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Original Article

Stability Study in Accelerated Conditions of Gentamicin-Glyceryl Monooleate-Water Based Gel Used in the Treatment of Chronic Osteomyelitis

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ABSTRACT

This work evaluated under accelerated conditions the stability of the biodegradable gentamicin-glyceryl monooleate water based-gel used in the treatment of chronic osteomyelitis. Three batches of 600.0 g of gentamicin -glyceryl monooleate -water based-gel (5/80/15% w/w) were manufactured. The samples were stored in a climatic test chamber for stability evaluation according to the standards published by the International Conference on Harmonisation and the World Health Oganization (25 ± 2°C / 60 ± 5%RH) for 6 months. Samples were evaluated at pre-determinate times 0, 3 and 6 months with regard to their physicochemical properties and *In Vitro* drug release characteristics. Gentamicin sulfate present in the freshly prepared gels was the same after three and six months of storage at 25°C / 60% RH. However the relative amount of gentamicin sulfate in the freshly prepared gel that was about 110% decreased by about 11% after six months of storage. The release profiles of the gels remained similar despite a partial hydrolysis of glyceryl monooleate and an increase in the residual amount of water and in the viscosity.

Keywords: Stability, Gentamicin, Glyceryl monooleate, Sustained-release, Chronic osteomyelitis.

INTRODUCTION

The biodegradable gentamicin-glyceryl monooleate (GMO) water-based gel previously developed by Ouedraogo and co-workers[1] showed suitable physicochemical and clinical characteristics for being used as an alternative in the treatment of chronic osteomyelitis. Indeed, the developed gel was homogeneous, transparent, semisolid, bioadhesive and was characterized by suitable rheological properties which allow it to be implanted inside a pathologic bone without any further manipulation. Moreover, it was able to swell upon contact with aqueous fluids, maintaining an *In Vitro* sustained-release of the drug for more than 20 days in phosphate buffer (pH 7.0). Such physicochemical properties allowed its application in the bone cavity after surgical curettage and also avoided the formation of hematomas.[1] Moreover, *in vivo* biocompatibility and toxicity assessment[2] and a phase II clinical trial performed on 19 patients demonstrated that the developed gel presented a suitable safety profile and efficacy. Indeed, eighteen patients recovered from chronic osteomyelitis without adverse events after follow-up ranging from 2 to 12 months.[3] Due to these promising results, this gel has potential to be used to improve the therapeutic management of chronic osteomyelitis, which still represents 5.3% of causes of hospitalization in the traumatology unit of the Centre Hospitalier Universitaire Yalgado Ouedraogo / Burkina Faso.[4]

However, one of the most persistent challenges in using these gels is to assure the stability of the gel during storage, especially in conditions which may be found in hot, dry climatic zones. Indeed, the potential instability issues may include modifications of the rheological and sustained-release properties, the apparition of impurities or microbiological growth.

The standards published by the International Conference on Harmonisation (ICH) and the World Health Organization (WHO) recommend carrying out stability studies to evaluate the stability of the finished pharmaceutical product under various environmental factors such as temperature, humidity and light. These tests aim to establish the most suitable storage conditions, the re-test periods and the maximal duration of storage in predetermined conditions. For a pharmaceutical product which is intended for storage at 2-8°C, these stability tests must be performed under the conditions of accelerated degradation (25 ± 2°C / 60 ± 5% RH for 6 months) and/or in long-term storage conditions (5 ± 3°C for 12 months). Accelerated degradation should be performed to increase possible chemical degradation and physical alteration of both the drug and the gel matrix.[5,6]

Therefore, the aim of this study was to evaluate the effect of accelerated conditions on the physicochemical properties of the developed gentamicin-monoolein water-based gel.

MATERIALS AND METHODS

Materials

Gentamicin sulfate was purchased from Yantsai Pharma (China). GMO (Rylo MG PHARMA 20) was used as an *in situ* gelling agent and was purchased from Danisco (Denmark). Ethanol 96% (w/w) denaturized with isopropanol was used as a solvent for dissolving GMO and was supplied by Fagron (Belgium). Distilled water was freshly prepared in the laboratory and sterilized by autoclaving at 121°C for 20 min. Amber borosilicate vials of 60 mL with wide mouth and black screw polypropylene caps (38 mm of diameter) were used as packaging

for the gels and were purchased from Fischer Scientific (France).

