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Journal of Chromatography A, 705 (1995) 396–399

JOURNAL OF CHROMATOGRAPHY A

Short communication

Adsorption of oxysterols on different microtube materials during silanyzation prior to gas chromatographic determination

Francesc Guardiola, Rafael Codony*, Magda Rafecas, Josep Boatella

University of Barcelona, Nutrition and Food Science Unit, Faculty of Pharmacy, Av. Joan XXIII s/n, 08028-Barcelona, Spain

First received 29 April 1994; revised manuscript received 8 September 1994; accepted 19 October 1994

Abstract

The observation of increasing variability in the GC determination of oxysterols when the material of the silanization microtubes was changed led to a comparative study of three kinds of microtubes (Pyrex glass, polypropylene homopolymer and polypropylene copolymer). The characteristics of the adsorption of each material on different oxysterols and their influence on the reliability of the determination are reported. The variability of the adsorption in the different materials is high for 5α-cholestane, one of the most widely used internal standards in the GC determination of oxysterols. This will affect the precision and accuracy of the relative response factors. Significant differences were also found for cholesterol and the five oxysterols analysed, especially between the polypropylene copolymer and the other two materials.

1. Introduction

There have been few reports on the adsorption of the sterols and oxysterols (OS) on the surface of the materials used during the analytical procedures involved in their determination [1,2]. However, this is not a trivial phenomenon, as variable results are obtained if changes in the material used are introduced without strict control. This variability can lead to large errors in the determination of some OS, owing to their low concentration in foods.

The need for this study was suggested by the observation of an unexpected variability of results in the GC determination of OS [3] when the nature of the plastic silanization microtubes was changed. We designed a study with the aim of establishing the degree of adsorption on glass and two different plastic materials for cholesterol, the main OS, and 5α-cholestane, the internal standard (I.S.) used in the determination.

2. Experimental

2.1. Reagents and standards

Ethyl acetate (ACS grade) and dried pyridine (maximum 0.01% water, for analysis) were obtained from Merck (Darmstadt, Germany) and Sylon BTZ [N,O-bis(trimethylisilyl)acetamide–trimethylchlorosilane–N-trimethylsilylimidazole (3:2:3), for research], in 0.1-ml glass ampoules, from Supelco (Bellefonte, PA, USA). Standard cholesterol (>99%, by GC) was supplied by Merck and 5α-cholestane (99%, by GC) by Supelco. All other standards were obtained from

* Corresponding author.

0021-9673/95/$9.50
SSDI 0021-9673(94)01018-8

Sigma (St. Louis, MO, USA): cholesterol-5α,6α-epoxide (α-CE) (99%, by TLC), 7β-hydroxycholesterol (7β-HC) (99%, by TLC), cholestanetriol (CT) (>97%, by GC), 7-ketocholesterol (7-KC) (>99%, by HPLC) and 25-hydroxycholesterol (25-HC) (>98%, by TLC).

All these standards were weighed in a Sartorius 2004 MP microbalance with an accuracy of 0.01 mg. The standards were handled as ethyl acetate solutions (2.5 mg per 10 ml for internal standards and 2.0 mg per 10 ml for the others). When dilutions of these solutions were needed, they were prepared with ethyl acetate immediately prior to use.

2.2. Gas chromatographic conditions

GC was performed on a Perkin-Elmer Sigma 2000 chromatograph equipped with a flame ionization detector and fused-silica capillary column (25 m × 0.25 mm I.D.), with a stationary phase film thickness of 0.13 μm methylsilicone (CP-Sil 5 CB) from Chrompack (Middelburg, Netherlands). Helium was used as the carrier gas and the chromatographic conditions were as follows: oven temperature, programmed from 210 to 240°C at 6°C/min, from 240 to 270°C at 4°C/min and from 270 to 290°C at 2°C/min with a 5-min hold at 290°C; injector temperature, 290°C; detector temperature, 350°C; splitting ratio, 1:40; inlet pressure, 15 psi; and sample volume injected, 2 μl.

