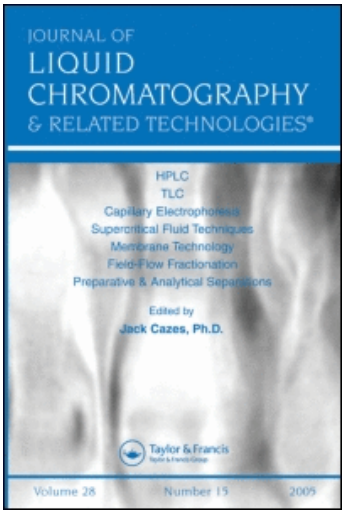

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Nozal, M. J. Del , Bernal, J. L. and Marinero, P.(1996) 'Simultaneous HPLC Determination of Cholesterol, α-Tocopherol, Retinol, Retinal and Retinoic Acid in Silicons Oils Used as Vitreous Substitutes in Eye Surgery', Journal of Liquid Chromatography & Related Technologies, 19: 7, 1151 — 1167
To link to this Article: DOI: 10.1080/10826079608006309
URL: http://dx.doi.org/10.1080/10826079608006309

J. LIQ. CHROM. & REL. TECHNOL., 19(7), 1151-1167 (1996)

SIMULTANEOUS HPLC DETERMINATION OF CHOLESTEROL, α-TOCOPHEROL, RETINOL, RETINAL AND RETINOIC ACID IN SILICONE OILS USED AS VITREOUS SUBSTITUTES IN EYE SURGERY

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the simultaneous determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in silicone oils, used as vitreous substitutes in eye surgery, is reported. The mixture components are removed from the matrix with recoveries close to 97% by solid-phase extraction with silica cartridges and subsequently separated on a C_8 column, using two different mobile phases consisting of acetonitrile/ammonium acetate and methanol/water that are passed sequentially.

The compounds are detected by UV-Vis spectroscopy at two different wavelengths (210 and 350 nm) that are also set sequentially. The detection limits thus achieved range from 0.19 to 7.48 $\mu g\ mL^{-1}$.

INTRODUCTION

Researchers have long strived to find a substitute for vitreous, which is indispensable for successful treatment of complex retinal detachments. So far, silicone oil appears to be the most suitable and least troublesome choice for this purpose.[1] However, some authors have noted the risk of complications such as cataracts, corneal opacity, glaucoma, *etc.* that arise from the operation itself or from the silicone or some impurity it may contain.

Nakamura et al.[2] studied the eye toxicity of low-molecular weight substances present in silicone oil and suggested the need to remove them prior to implantation of the oil.[3] Also, because of its chemical nature, silicone oil can dissolve fat-soluble compounds while residing in the vitreous cavity, as pointed out by Refojo et al.[4] who reported the presence of cholesterol and retinol, in addition to a large number of unidentified compounds. The identification of such compounds and their determination is of great interest, with a view to expanding available knowledge on the interaction of silicone oil with its receptacle (the vitreous cavity) and optimizing procedures for the treatment of retinal detachment (one of several uses of silicone oil). This entails the prior extraction of the compounds to be identified from the oil and their subsequent analytical determination.

There appears to be only a single reported procedure for the extraction of cholesterol and retinol from silicone oil,[4] and none for the other compounds. From other types of matrices, these compounds are extracted preferentially by saponification,[5-11] which can result in degradation of the extract, or by direct solvent extraction,[4,12-20,23,25] solid-phase extraction[21,22] or supercritical fluid extraction.[23,24] These procedures usually employ fat-soluble anti-oxidants (e.g., hydroxytoluene butylate, ascorbic acid, pyrogallol) in order to reduce potential losses through oxidation.

There is also no single available method for the determination of all the above mentioned compounds simultaneously. There are, however, a number of methods for the separation and quantitation of some, most of which rely on either gas chromatography,[11,18,25] gel permeation chromatography,[11,25-27] or high performance liquid chromatography in its normal phase mode with an amino or silica column[7-10,17,19,22,29] or the reverse phase mode with a C_8 or C_{18} column.[5-7,12-16,19,24,26,28] Detection is preferentially done by UV-Vis[4,6,7,10,11,13,15-17,22,24,26] or fluorescence spectroscopy,[4,6-10,27] but can also be electrochemical,[5,14] chemiluminescent,[29] refractometric[19] or even by measurements of diffuse radiation.[28]

Based on the above considerations, we developed a reverse phase HPLC method with UV detection at two different wavelengths which allows the determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in ophthalmic silicone oils from a single chromatogram.

