

CHOLESTEROL, α -TOCOPHEROL, AND RETINOID CONCENTRATIONS IN SILICONE OIL USED AS A VITREOUS SUBSTITUTE

CONCENTRACIONES DE COLESTEROL, α -TOCOFEROL Y RETINOIDES EN ACEITE DE SILICONA TRAS SU UTILIZACIÓN COMO SUSTITUTIVO VÍTREO

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ABSTRACT

Objective: To verify the existence of organic lipophylic compounds in silicone oil extracted from human eyes following its use for previous retinal detachment, and to determine the intraocular permanence time of these substances in the oil.

Methods: Concentrations of retinoic acid, retinol, retinal, cholesterol and α -tocopherol were detected by HPLC in 23 samples of silicone oil extracted from patients with complicated retinal detachments. The time interval between the time of injection of the silicone oil and the subsequent assessment varied from 3 to 50 months (the permanence time).

Results: All tested compounds were found in the samples, but these were most commonly cholesterol and less frequently α -tocopherol. There was an inverse relationship between retinoic acid concentration and age ($p=0.023$), and a direct relationship

RESUMEN

Objetivo: Determinar si existen concentraciones de compuestos lipofílicos de origen orgánico en muestras de aceite de silicona extraídas de ojos humanos y comprobar si existe alguna relación con el tiempo de permanencia intraocular del aceite.

Métodos: Por cromatografía líquida de alta resolución (HPLC) se han determinado las concentraciones de ácido retinoico, retinol, retinal, colesterol y α -tocoferol en 23 muestras de aceite de silicona de 1.000 cs, extraídas de pacientes con desprendimientos complejos de retina con tiempos de permanencia intraocular variables (3 a 50 meses).

Resultados: Se han encontrado concentraciones de todos los compuestos, sobre todo de colesterol, y en menor medida de α -tocoferol. Se ha observado una correlación inversa de la edad y las concentraciones de ácido retinoico ($p=0,023$). Y una correlación

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between cholesterol concentration and permanence time ($p=0.0008$) at least up to 20 months.

Conclusions: These findings confirm that silicone oil is not an inert substance but is capable of extracting lipophilic compounds from the intraocular tissues. There is a clear linear elevation of cholesterol levels with increased intraocular permanence time. This finding could be used to further establish a safe permanence time for intraocular silicone oil used in ophthalmologic surgery. More studies with larger samples are warranted to evaluate this further (*Arch Soc Esp Oftalmol* 2006; 81: 13-20).

Key words: Retinal, retinol, retinoic acid, cholesterol, α -tocopherol, silicone oil, vitreous substitute.

directa entre el tiempo de permanencia intraocular del aceite y los niveles de colesterol ($p=0,0008$) que se mantiene hasta los 20 meses.

Conclusiones: Se confirma que el aceite de silicona no es una sustancia tan inerte ya que es capaz de disolver compuestos lipídicos procedentes de tejidos intraoculares. Existe una elevación lineal de los niveles de colesterol que podría utilizarse como orientación para decidir cuándo extraer el aceite de silicona, aunque hacen falta estudios con series más grandes.

Palabras clave: Retinal, retinol, ácido retinoico, colesterol, α -tocoferol, aceite de silicona, sustituto vítreo.

INTRODUCTION

Silicone oil (SiO) is a term utilized to designate any of the hydrophobic polymeric compounds based on the chemistry of siloxane which are utilized as vitreous substitutes since the Sixties (1).

Although these substances have been considered to be relatively inert, a number of adverse effects have been published throughout these years with corresponding histopathological correlations (2). However, there are studies which deny this toxicity or attribute it to circumstances not directly related to the use of SiO. In any case, it is not easy to find alternatives for this long-term vitreous substitute, and to date SiO continues to be considered as the best option for repairing complex retina detachments.

To improve the biocompatibility of SiO, highly purified oils are utilized in order to reduce as much as possible the presence of the so-called LMWCs (Low Molecular Weight Components) (3-6).