Manufacturing of the gels

Three batches of 600.0 g of gentamicin-GMO-water-based gel (5/80/15% w/w), referred to as Lot 1, Lot 2 and Lot 3, were manufactured in aseptic conditions in a sterile room. All the materials used for the manufacturing of these gels were previously sterilized by autoclaving at 121°C for 20 min or using a validated procedure consisting of heating the materials at 180°C for 1 hour in an oven.

All the gels were prepared at low pressure. GMO was gently melted in a thermostated bath at 50°C and manually mixed with 150.0 ml of denaturized ethanol until homogeneity. On the other hand, gentamicin sulfate was dispersed in 150.0 ml of distilled water. An excess of water (60.0 ml) was added to facilitate the subsequent elimination of ethanol. Indeed, the water-ethanol mixture formed an azeotropic mixture that allowed both ethanol and water to be evaporated at similar temperatures. The dispersion was magnetically stirred to get a clear solution of gentamicin sulfate. The dissolved active drug and the mixture of GMO and ethanol were separately filtered through a 0.22 µm cellulosic membrane (VWR, Belgium). The filtrate of the GMO-ethanol mixture was then transferred into a rotary evaporator flask and the filtered solution of gentamicin sulfate was slowly added under manual stirring. The excess of water and ethanol was removed from the mixture under vacuum in a rotary evaporator (Büchi Rotavapor R-205 LABORTECHNIK, Switzerland) at 50°C for 140 min. The rotation per minute ranged between 150 and 235 rpm and the pressure was set from 300 to 80 bars until the desired quantity of final product (600.0 g) was obtained. The products were immediately conditioned in brown glass bottles at a unit dose of 25.0 g and stored at 2-8°C.

Study protocol

An accelerated stability study was conducted on three batches of the product. The samples were stored in a climatic test chamber for stability evaluation (Pharm 600 WEISS, Germany) within the ICH and the WHO parameters recommended for this type of preparation (25 ± 2°C / 60 ± 5%RH) for 6 months. Samples were evaluated at pre-determinate times (0 (M_0), 3 (M_3) and 6 (M_6) months).

Macroscopic observation

The gels were visually observed to evaluate their color and turbidity.

Qualitative and quantitative evaluation

The effective gentamicin content in each GMO water-based gel was determined at 0, 3 and 6 months of storage, in order to appreciate its stability. Gentamicin sulfate was extracted from the gel using a liquid-liquid extraction method.

Approximately 1500.0 mg gel containing 75.0 mg active ingredient was dissolved in 40.0 ml chloroform (Sigma-Aldrich, Germany). Gentamicin sulfate was extracted 3 times with 5.0 ml distilled water to obtain a concentration of 5 mg/ml before being filtered through a 0.45 µm cellulosic membrane (VWR, Belgium). The identification and the determination of the gentamicin sulfate were then performed on the extracted solutions.

Gentamicin sulfate was identified by thin layer chromatography (TLC), as recommended by the European Pharmacopoeia 7th Ed.[7] A thin plate of silica gel G60 F254 (Merck, Germany) was used as the stationary phase. The mobile phase consisted of the lower layer of a mixture of equal volumes of concentrated ammonia, methanol and methylene chloride. Gentamicin

sulfate Chemical reference substance (CRS) was used as a reference. The development was done over 2/3 of the plate and the gentamicin was visualized after spraying a solution of ninhydrin (1.0 g ninhydrin in 50 ml ethanol (96% v/v) and 10 ml glacial acetic acid). Immediately after spraying, the samples were heated at 110°C for 5 min to be revealed.

Gentamicin sulfate content was determined by UV spectrophotometry. However, as gentamicin does not absorb light in the UV domain, a derivatization step was previously performed at pH 10.4. The derivatization reagent was prepared as follows: 400.0 mg orthophthalaldehyde (Sigma-Aldrich, Germany) was dissolved in 2.0 ml methanol; 38.0 ml borate buffer (pH 10.4) and 0.8 ml thioglycolic acid (Sigma-Aldrich, Germany) were added and the pH was adjusted to 10.4 with a solution of potassium hydroxide at 45% (w/v).