EXPERIMENTAL

Apparatus and Chromatographic Conditions

The chromatographic instrumentation consisted of a Hewlett-Packard HP-1050 liquid chromatograph (Waldbronn, Germany) furnished with a multi-solvent partitioning pump, a UV-Vis variable wavelength detector and an integrator.

The column used was a 15 cm x 0.46 cm ID Zorbax C_8 from Jones Chromatography (Lakewood, CO) packed with particles of 5 μm diameter. Samples were injected via a Rheodyne 7125 injector (Berkeley, CA) furnished with a 20-μl loop.

The compounds of interest were eluted with either of two solvents. Eluent A consisted of 72:25 acetonitrile/0.2M NH4OAc and Eluent B of 95:5 methanol/water. The former was passed at a rate of 2 mL/min for 10 min and followed by a linear gradient of the latter for 1 min, which was maintained at 1.5 mL/min until the end of the run.

For detection, a wavelength of 350 nm was used for the first 14 min; it was subsequently changed to 210 nm.

Figure 1 shows the chromatogram obtained for a mixture of standards. Retention times were highly reproducible between chromatograms. The coefficients of variation obtained in 10 consecutive runs with the standards ranged from 0.25% for α-tocopherol to 2.16% for retinoic acid.

Reagents

The silicone oil used was supplied by IOBA-Domilens (Barcelona, Spain) in a 2000 cs viscosity. All solvents used were HPLC grade. Acetonitrile, methanol, *n*-hexane and *n*-propanol were purchased from Scharlau (Barcelona, Spain). Methylene chloride was supplied by Merck (Darmstadt, Germany).

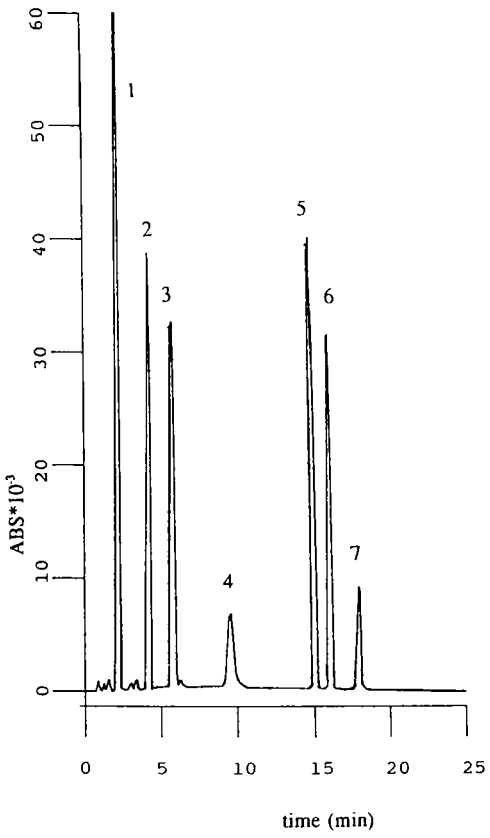

Figure 1. Chromatogram obtained from a silicone oil spiked with a mixture of standards (0.01 mg mL^{-1}). Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate .

The water was purified by passage through Compact Milli-RO and Milli-Q water systems from Millipore (Milford, MA).

Ammonium acetate, sodium acetate, ammonium hydrogen phosphate, anhydrous sodium sulphate and all other chemicals used to prepare the buffers were analytical-reagent grade and provided by Merck.

Retinoic acid, retinol, retinal, α-tocopherol, cholesterol, retinol acetate, α-tocopherol acetate (used as internal standards), and hydroxytoluene butylate (BHT, used as anti-oxidant) were supplied by Sigma Chem Co. (St Louis, MO).