2.3. Design of the experiments

We designed two experiments to study the use of microtubes made of three common materials: Pyrex glass, polypropylene (PP) homopolymer and polypropylene copolymer.

In experiment I, 5 μg of each OS and 5 μg of cholesterol were added to six microtubes of each type of material. Then 50 μl of pyridine were added as solvent and 50 μl of Sylon BTZ as silanization reagent, and the blend was kept for 20 min to complete the reaction. Then, 50 μl of the blend were transferred into a glass microtube and 37.5 μg of 5α-cholestane (I.S.) were added. After 20 min, 2 μl of this blend were injected into the chromatograph. In order to check the adsorption of 5α-cholestane, 50 μl of Sylon BTZ were added to six microtubes of each type containing 75 μg of 5α-cholestane dissolved in 50 μl of pyrine. After 20 min, 50 μl of the blend were transferred into a glass microtube containing 37.5 μg of cholesterol, which was used as the I.S. After 20 min of reaction, 2 μl were injected into the chromatograph. In all instances, as can be observed, the I.S. was added directly to the final glass microtube, so that its adsorption should not affect the results.

In experiment II, four microtubes of each material were filled with 75 μg of 5α-cholestane (I.S.), 5 μg of each OS standard, 5 μg of cholesterol and 50 μl of pyridine as solvent. The blend was silanized with 50 μl of Sylon BTZ and, after 20 min, 2 μl of the reaction mixture were injected into the chromatograph.

3. Results and discussion

3.1. Results of experiment I

From the chromatograms obtained, the ratio between the areas of the standard and of the internal standard (A_{xs}/A_{is}) was calculated for each compound. In all instances, mean values of these ratios were higher in glass microtubes, owing to the higher degree of adsorption of standards in the plastic materials than in the glass (Table 1).

An analysis of variance (ANOVA) applied to these results showed that there are significant differences in the area ratio (A_{xs}/A_{is}) for cholesterol ($P = 0.0035$), 5α-cholestane ($P < 0.0001$) and α-CE ($P < 0.0001$), depending on the microtube used. For these three compounds, in order to know which mean ratios differ, we applied the Scheffé's test for a posteriori contrasts ($\alpha = 0.05$). The results obtained are given in Table 2.

Table 2 shows that 5α-cholestane was adsorbed in PP homopolymer more than in glass because, on average, A_{xs}/A_{is} in glass was between 0.0392 and 0.1217 higher than in PP homopolymer. In addition, this compound in PP

Table 1
Mean values for the ratio A_{xs}/A_{is} of 5α-cholestane, cholesterol and the five OS in the different microtube materials assayed ($n = 6$)

Material	5α-Cholestane	Cholesterol	α-CE	7β-HC	CT	7-KC	25-HC
Pyrex glass	0.8743	0.0776	0.0681	0.0698	0.0693	0.0569	0.0559
PP homopolymer	0.7939	0.0711	0.0604	0.0586	0.0667	0.0505	0.0538
PP copolymer	0.7047	0.0686	0.0628	0.0624	0.0692	0.0519	0.0551

Table 2
Significance level and confidence interval of the a posteriori contrasts (Scheffé's test, $\alpha = 0.05$)

Standard	Material	PP homopolymer	PP copolymer
5α-Cholestane	Pyrex glass	$P < 0.0001$ (0.0392–0.1217)[a]	$P < 0.001$ (0.1283–0.2109)
	PP homopolymer		$P < 0.0001$ (0.0435–0.1184)
Cholesterol	Pyrex glass	NS[b]	$P = 0.0035$ (0.0275–0.1756)
	PP homopolymer		NS
α-CE	Pyrex glass	$P < 0.0001$ (0.0567–0.2250)	$P = 0.0311$ (0.0094–0.1759)
	PP homopolymer		NS

[a] P = Significance level of the contrast; the confidence interval is given in parentheses.
[b] For this standard the difference between the mean ratios (A_{xs}/A_{is}) using these two kinds of microtube is not statistically significant.

copolymer was highly adsorbed and presented a value for A_{xs}/A_{is} between 0.1283 and 0.2109 lower than in glass and between 0.0435 and 0.1184 lower than in PP homopolymer. Cholesterol and α-CE presented higher adsorption in PP copolymer than in glass and, moreover, α-CE presented higher adsorption in PP homopolymer than in glass.