However, even though at present SiO is utilized with the highest possible degree of purity, complications continue to arise the pathology of which is not yet fully understood, such as band keratopathy, chronic hypotonia or intraocular inflammations (2).

Some authors have attributed said complications to purely mechanical effects of the oil (7), which is sometimes not easy to admit on the basis of the anatomic and pathological findings in human eyes (6).

One aspect which has not been given due attention by researchers is the lipophilic nature of these compounds which can cause damage due to their

ability to dissolve intraocular lipids. In theory and depending of their solubilization parameters, phospholipids or proteins would not dissolve in SiO whereas cholesterol and its esters would, together with liposoluble vitamins such as vitamin A. This possible pathogenic capacity was explored by Miguel F. Refojo in 1988 (8) in experimental research with rabbits and analysing the lipids concentration in SiO samples which had remained in two human eyes for 51 and 96 weeks respectively. The result shows the presence of measurable amounts of cholesterol and retinol in the oil (silicone and fluor-silicone), which was interpreted as a proof that these substances are not as inert as had been believed. The same author carried out similar experiments with a silicone-fluorsilicone copolymer called SiFo (9,10), and also found retinol and cholesterol.

The purpose of this research is to analyze the concentrations of cholesterol, α -tocoferol and retinoids (retinoic acid, retinol, retinal) in samples obtained from patients with whom it was necessary to utilize intraocular SiO extracted after a given period of permanency in the eye, when the surgeon deduced that the time was right. An additional purpose is to try to establish the possible correlations of said concentrations with the time of permanency within the eye.

SUBJECTS, MATERIAL AND METHODS

After obtaining the approval of the research committee of our hospital (Valladolid University Clini-

cal Hospital), 23 highly purified SiO samples were extracted from 1.000 cs of patients in whom it was necessary to utilise this substance to repair complex retina detachments, complicated mainly by proliferating vitreous retinopathy (VRP). The procedures were carried out as per the 1983 version of the Helsinki Declaration.

The characteristics of the sample are shown in Table I.

Analytical Methods

The SiO was extracted from the patients eye with a glass aspiration syringe, deposited in a flask of the same material, sealed with a Teflon top and wrapped in aluminium paper to be sent to the Analytical Chemistry Department without any further manipulation. The samples remained in a refrigerator and were isolated from light before the analysis.

In order to determine simultaneously the existence of retinoic acid, retinol, retinal, cholesterol and α -tocopherol, high-resolution liquid chromatography (HPLC) was utilised (Hewlett-Packard HP-1050, Waldbronn, Germany).

For separation, a C8 Zor-bax column was used

(Jones Chromatography, Lakewood, Colorado, USA) (15 x 0.46 cm) with a 5 mm particle diameter. To carry out the simultaneous analysis of the five compounds with their respective internal patterns, a mobile phase change was carried out. Acetonitrile was utilized: 0.2 M ammonia acetate (75:25, v/v) with a flow of 2 ml min⁻¹ during 10 minutes. Subsequently, by means of a one-minute linear gradient, a mixture of methanol and water (95:5, v/v) was flushed at a flow rate of 1.5 ml min⁻¹ up to the end of the separation. To increase the sensitivity of the analysis, we also made a change in the detection wavelength, utilizing 350 nm for the first 14 minutes and then 210 nm up to the end of the analysis.

Figure 1 shows the chromatogram for a 20 ml injection of a mixture of patterns in the above-mentioned conditions, and figure 2 shows the chromatogram corresponding to the sample of patients (No 13 of the series). The retention times were highly reproducible and the variation coefficients were comprised between 0,25% for α -tocopherol and 2.16% for retinoic acid.