Individual solutions of the analytes were prepared at a 0.1 mg mL^{-1} concentration in *n*-hexane (0.2 and 1 mg mL^{-1} for retinol and BHT, respectively). The solutions were degassed and stored refrigerated (-18°C) in topaz vials. Working-strength standards were prepared from these solutions by diluting 25 μL of 1 mg mL^{-1} BHT, 25 μL of 0.2 mg mL^{-1} and 25 μL of 0.1 mg mL^{-1} in *n*-hexane.

Reagent Stability

Because the compounds to be determined were labile and prone to oxidation, heat and UV radiation, we studied the temporal effect of light and heat on them. From the results obtained, it was decided that the standards could be used for 21 days with no appreciable alteration, provided they were kept refrigerated and in the dark.

We also studied changes in the compounds caused by the addition of variable amounts (0 - 100 μg/mL) of hydroxytoluene butylate (an anti-oxidant). It was observed that the presence of BHT extended the service life of the standards (a BHT concentration of 50 μg mL^{-1} preserved them unaltered for at least 60 days).

Calibration

Calibration was done with 7 different concentrations of the mixed standard that were added to 1 g of purified silicone oil. After stirring in a vortex mixer, (Fisher Scientific, Pittsburgh, PA), the mixture was passed through an SPE Si-Bond Elut cartridge (Varian, Harbor, CA) packed with 1 g of solid phase that was pre-activated with 5 mL of *n*-hexane. The compounds of interest were eluted with 0.5 mL of methanol and injected into the chromatograph following passage through 0.45-μm Millipore filters (Millipore, Bedford, MA). All samples were prepared and injected at least in triplicate in order to calculate coefficients of variation and the chromatographic reproducibility.

Sample Preparation

Prior to extraction, water was removed as follows: 1 g of silicone oil was mixed with 2 mL of methylene chloride, stirred in a vortex mixer for 2 min and centrifuged at 3000 G. Then, the supernatant was withdrawn with a Pasteur pipette. The procedure was repeated twice more. The sample was subsequently

passed through a 0.45 μm filter and the filtrate heated at 50°C and exposed to a helium stream for 30 min. Finally, the internal standards and BHT were added (retinol acetate 5 μg, α-tocopherol acetate 2.5 μg and BHT 25 μg) and the procedure described above for the standards was applied.

RESULTS AND DISCUSSION

Chromatographic Conditions

Wavelength

While some of the compounds studied exhibit native fluorescence, we opted for UV-Vis detection as it allowed all of them to be identified with no derivatization. From the absorption spectra for the analytes it was noted that they exhibit absorption maxima at different wavelengths. Thus, retinal, retinol and retinoic acid can be determined at 350 nm, where they possess a high absorbance. On the other hand, cholesterol and α-tocopherol do not absorb at this wavelength. Therefore, even though some authors detect α-tocopherol at 290 nm, we chose to use S = 210 nm, where both this compound and cholesterol absorb maximally.

Chromatographic column

We tested both normal and reverse phase columns, loaded with different functionalities to separate the analytes. Initially, we used 25 cm x 0.46 cm ID IB-Sil-Silica columns and 15 cm x 0.46 cm ID Spherisorb amino-modified columns, both from Phenomenex (Torrance, CA) and packed with particles of 5 μm. The two types of columns were used with various mobile phases consisting of hexane/*n*-propanol mixtures. The results thus obtained were unsatisfactory (retinol and α-tocopherol could never be separated). We also tested various C_{18} columns, but also in vain, since retinal and retinol could not be resolved in a reasonably short time (see Table 1, which shows the retention times for these compounds). We then switched to a C_8 column, which afforded the separation of all the analytes in a fairly short time and was, therefore, adopted for subsequent experiments.

Mobile phase

The mobile phase was optimized as regards both composition and the chromatographic conditions leading to maximal resolution.

Table 1

Retention Time Obtained for Retinol and Retinal in Different Columns.*

Column	Retinol	Retinal
Lichrosorb KAT, 10μm (12.5x0.4cm)	1.40	1.41
Pinkerton 5μm (25x0.46cm)	2.89	2.88
Hypersil SAS, 5μm, C_{18}, (25x0.46cm)	3.08	3.13
T-Bondapak, 10μm, C_{18}, (30x0.39cm)	5.16	5.26
Hypersil ODS, 5μm, C_{18}, (13x0.32cm)	7.49	7.64
Spherisorb ODS-1, 5μm (15x0.46cm)	11.97	13.73
Bonclone, 10μm, C_{18}, (30x0.39cm)	12.91	13.40
Sphere ODS, 10μm, (20x0.46cm)	15.08	15.96
Novapak C_{18}, (15x0.39cm)	17.12	18.49
Lichrospher 100 rp-18, 5μm (25x0.46cm)	19.47	20.38
Spherisorb ODS-2, 5μm (25x0.46cm)	25.91	30.19

* Mobile phase, MeOH:H_2O (90:10); flow rate, 1mL/min.