3.2. Results of experiment II

As the I.S. (5α-cholestane) showed a higher adsorption in the plastic materials, we carried out a second experiment, in which the I.S. was added to the same microtube as the other standards, to simulate the real procedure followed for calibration. From the chromatograms obtained, the ratio A_{xs}/A_{is} was calculated for each standard and the mean values are given in Table 3.

The results in Table 3 indicate that the mean ratio A_{xs}/A_{is} is maximum in the PP copolymer and minimum in the Pyrex glass, for all standards. This is Because the 5α-cholestane is more adsorbed in the PP copolymer than in the PP homopolymer, and much more so than in the glass. The application of the ANOVA to the mean values of A_{xs}/A_{is} for each standard in the

Table 3
Mean values of A_{xs}/A_{is} for each standard in the different microtubes ($n = 4$)

Material	Cholesterol	α-CE	7β-HC	CT	7-KC	25-HC
Pyrex glass	0.0862	0.0582	0.0751	0.0710	0.0556	0.0489
PP homopolymer	0.0918	0.0622	0.0801	0.0755	0.0601	0.0567
PP copolymer	0.1129	0.0843	0.1084	0.1082	0.0795	0.0783

Table 4
Significance level and confidence interval of the a posteriori contrasts (Scheffé's test, $\alpha = 0.05$)

Standard	Material	PP homopolymer	PP copolymer
Cholesterol	Pyrex glass	NS[a]	$P = 0.0005$ (0.014–0.039)[b]
	PP homopolymer		$P = 0.0024$ (0.009–0.033)
α-CE	Pyrex glass	NS[a]	$P < 0.0001$ (0.019–0.034)
	PP homopolymer		$P < 0.0001$ (0.015–0.030)
7β-HC	Pyrex glass	$P = 0.0130$ (0.001–0.010)	$P < 0.0001$ (0.029–0.037)
	PP homopolymer		$P < 0.0001$ (0.024–0.032)
CT	Pyrex glass	NS[a]	$P = 0.0003$ (0.022–0.053)
	PP homopolymer		$P = 0.0007$ (0.017–0.048)
7-KC	Pyrex glass	NS[a]	$P = 0.0004$ (0.013–0.035)
	PP homopolymer		$P = 0.0017$ (0.009–0.030)
25-HC	Pyrex glass	$P = 0.0254$ (0.001–0.015)	$P < 0.0001$ (0.023–0.036)
	PP homopolymer		$P < 0.0001$ (0.015–0.028)

[a] For these standards the difference between the mean ratios (A_{xs}/A_{is}) using these two kinds of microtube is not statistically significant.
[b] P = significance level of the contrast: the confidence interval is given in parentheses.

different materials showed that statistically significant differences exist for cholesterol ($P = 0.0003$), 7β-HC ($P < 0.0001$), α-CE ($P < 0.0001$), CT ($P = 0.0001$), 7-KC ($P = 0.0003$) and 25-HC ($P < 0.0001$). Therefore, applying the Scheffé's test for a posteriori contrasts ($\alpha = 0.05$), we can conclude that, for all standards, the mean value of A_{xs}/A_{is} is significantly higher in the PP copolymer than in glass and than in the PP homopolymer. Moreover, these mean values are significantly higher in the PP homolymer than in glass only for 7β-HC and 25-HC (Table 4).

The main conclusion was that the type of microtubes used in the method can significantly affect the results obtained, mainly in the calculation of the relative response factors of sterols and in the calibration graph because one of the most usual I.S., 5α-cholestane, shows significantly different adsorption rates on each kind of microtube. For this reason, we think that the most reliable results can be obtained using the Pyrex glass microtubes for derivatization, as they offer lower and less variable adsorption of all compounds.

Acknowledgements

This work was supported in part by a research grant from the Comissió Interdepartamental de Recerca i Innovació Tecnològica (CIRIT). C. Sala-Villaplana provided technical assistance.

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