The derivatization reaction was done by adding 10.0 ml of the filtered extract to a 25.0 ml volumetric flask containing 800.0 µL derivatization reagent. The volume was then completed to 25.0 ml with methanol. The mixture was vortexed and heated in a water bath at 60°C for 15 min. Finally, the samples were cooled at room temperature for 25 min.

Absorbance of gentamicin was measured using a HP 8453 UV-Visible spectrophotometer (Hewlett Packard, Germany) with 1 cm quartz cells at 325 nm.

Residual water

The residual amount of solvent remaining in the gels after their preparation was determined in triplicate using a moisture analyzer equipped with a halogen heating system (MB35 Halogen Ohaus, Switzerland). An exact amount of about 1.000 g gel was heated at 100 °C for 10 min and the loss of solvent (e.g. water/ethanol) was continuously recorded.

Rheological Studies

Rheological properties of the gels were evaluated in triplicate at 37.0 ± 0.1°C using a Brookfield viscometer LVDV-II + (Brookfield Engineering Laboratories, Inc., USA) mounted with a small sample adapter Spindle SC-25. The viscosity (mPa.s) was recorded from 2 to 75 rpm after stabilization of values. The rheological properties of the developed gels were evaluated by drawing the rheograms representing the evolution of the viscosity as a function of the shear rates (s-1).

Evaluation of the free fatty acid content

The free fatty acids contained in the freshly prepared gels and in those subjected to accelerated degradation were extracted with hexane. They were then evaporated under a flow of nitrogen, esterified with 0.5 N chlorhydric acid in methanol (Supelco, USA), and identified and assayed in triplicate using a validated gas chromatography method (Carlo Erba Instruments AUTO/HRGC/MS, Italy). Esterified fatty acids (e.g. methyl stearate, methyl palmitate, methyl linoleate, and methyl oleate) and methyl cis-10-heptadecenoate (Sigma-Aldrich, Germany) were used as the external and internal standards, respectively. The GC column (Chrompack N.V., Belgium) was a WCOT 25 m × 0.32 ID coating FFAP CB (with 0.32-µm film thickness) column, the injection volume was set at 1 µl, helium was used as the carrier and the pressure was maintained at 0.59 bars. The working temperature was set as follows: 80°C was maintained for 1min, then the temperature was increased to 230°C at a heating rate of 8°C/min.

In Vitro dissolution test

In Vitro dissolution studies were conducted in triplicate at 37.0 ± 0.5°C, using the USP24 (2 000) no. 2 (paddle) apparatus (60 rpm). The dissolution media consisted of a phosphate buffer (pH 7.0) [50 mM dipotassium hydrogen phosphate and 50 mM

glacial acetic acid) supplemented with 0.05% polysorbate 20 (Sigma Aldrich, USA) and 0.02% (w/v) sodium azide (Sigma Aldrich, USA)]. Topical dissolution cells (Distek, Netherlands) were filled with a weighed amount of about 1.5 g gel containing about 75.0 mg gentamicin sulfate before being placed into the dissolution vessels. The dissolution baths were filled with 500.0 ml buffered medium. Then 15 mL dissolution medium were respectively withdrawn at 3, 6, 24, 96, 168, 240, 336 and 480 hours. To preserve sink conditions, fresh dissolution medium maintained at 37°C was added to the dissolution bath immediately after sampling. The samples were filtered through a 0.45 µm cellulosic membrane and measured at 325 nm to evaluate the cumulative amounts of gentamicin that were released. At the end of the dissolution test, the residual amounts of gentamicin sulfate that still remained in the gels were extracted using the previously-described extraction method before being assayed.

Statistical analysis

Data were expressed as a mean ± Standard Deviation (m ± SD).

As recommended in the FDA Guidances for Industry, the similarity factor f_2 was used to determine the similarity of release profiles.[8,-10] The release profiles were compared under similar conditions of dissolution (3, 6, 24, 96, 168, 240, 336 and 480 hours). As indicated by Shah and coworkers, the similarity factor value has to be higher than 50 for two dissolution profiles to be similar. Moreover, the Kruskal-Wallis statistic test was used to compare two and more than two means, respectively. Variation was statistically significant when the p value was lower than 0.05. Statistical analysis was carried out using Graph Pad PRISM version 5.01 software (Graph Pad software Inc., USA).