Regarding composition, we initially considered using methanol/water mixtures that were tested in variable proportions. The mixture afforded the separation of cholesterol, α-tocopherol and α-tocopherol acetate (see Fig. 2.a), but failed to resolve retinol and retinal, which gave strongly overlapped

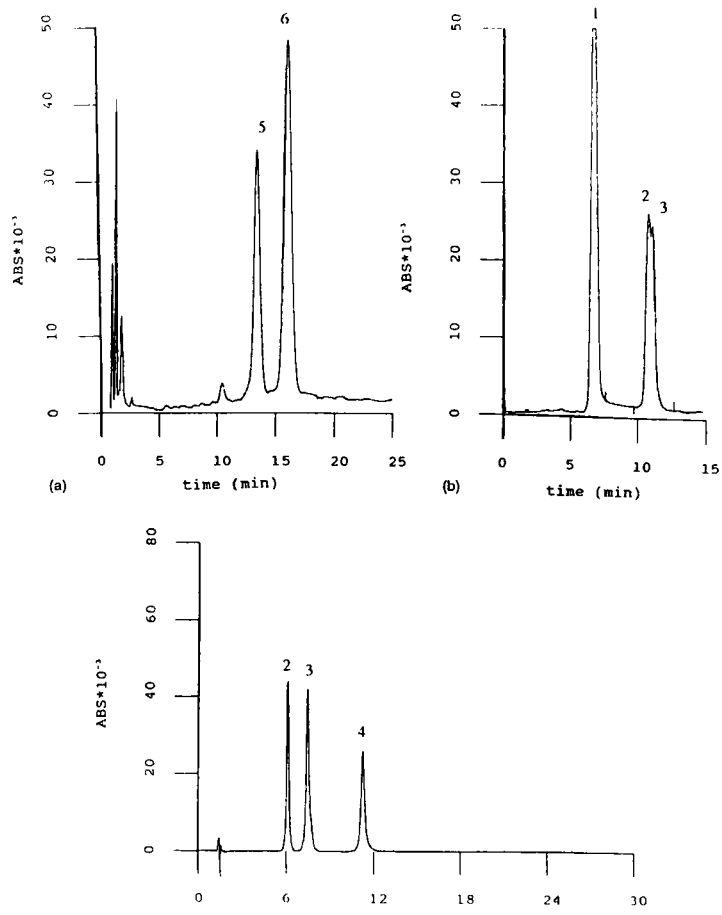

Figure 2. Chromatograms of a silicone oil, spiked with a mixture of standards, in different mobile phases. Conditions: column, Zorbax C_8 (5μm, 15 cm x 0.46 id); flow rate 1mL/min. Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate . **2a.** Mobile phase: Methanol-water (85:15); **2b.** Mobile phase: Methanol-water (92.5:7.5); **2c.** Mobile phase: Acetonitrile-water (85:15).

chromatographic peaks (Fig. 2.b) whatever the methanol content in the mixture. We then tested acetonitrile/water mixtures, which resolved retinol, retinal and retinol acetate; however, retinoic was eluted almost immediately and the other compounds, with adequate retention times, could not be resolved

Table 2
Variation of Analytes Recovery as a Function of Hexane Volume used to Dissolve the Samples

n-Hexane (ml)	Retinoic Acid	Retinol	Retinal	Cholesterol	α-Tocopherol
10	93.93	57.64	15.34	54.67	14.77
5	94.42	93.38	16.33	77.19	19.83
2	94.83	94.53	20.58	78.85	27.64
1	95.63	101.25	97.85	98.99	99.65
0.5	94.85	95.55	41.13	89.50	60.75

from one another (See Fig. 2c). We considered altering solute retention by changing the ionic strength. Of all the salts tested for this purpose, ammonium acetate provided the best results; while the most strongly retained compounds could not be resolved, the retention of retinoic acid was indeed increased. Studying the influence of the ammonium acetate concentration on solute retention, it was observed that the retention of retinoic acid increased with increasing NH_4OAc concentration up to 0.2M, with no appreciable effect on the other compounds. Above 0.2M, the peak for retinoic acid was considerably broadened, so such a concentration was adopted for further experiments. The pH was found to have no appreciable effect on the separation, so the value provided by the salt solution was accepted.

