were analytical reagent grade: hydrochloric acid (37%), methanol (Merck, Germany), toluene and chloroform (Riedel-de-Haën, Germany).

To prepare the stock solution 0.75 mg 1-tetracosanoic ($C_{24:0}$), 0.57 mg 1-pentacosanoic ($C_{25:0}$), 1.55 mg 1-hexacosanoic ($C_{26:0}$), 1.48 mg 1-heptacosanoic ($C_{27:0}$), 17.79 mg 1-octacosanoic ($C_{28:0}$), 0.93 mg 1-nonacosanoic ($C_{29:0}$), 10.00 mg 1-triacontanoic ($C_{30:0}$), and 0.60 mg 1-hentriacontanoic ($C_{31:0}$) acids; all $>99\%$ GC (Sigma, USA) were weighed into a 25 ml volumetric flask, the volume was completed with chloroform and it was mixed. 1-nonadecanoic acid ($C_{19:0}$, Sigma, USA), 1 mg/ml in chloroform, was used as internal standard. These solutions were found to be stable at least for 1 month, when stored at +10°C. The methylating solution (MSoln) was prepared with hydrochloric acid–methanol (5:95, v/v), it was weekly prepared and stored at + 10 °C. A 10 mg/ml solution of acacia gum (UCB, Belgium) in distilled water was used as vehicle.

2.3. Suspensions formulation

The test procedure was applied to D003 suspensions of different concentrations (10.00, 27.18, 50.08, 104.14, 160.49 and 251.79 mg/ml). In each case, the weighed quantity of D003 was placed in a 150-ml beaker and the vehicle was added drop by drop, with continuous stirring until the powder was moisturized. Then, the product was transferred to a 250 ml volumetric flask with continuous washing of the beaker and the volume was completed with the same vehicle. These solutions were found to be stable at least for 1 month, when stored at +10 °C.

2.4. Test procedure

From the suspensions of 27.18, 50.08, 104.14, 160.49 and 251.79 mg/ml the next volumes were transferred to 100 ml volumetric flasks (40.0, 20.0, 10.0, 6.0 and 4.0 ml respectively). The volumes were completed with water to form new suspensions of approximately 10 mg/ml. The suspension of 10 mg/ml was used as such. From these new suspensions 1 ml was transferred to a test tube with screw cap, 1 ml of the internal standard solution and 3 ml of chloroform were added and it was heated at 80 °C for 30 min with occasional stirring. This sample was transferred to a separator funnel and the organic phase was isolated. It was evaporated to dryness at 80 °C with a nitrogen flow. MSoln (1 ml) was added, the tube was closed and it was heated at 80 °C for 90 min. Afterwards, the content was evaporated to dryness at 80 °C with the help of a nitrogen flow; 1 ml of toluene was then added and vial was closed again and heated at 80 °C for 3 min. Portions of 1 μl were analyzed by GC.

The mass (mg) of each acid was obtained by the internal standard method [11], according to the following equation:

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

where f_i^m is the relative mass response factor for compound i.

In order to determine f_i^m, 1 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, 0.5 ml of the MSoln were added, the vial was sealed and the mixture was heated at 80 °C for 90 min. Content was evaporated to dryness, 0.25 ml of toluene were added and the mixture was heated at 80 °C for 3 min. Portions of 1 μl were analyzed by GC. This procedure was performed in triplicate, and f_i^m was calculated as follows:

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

Commercial standards of $C_{32:0}$, $C_{33:0}$, $C_{34:0}$, $C_{35:0}$, and $C_{36:0}$ are not available, then the f_i^m of $C_{30:0}$ was used for $C_{32:0}$, $C_{34:0}$ and $C_{36:0}$, and f_i^m of $C_{31:0}$ was used for $C_{33:0}$ and $C_{35:0}$. The content of D003 in these suspensions was calculated by the summation of the contents from $C_{24:0}$ to $C_{36:0}$. For obtaining the concentration of D003 in the original suspensions, the found content in the suspensions of approximately 25, 50, 100, 150 and 250 mg/ml were multiplied by the next dilution factors 2.5, 5, 10, 15 and 25, respectively.

2.5. Validation of test procedure

In order to know the specificity of the method, the chromatographic profiles of: suspension of 250 mg/ml, D003 active principle, blank of acacia gum solution, and a solution of the internal standard were studied.

Linearity of the method was assessed over a range 10–250 mg/ml, with this aim the six prepared suspensions were analyzed to determine the content of D003 ($n=5$). The regression line ($y=a+bx$) was calculated by the method of least squares based on the amount found (y) versus the amount added (x). Evaluation was made by linearity and proportionality test for $P=0.05$ [12]. Acceptance criteria were as follows: correlation coefficient (r) > 0.99; relative standard deviation of response factors (RSD_f) $\leq$ 5%, where response factor is defined as y/x; relative standard deviation of slope (RSD_b) $\leq$ 2%; and the zero value should be included in the confidence interval of the intercept (CI = Intercept $\pm$ $t \times$ SD). The accuracy was assessed for each suspension by a recovery study. Recovery was 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 t-test. The experimental value of t was calculated as follow:

$$t = \frac{|100 - \text{Recovery}|\sqrt{n}}{\text{RSD}}$$

where RSD is the relative standard deviation of the analyses.

The null hypothesis (the recovery is close 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.