RESULTS AND DISCUSSION

Immediately after preparation, the gels had a homogeneous, transparent, semi-solid appearance that was characterized by a pale yellow tone. After 6 months at 25°C, the color became darker, rising from straw yellow to deep yellow straw. This phenomenon could possibly result from an interaction between gentamicin sulfate and GMO and/or a chemical degradation of GMO or gentamicin sulfate. It should be noticed that a similar observation has been reported with gentamicin sulfate solubilized in a 5% (w/w) dextrose solution.[11] In this study, the evolution of the color should not be attributed to an interaction between the GMO and the active drug. Indeed, it has previously been demonstrated that samples of gels stored at 2-8°C for 10 months conserved the same DSC thermal profile analysis[1] so it was concluded that there was no interaction between the different components of the gel.

Physicochemical properties

The identification of the incorporated active drug was performed in triplicate by TLC after extraction. The samples revealed 3 spots corresponding to the three major components of gentamicin (Gentamicins C1, C1a and C2). Indeed, gentamicin is not a single molecule but a complex of three major and several minor components. The 3 spots revealed were similar in position, color and size to the three main spots obtained with the raw gentamicin sulfate used as the reference (Fig.1). The data matched with the recommendation of the European Pharmacopoeia 7th Ed.[7] This result attested to the presence of gentamicin sulfate in the freshly prepared gels and was the same after three and six months of storage at 25°C / 60% RH.

The relative amount of gentamicin sulfate in the freshly prepared gel determined by UV spectrophotometry was about 110%

(Table 1). After 6 months of storage at 25°C/60% RH, the relative amount had decreased to 95.5 ± 1.3 (Lot 1), 101.02 ± 0.3 (Lot 2) and 99.8 ± 0.5 (Lot 3) % (Table 1). A decrease in the relative amount of gentamicin sulfate from 103.8 ± 0.4 to 100.6 ± 0.9 (% w/w) was also observed with gels stored for ten months at 2-8°C.[1] The decrease in the content of gentamicin sulfate resulted from a degradation. However, the assay performed by UV spectrophotometry was not able to identify or quantify the three components of gentamicin or their potential degradation products.[12] These products might be sisomicin, deoxystreptamine or garamine.[11- 14] Furthermore, the degradation products did not seem to be present in significant concentrations since the identification of gentamicin sulfate by TLC showed that the spots of gentamicin contained in the gel were similar to those obtained with gentamicin sulfate SCR.[7] Moreover, the relative amounts of gentamicin sulfate still present in the gels after the stability study were in the range recommended by the USP NF32 (90-135%).[15]

The residual amount of water in the freshly prepared gels was about 15% (w/w) (Table 1). After 6 months of storage at 25°C, 60% RH, the residual amount slightly increased to 15.0 ± 0.2 (Lot 1), 17.0 ± 0.3 (Lot 2) and 17.1 ± 0.4 (Lot 3). As the packaging container was not waterproof, the gels could absorb external water. However, the increase in the residual amount of water was not statistically significant (Table 1, Kruskal-Wallis statistic test, $P<0.05$). The taking up of water of by the gels during the study shows that the packaging was not suitable for this kind of preparation and exposes the gel to risk of microbial contamination. Therefore, the ambers borosilicate vials crimp with stopper of penicillin type and the inviolable caps should be used to avoid the risk of microbial contamination because the gel must remain sterile for the specified period of use.[16]

The viscosity of the gels (mPa.s) measured at 0.44 s^{-1} was about 30 000 at M_0. After 6 months of storage at 25°C/60% RH, the viscosity increased (Table 1). The viscosity of the gels increased with an increased amount of residual water (Table 1). This observation may be attributed to the GMO, which became more viscous when the water content increased.[17,18]

However, despite an increase in their viscosities, all the gels were characterized by a non-Newtonian pseudoplastic rheological behavior (Fig. 2). This behavior was similar to that observed on a biphasic mixture of GMO and water.[1,19,20]

At M_0, the free fatty acids that were mainly present in the gels were palmitic, stearic, oleic and linoleic acids. At M_3 and M_6, the qualitative composition remained similar but their amounts increased in different proportions (Table 1). The amount of oleic acid was the largest. Its increase may be due to a partial hydrolysis of GMO in glycerol and oleic acid, which may a consequence of its interaction with the residual amount of water present in the gel. Indeed, GMO is a mixture of glycerides of oleic acid and other fatty acids, consisting mainly of the monooleate.[21] A similar increase had already been observed in the gels after storage at 2-8°C for ten months.[1] However, this increase was not significant (Table 1, Kruskal-Wallis statistic test, $P < 0.05$).