The inability to obtain a mixture allowing all the analytes to be resolved and the fact that two of the mixtures tested afforded the full resolution in two groups, led us to consider using them in sequence. In order to optimize resolution, the best proportion of organic modifier in both was established. For this purpose, we first changed the proportion of acetonitrile and examined its effect on the retention times of the analytes. As can be seen in Fig. 3a, increasing the amount of acetonitrile in the mobile phase decreased the resolution. We chose a proportion of 75%, which resulted in acceptable separation and elution in a fairly short time. The methanol-water mixture was then optimized similarly.

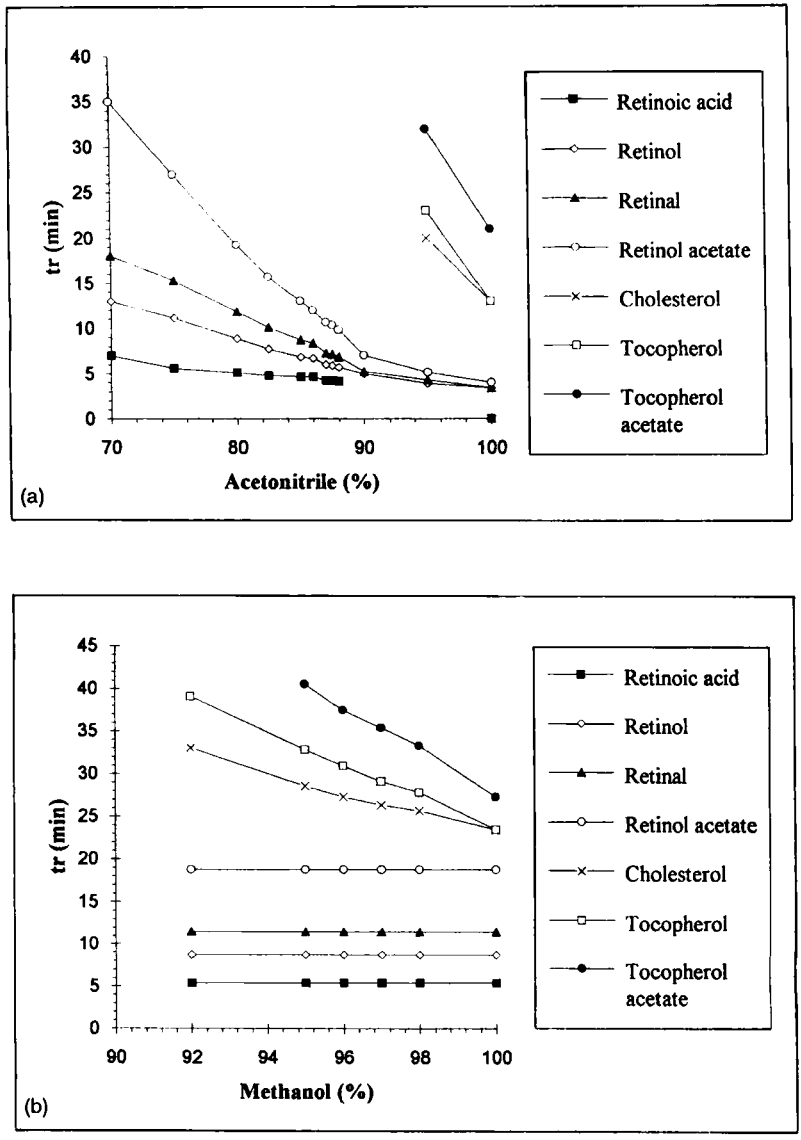

Figure 3. Plots of t_r values versus percentage of organic modifier. Flow rate, 1mL/min. **3a.** Variation of the percentage of Acetonitrile; Mobile phase ACN-NH_4Ac; **3b.** Variation of the percentage of methanol; Mobile phase ACN:NH_4Ac 0.2 M (75:25) during 10min and MeOH:H_2O at 11min.