Repeatability of the method was assessed for each suspension ($n=8$) and confidence limits: $\bar{x} \pm t \times \text{SD}$, were determined for $P=0.05$, where t is the value of Student distribution for $n-1$. The intermediate precision was assessed for suspensions with the lower, middle and upper concentrations (10.00, 50.08 and 251.79 mg/ml), which were analyzed by two technicians in different equipments ($n=8$). In the second equipment (GC14A, Shimadzu) there were changed the following conditions: it was used a DB5 wide-bore fused-silica capillary column (30 m, 0.53 mm id, 1.5 μm D_f; J&W Scientific, USA), operated from 250 to 320 °C at 5 °C/min, while injector and detector temperatures were 300 and 320 °C, respectively. Carrier gas (H_2) flow was 10.25 ml/min.

3. Results and discussion

3.1. Specificity

A good resolution between D003 components, and no chromatographic interference between the internal standard, D003, and other components of the suspensions was observed (Fig. 1). Then, this technique is specific for the quality control of these suspensions.

3.2. Linearity and accuracy of the method

The obtained regression line was $y=(1.00\pm0.01)x-(0.14\pm1.07)$. It was observed that r value (0.9998) is higher than the acceptation limit, indicating a positive correlation for a probability higher than 99.9%. The zero value was included in the confidence limits of the intercept, therefore there is no bias. The RSD_f (1.75%) and the RSD_b (0.39%) were lower than the acceptance criteria, indicating a good linearity.

The average recoveries for all suspensions were between 98.5 and 101.28% (Table 1). These recoveries and 100% value were not significantly different according to the calculated t values, which were lower than the tabulated t for $P=0.05$ (2.776). Then, it can be considered that this method is accurate. Experimental G (0.2811) was lower than the critical value too (0.4803), indicating that the original concentration of the suspension does not affect the dispersion of results.

Table 1
Results of the accuracy study

Real conc. (mg/ml)	Statistic	Obtained conc. (mg/ml)	Recovery (%)	t_{exp}
251.80	Mean	251.62	99.93	0.110
	SD	3.57	1.42	
	RSD (%)	1.42	1.42	
160.49	Mean	161.02	100.33	0.568
	SD	2.10	1.31	
	RSD (%)	1.30	1.30	
104.14	Mean	103.32	99.22	0.997
	SD	1.81	1.74	
	RSD (%)	1.75	1.75	
50.08	Mean	49.35	98.55	1.544
	SD	1.04	2.07	
	RSD (%)	2.10	2.10	
27.18	Mean	27.47	101.07	2.573
	SD	0.26	0.94	
	RSD (%)	0.93	0.93	
10.00	Mean	10.13	101.28	1.599
	SD	0.18	1.82	
	RSD (%)	1.79	1.79	

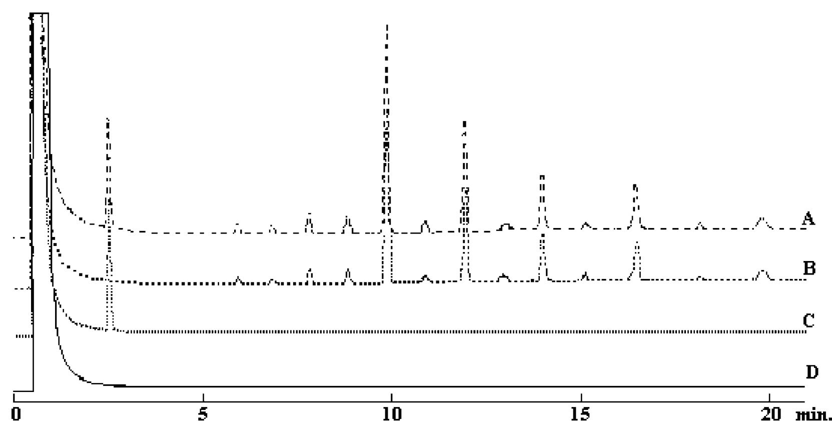

Fig. 1. Chromatograms of: (A) 250 mg/ml suspension of D003 with the internal standard; (B) sample of D003 (active principle); (C) internal standard; and (D) blank of acacia gum solution.

Table 2
Results of the repeatability ($n = 8$) and intermediate precision ($n = 16$) study

Real conc. (mg/ml)	Repeatability		Intermediate precision	
	Mean $\pm t \times$ SD (mg/ml)	RSD (%)	Mean $\pm t \times$ SD (mg/ml)	RSD (%)
251.80	253.12 ± 8.12	1.35	253.48 ± 6.46	1.20
160.49	161.80 ± 4.65	1.21		
104.14	103.46 ± 3.36	1.37		
50.08	49.34 ± 2.34	2.00	49.89 ± 2.25	2.10
27.18	27.39 ± 0.82	1.25		
10.00	10.07 ± 0.43	1.82	9.98 ± 0.37	1.76

t_{tab} (0.05; 7) = 2.37, t_{tab} (0.05; 15) = 2.12.

3.3. Precision

Repeatability was determined for each suspension, whereas for assaying the intermediate precision three suspensions were analyzed by two technicians in different equipments. It can be observed (Table 2) that in both series the RSDs relative standard deviations for the different suspensions were lower than the established acceptance criteria: 2.2% for suspensions with 250 and 150 mg/ml, 2.8% for suspensions from 100 to 25 mg/ml, and 4% for suspension with 10 mg/ml; therefore, it can be ensured that the method has a good precision [12,13].

4. Conclusions

The validated method gave successful results for the determination of D003 in aqueous suspensions containing from 20 to 200 mg/ml. This procedure can be used for routine analyses of these suspensions.

Acknowledgements

Thanks are extended to Haydeé García, Department of Pharmacology, Center of Natural Products, for her kind assistance with the preparation of the D003 suspensions.

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