Dissolution test

The release of gentamicin sulfate was sustained for 480 hours. The burst effect observed at the early stage of the test was moderate (Fig. 3), as was previously reported by other authors with gentamicin-loaded GMO-based gels.[1,20] The dissolution profiles remained similar. Indeed, all similarity factor values were higher than 50 (Table 1). Thus, despite the increase of the viscosity of gels during the storage, the sustained-release

properties were not altered. This may be due to the cubic phase of the gel as the monoolein water-based gel remains stable in the presence of excess of water. However, the increase of water increases hydrophilic of the matrix which can increase the drug release despite an increase in matrix viscosity.[18]

CONCLUSION

Three batches of biodegradable gels made of Gentamicin-GMO-water (5/80/15% w/w) and manufactured in aseptic conditions were submitted to accelerated aging according to the requirements of ICH and WHO. The content of Gentamicin sulfate decreased by about 11%. The release profiles of the gels remained similar despite a partial hydrolysis of GMO and an increase in the residual amount of water and in the viscosity.

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This paper is dedicated to the memory of Moustapha Ouedraogo.

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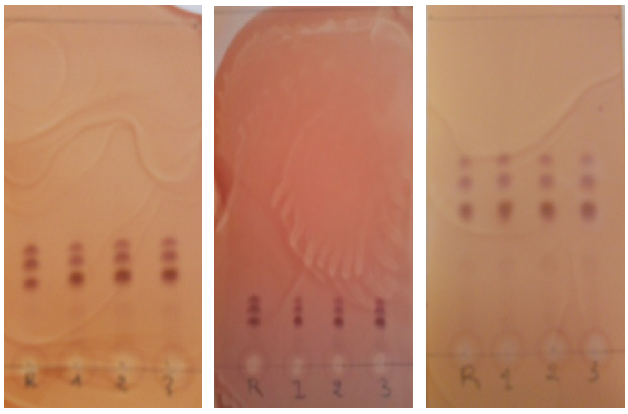

M_0 M_3 M_6

Figure 1. Chromatographic pictures of Gentamicin sulfate CRS® (R), gentamicin-GMO-water-based gel (5/80/15% w/w) batches 1 (Lot 1), 2 (Lot 2) and 3 (Lot 3), at 0 (M_0), 3 (M_3) and 6 (M_6) months of storage à 25 ± 2°C / 60 ± 5% RH

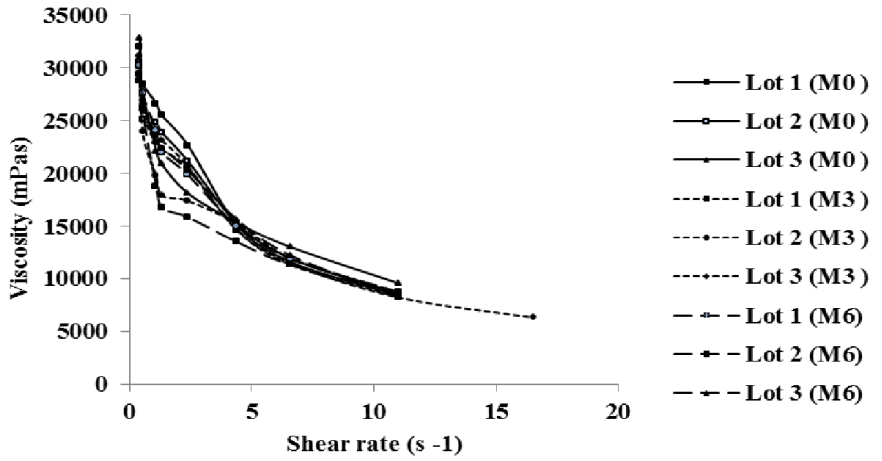

Figure 2. Rheograms of the three batches (Lot1, Lot2 and Lot 3) of the gentamicin-GMO- water-based gel (5/80/15% w/w) at 37.0 ± 0.1°C, measured with a SC4-25 spindle (mean ± SD, n = 3) after 0 (M_0), 3 (M_3) and 6 (M_6) months of storage à 25 ± 2°C / 60 ± 5% RH