To this end, a 75:25 acetonitrile/NH_4OAc mixture was passed for 10 min and then replaced with a methanol/water phase of variable composition. As can be seen from Fig. 3b, 95% MeOH ensured acceptable separation in a reasonably short time.

We also examined the influence of the mobile phase flow-rate and column temperature on the separation and found increases in both variables to decrease the retention times for all the solutes. Increasing the flow-rate shortened the analysis time, so 2 mL/min was adopted for the acetonitrile/NH_4OAc mixture and 1.5 mL/min for the methanol/water mixture.

Extraction/Clean-up

Prior to analyses proper, the silicone oil samples required developing a procedure for extracting the analytes from the matrix. We evaluated two choices for this purpose: liquid-liquid and solid-liquid extraction.

Liquid-liquid extraction

We first attempted extraction with solvents such as methanol, acetonitrile, isopropyl alcohol and acetone, all of which dissolved the analytes but not the silicone (except acetone, which dissolved the latter slightly). The procedure used was the same in every instance: an amount of 1 g of purified oil was spiked with known amounts of the analytes and the internal standards (all in *n*-hexane in order to facilitate dissolution in the sample) and the solvent was then removed in a rotary evaporator. Next, 10 mL of extractant was added with stirring for 10 min, the two phases were separated and the solvent was concentrated to dryness. The resulting residue was dissolved in methanol and filtered, and the filtrate was injected into the chromatograph.

All the solvents provided very high recoveries (about 97%) at every concentration tested except for isopropyl alcohol, which recovered only *ca.* 50% of the analytes. We then studied the influence of experimental variables potentially affecting the extraction process, *viz.* the extractant volume, addition of salts, pH and agitation time. Recoveries hardly changed above a volume of 10 ml, which was, therefore, adopted since larger volumes made the sample cumbersome too handle. The addition of salts had no appreciable effect. As regards pH, a neutral or alkaline medium had no effect on the separation, whereas an acid medium was detrimental as it caused retinol and retinal to decompose.

Table 3

Recovery and Reproducibility for Analytes, from Spiked Silicone Oil Samples, after Solid-Liquid Extraction (n=7). Detection and Quantification Limits, Applying the Method with Solid-Liquid Extraction

	Retinoic Acid	Retinol	Retinal	Cholesterol	α-Tocopherol
%Recovery	94.34	100.45	96.05	97.97	98.70
%CV	1.26	0.99	1.61	0.85	0.95
Detection limit (μg/ml)	0.0792	0.0514	0.2350	7.4789	0.6049
Quantification limit (μg/ml)	0.2641	0.1712	0.7835	24.9295	2.0163

In order to test the above procedure, several identically treated samples were injected into the chromatograph. But, the results were scarcely reproducible, possibly because of the thermolabile nature and ready oxidation of the analytes, facilitated by some of the conditions used in sample preparation procedure.

Solid-liquid extraction

We tested various extraction cartridges, loaded with different functionalities. We initially tried C_{18} cartridges. Samples were spiked with the analytes, supplied with 10 mL of methanol and agitated in a vortex mixer for 2 min. The two phases were then separated and the organic one was passed through the cartridge, previously activated with a solvent (methanol, acetonitrile or isopropyl alcohol). Next, the cartridge was dried with a nitrogen stream and eluted. In no case was full retention of the compounds achieved, so the packing was replaced with a more polar material: silica. Thus, we used Si-Bond Elut cartridges that were also pre-activated with various solvents and loaded with samples treated in different ways. Based on the results, the highest retention was obtained by activating the cartridge with 5 mL of *n*-hexane. As regards sample preparation, if 1 g of silicone oil was spiked with standards dissolved in *n*-hexane, the volume of solvent used had a dramatic effect on analyte recovery, as shown in Table 2 (1 mL of *n*-hexane resulted in the highest recoveries for all the analytes). The cartridge was then desiccated and the analytes eluted with 0.5 mL of methanol (larger volumes did not raise recoveries and resulted in superfluous dilution).