Preparation of the samples

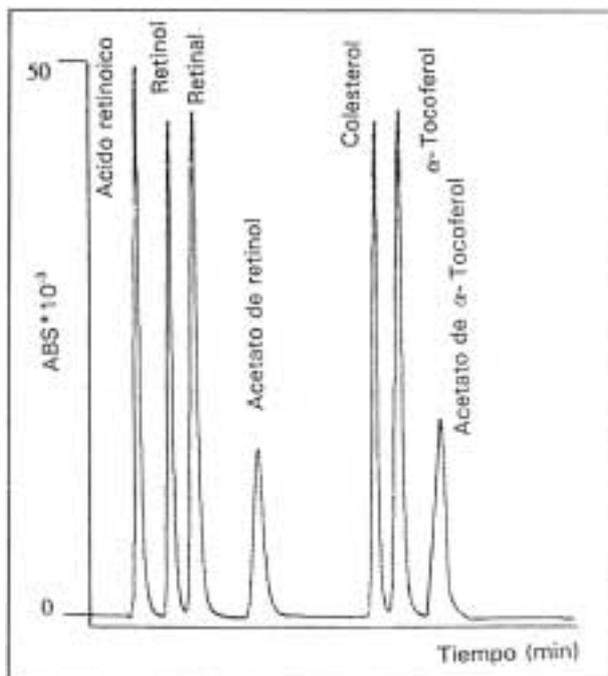
Prior to extraction, water was totally removed with methylene chloride (2 ml per gram of silicone). Subsequently, anhydrous sodium sulphate was added to complete the removal. Thereafter, the sample was passed through 0.45 mm filters to eliminate sodium sulphate and helium was bubbled over the liquid (raised up to 50°C) to eliminate the solvent completely.

In order to isolate the target compounds from the silicone oil matrix, solid phase extraction with silicon cartridges was utilized (Si-Bond Elut, Varian) with 1g filling. To 1g of solid sample were added 25 μ l of retinole acetate and tocopherol acetate, utilized as internal patterns for quantification, with a concentration of 0.1 mg ml⁻¹, 25 μ l of BHT of 1 mg ml⁻¹, and 1 ml of n-hexane. Subsequently, the resulting solution was passed through a previously activated cartridge with the passage of 5ml of n-hexane, and the compounds were eluted with 0.5ml of methanol, filtered and injected in the chromatograph.

The treatments described above provided high rates of recovery (exceeding 93%) for all compounds, as well as good reproducibility.

Table I. Characteristics of some patients and Time of permanency of SiO in the eye

Sample	Age	Gender	Time of permanency (months)
1	66	V	2.0
2	58	V	3.0
3	25	V	4.0
4	23	Y	5.4
5	50	V	5.8
6	61	V	7.2
7	45	H	7.4
8	30	V	11.5
9	64	V	11.7
10	54	V	12.5
11	45	V	13.0
12	59	H	13.1
13	67	V	13.3
14	14	H	13.5
15	47	V	15.0
16	40	H	15.0
17	16	V	15.7
18	41	V	17.5
19	28	H	17.9
20	70	H	19.6
21	59	V	19.8
22	14	H	20.2
23	56	V	47.9



tr (Acido Retinico):	2,26 min
tr (Retinol):	4,40 min
tr (Retinal):	5,93 min
tr (Acetato de retinol):	9,85 min
tr (Colesterol):	17, 03 min
tr (α-Tocoferol):	18,24 min
tr (Acetato de α-Tocoferol):	21,38 min

Fig. 1: Chromatogram corresponding to the injection of a mixture of patterns.

Reactants

All utilized solvents were of HPLC quality. Acetonitrile, methanol, n-hexane and n-propanole were supplied by Scharlau (Barcelona, Spain). Methylene chloride was supplied by Merck (Darmstadt, Germany). Ammonium acetate, sodium acetate, mono-based ammonia phosphate, anhydrous sodium sulphate were of analytical grade and supplied by Merck (Darmstadt, Germany).