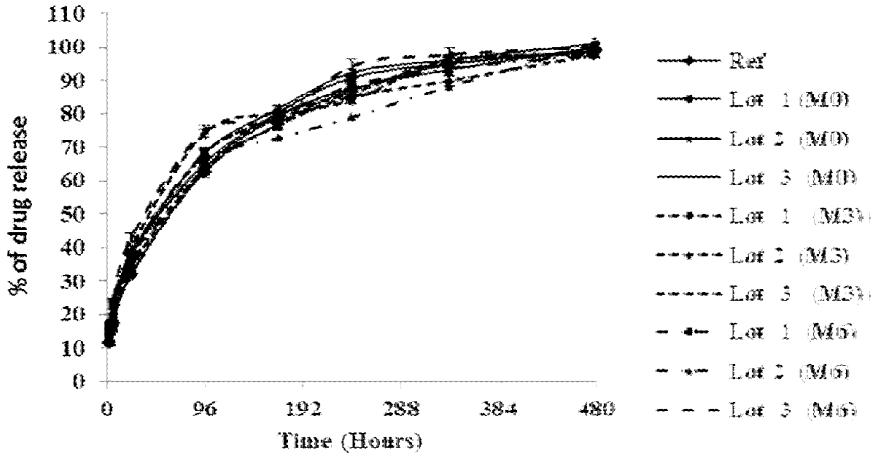

Figure 3. Dissolution profiles of gentamicin contained in the three batches (Lot1, Lot 2 and Lot 3) of the gentamicin-GMO-water-based gel (5/80/15% w/w), in phosphate buffer pH 7 (mean ± SD, n = 3) after 0 (M_0), 3 (M_3) and 6 (M_6) months of storage à 25 ± 2°C / 60 ± 5% RH

Table 1. Evolution of the physicochemical characteristics of the three batches (Lot 1, Lot 2 and Lot 3) of the gentamicin-GMO-water-based gel (5/80/15% w/w), after 0 (M_0), 3 (M_3) and 6 (M_6) months of storage à 25 ± 2°C / 60 ± 5% RH

	M_0			M_3			M_6		
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
Gentamicin Sulfate Content (% w/w, m ± SD, n = 3)	108.2 ± 0.7	110.8 ± 1.2	111.1 ± 0.9	98.5 ± 0.1	103.8 ± 0.2	104.2 ± 0.1	95.5 ± 1.3	99.3 ± 0,3	99.8 ± 0.5
Residuel water amounts (% w/w, m ± SD, n = 3)	14.0 ± 0.5	15.4 ± 0.8	15.3 ± 0.6	14.15 ± 0.9	16.4 ± 0.9	16.5 ± 0.3	15.0 ± 0.2	17.0 ± 0.3	17.1 ± 0.4
Viscosity s) (mPa.s, m ± SD, n=3) at Shear rate 0.44 $^{-1}$ s^{-1}	28781 ± 874	30562 ±1016	30141 ± 711	29433 ± 1364	31066 ± 867	31213 ± 884	30254 ± 541	31983 ± 533	32804 ± 256
Free fatty acid content (% w/w, mean ± SD, n = 3)									
Oleic acid	5.27 ± 0.18	4.93 ± 0.24	4.91 ± 0.26	5.35 ± 0.14	5.25 ± 0.38	5.16 ± 0.07	5.62 ± 0.23	5.38 ± 0.05	5.36 ± 0.12
Palmitic acid	0.26 ± 0.04	0.27 ± 0.03	0.29 ± 0.02	0.28 ± 0.01	0.30 ± 0.03	0.28 ± 0.01	0.29 ± 0.01	0.31 ± 0.02	0.33 ± 0.01
Stearic acid	0.18 ± 0.03	0.18 ± 0.04	0.17 ± 0.03	0.17 ± 0.01	0.21 ± 0.04	0.20 ± 0.00	0.19 ± 0.01	0.19 ± 0.00	0.20 ± 0.00
Linoleic acid	0.49 ± 0.02	0.46 ± 0.03	0.45 ± 0.02	0.50 ± 0.01	0.48 ± 0.03	0.50 ± 0.00	0.56 ± 0.02	0.50 ± 0.00	0.51 ± 0.01
F_2	76.22	84.72	69.68	75.91	80.57	64.94	69.3	66.01	58.03