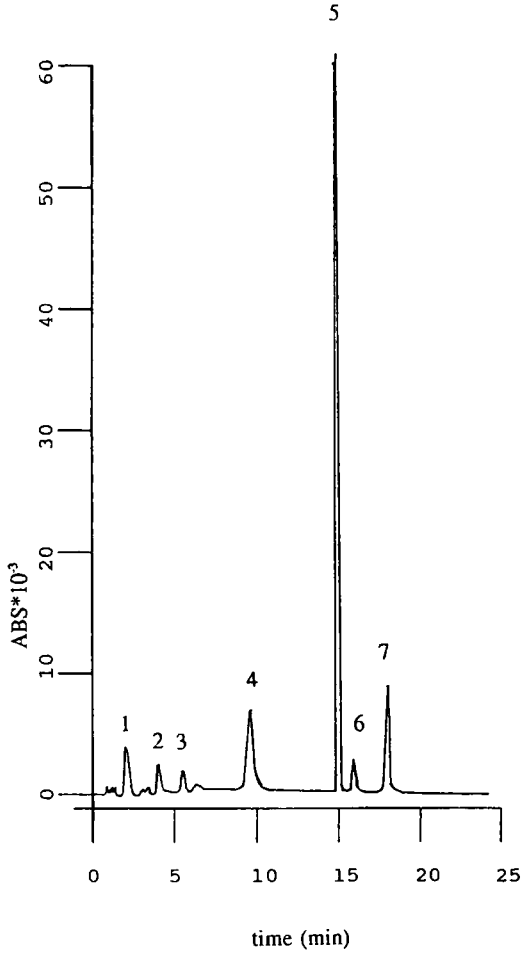

Figure 4. HPLC chromatogram of a silicone oil sample extracted from patient's eye, after surgery treatment. Peaks: 1=Retinoic acid, 2=Retinol, 3=Retinal, 4=Retinol acetate, 5=Cholesterol, 6=α-Tocopherol, 7=α-Tocopherol acetate .

The reproducibility of the procedure was tested by applying it to samples spiked with variable concentrations of all the analytes in the range 0.15-20 1g mL^{-1}. As can be seen from Table 3, the reproducibility was quite good throughout the concentration range studied.

Table 4

Results Obtained Applying the Proposed Procedure to Samples of Silicone Oil Extracted from Patients (μg/mL)

Sample	Retinoic Acid	Retinol	Retinal	Cholesterol	α-Tocopherol
1	0.03	0.16	0.02	33.96	0.08
2	0.02	0.08	0.02	53.78	0.11
3	0.03	0.17	0.14	61.98	0.12

Quantitation

The analytes were quantified in spiked samples subjected to the above-described treatment using the internal standard method. Calibration curves were obtained from replicate injections of a fixed volume of 20 μL containing the analytes at concentrations up to 50 mg mL^{-1} and were linear for all the compounds.

Table 3 gives the detection and quantification limits obtained, as calculated from the expression $3\sigma_{y/x}/s$ and $10\sigma_{y/x}/s$ (where $\sigma_{y/x}$ is the deviation from the fitting and s the slope of the calibration curve).

The proposed method was validated by using the standard addition method to spiked samples containing variable amounts of the analytes. Comparing the results obtained by applying both methods it was observed that the two sets are quite consistent.

Sample Analysis

The proposed method was applied to the determination of the analytes in 3 samples of silicone oil obtained from patients in which they have been implanted to repair retinal detachment. Figure 4 shows the chromatogram obtained for one of the samples. The results are given in Table 4. As can be seen, the compounds underwent some dissolution, which was maximal for cholesterol and minimal for retinoic acid.

CONCLUSIONS

The proposed method is a straightforward, expeditious means for the simultaneous determination of cholesterol, α-tocopherol, retinol, retinal and retinoic acid in silicone oils used as substitutes for vitreous in eye surgery. The sequential use of two different eluents and two wavelengths for detection results in high sensitivity and reproducibility. Also, subjecting the samples to solid-liquid extraction with silica cartridges affords analyte recoveries close to 97%.

ACKNOWLEDGMENTS

The authors wish to thank Castilla y León Government (Spain) for financial support to this project.

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Received October 20, 1995
Accepted November 21, 1995
Manuscript 4016