The retinoic acid, retinole, retinal, α-tocopherol, cholesterol, retinole acetate, α-tocopherol acetate, utilized as internal patterns and butylhydroxitoluene (BHT) were supplied by Sigma-Aldrich (Madrid, Spain). BHT was utilised as antioxidant

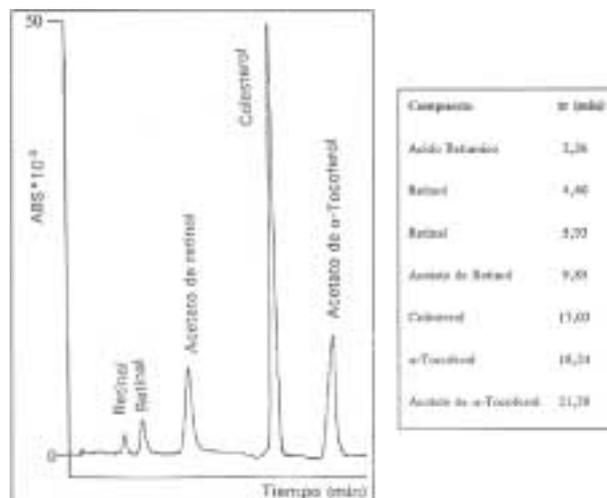


Fig. 2: Chromatogram corresponding to the injection of a sample extracted from the eye of a patients (sample 13).

after it was established with specific experiments that the utilization of 50 ppm of this compound allows the preservation of patterns with an error below 3% for a 60 day period.

Calibration

Individual dissolutions of each compound in n-hexane were prepared and utilized as a basis for preparing a pattern mixture of known concentration in each target compound.

For the quantification of the compounds, the internal pattern method was utilised. The calibration of the compounds was made by adding different volumes of the pattern mixture to 1g of purified silicone and subjecting them to the same treatment as the samples to be analysed. The calibration curves were obtained by repeated injection of a fixed volume of 20 µl in a concentration range of up to 50 mg l⁻¹, which proved to be linear for all compounds with low detection and quantification limits being obtained.

By means of regression analysis, the influence of the gender, age and permanency time of patients on the concentration of the different compounds was studied.

The statistical programme utilized is Statgraphics version Plus 5.1 (Manugistics, Inc, Dallas, EEUU).

RESULTS

The concentration of the different lipid compounds are shown in table II. In some cases values were not recorded in the table because the concentrations were below the detection limit. This was particularly marked in the case of α -tocopherol, where concentrations were detected in only seven of the 23 samples.

No significant differences were observed in what concerns gender in relationship to the different compounds. As regards the age of patients, significant differences were found only in the retinoic acid concentrations, which decreased with the increase of age ($p = 0.023$) (fig. 3). As regards the concentrations depending on the permanency time, significant differences were found only in cholesterol ($p = 0.0008$) (fig. 4), which raised gradually up to 20 months.

DISCUSSION

The lipid compounds studied in this research were selected on the basis of the indications of the initial work by Refojo (8) and on the solubility

parameters of lipophilic substances present in the retina and therefore presumed targets for being dissolved by SiO during its permanency in the eye. Retinoids (retinol, retinal and retinoic acid) are found in vertebrates mainly in the retina pigmentary epithelium and in light receptors (11). The role of retinol in the visual cycle is well known, but it must be taken into account that retinol, in the form of retinal 11cis, is found in the chromophore and that,

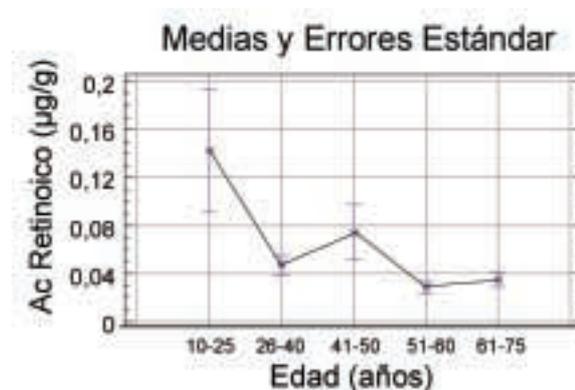


Fig. 3: Retinoic acid concentrations for different patients age groups. A significant reverse relationship was found $p=0.023$.

Table II. Concentrations of different compounds, in $\mu\text{g/g}$

Sample	Retinoic Acid	Retinol	Retinal	Cholesterol	Tocopherol
1	0.02	—	0.07	7.67	—
2	0.03	0.01	0.11	9.89	—
3	0.06	3.78	1.59	36.13	—
4	0.13	—	0.03	5.54	—
5	0.03	0.16	0.02	33.96	0.08
6	0.04	1.64	0.32	49.19	—
7	0.03	0.17	0.09	23.39	—
8	0.03	1.43	—	49.05	—
9	0.03	0.07	—	38.24	—
10	0.02	0.08	0.02	53.78	0.11
11	0.04	0.12	0.04	28.69	0.29
12	0.02	0.12	0.07	24.57	—
13	—	0.65	1.07	87.22	—
14	0.13	0.86	2.74	90.29	—
15	0.08	—	0.05	69.30	0.03
16	0.06	0.03	0.46	69.48	0.77
17	0.03	0.17	0.14	61.98	0.12
18	0.16	0.42	0.35	98.88	—
19	0.05	—	0.01	58.40	0.25
20	0.05	1.09	0.80	94.21	—
21	0.05	0.14	0.12	30.96	—
22	0.28	0.99	1.53	33.13	—
23	0.02	0.12	0.12	61.15	—

—: amounts below detection limit.



Fig. 4: Cholesterol concentrations based on the intraocular permanency time of silicone oil. A statistically significant relationship was found, $p=0.0008$.

after its transformation to «all-trans retinol», it is transported to the pigmentary epithelium of the retina where the regeneration process takes place (11). Internal, retinoic acid plays an important role in the functions of many cells (12) where it seems to regulate the expression of given genes. Vitamin A, in the form of retinol, is absorbed by the cell and oxidised into retinoic acid, which is capable of penetrating the nucleus and joining nuclear receptors (12). This substance, dissolved in SiO, has been utilised in an experimental proliferating vitreous retinopathy model to inhibit cellular proliferation, precisely due to its relatively high solubility in lipids (13). As regards α -tocopherol, which is also part of the liposoluble vitamins, it is essential for the integrity of the cellular membranes and functions as an antioxidantizing agent (14).

On the other hand, cholesterol and its esters with long chain fatty acids are important components of lipoproteins of all cellular membranes and not only of the retina (11).

The results of this research confirm that SiO is capable of extracting said lipids from intraocular tissue. This extraction may affect not only the physical properties of the oil but also the functioning of the retina and confirms that silicone is not as inert as described in literature (8).

As mentioned previously, the toxicity of silicone for the retina has been controversial from the beginning of the utilization of this substance as a vitreous substitute, and although there is no consensus in literature, at this time it is accepted that silicone oil produces a sustained intraocular inflammatory res-

ponse (6,15,16) and our group has published other papers which indirectly involve this substance in other more generalized inflammatory expressions (17,18).

In addition and as commented above, experimental research has proved the loss of the external layers of the retina, lesions in the ganglionic cell layer and even the presence of SiO at the level of the optic nerve (9,19). Some papers have proved reductions in the a and b waves of the electroretinogram (20), all of which supports the idea of retinal toxicity of this substance.

Notwithstanding the above and the recent appearance of the so-called «heavy» silicones, SiO continues to be at this time the only valid alternative for treating specific complex vitreous-retinal pathologies, and even though it is important to be aware of its adverse effects, in our view it would be more interesting to have clear guidelines about the time silicone can remain inside the eye without causing irreversible damages. In this respect, at least in what concerns cholesterol, there is an increase of its concentrations matching the permanency time, although this study does not allow to establish the pathological significance thereof.

No explanation has been found for the inverse relationship between the age of patients and the levels of retinoic acid. This finding should be confirmed with additional research.

In summary, it has been confirmed that SiO is capable of extracting lipid compounds from inside the eye, mainly from the retina, which allows us to state that it is not an inert substance, although this does not imply it is equally toxic. More research in this area is needed, at least while SiO continues to be the most utilized long evolution vitreous substitute.

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