



Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation

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Abstract

The present work compares six biochemical methods for extraction of lipids from human serum. Although some organic solvents were good lipid extractors, they precipitated most of the total proteins and albumin. On the other hand, methodologies using Triton X-114 and silica were efficient for extraction of lipids, while sparing the protein fraction.

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1. Introduction

The mammalian serum, i.e. the fluid phase of the coagulated blood, is constituted by water, electrolytes, proteins, lipids, carbohydrates, amino acids, vitamins, hormones and excreted metabolites, such as urea and uric acid [1].

The study of serum or plasma components usually demands either methods of purification or depletion of one or more molecules. Some methods can be used with this aim, such as affinity chromatography on sorbent columns and chromatography based on differences in solvent solubilities. However, the latter methods can modify the ratio of more than one serum constituent, as these constituents possess, in varying degrees, both polar and non-polar groupings.

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Biological lipids constitute a group of substances that have insolubility in water as a characteristic feature [2] and play many important physiologic roles, such as functioning as a substrate for synthesis of bile acid and steroid hormones [3]. They are also a source of energy, mainly as chylomicrons and very low density lipoproteins (VLDL), which have relatively large proportions of triacylglycerols in their composition [4].

As lipids are insoluble in water, a chloroform–methanol mixture is the solvent commonly used to extract them [5–7]. Chloroform extracts mainly neutral lipids (triglycerides, waxes and pigments), while methanol extracts cell-membrane lipids (phospholipids, glycoproteins and cholesterol). Hexane or hexane–isopropanol can also be used to remove neutral lipids [8–10]. In addition to extraction using organic solvents, absorption on hydrophobic affinity columns and detergent precipitation methods can also be used to remove lipids from biological samples [11–12].

The objective of this work is to compare six methods for extracting lipids from human serum, in terms of their efficacies and effect on total protein content.

2. Materials and methods

2.1. Human serum

Serum samples were prepared from blood samples collected from informed healthy laboratory volunteers.

2.2. Serum lipid extraction with organic solvents

Extraction with a chloroform–methanol mixture was based on the methodology of Folch et al. [7]. Briefly, 1 ml of normal human serum was added to 10 ml of chloroform–methanol 2:1 (v/v). The mixture was agitated manually for 20 s and centrifuged at $2500 \times g$ for 10 min at 20 °C. After centrifugation, the aqueous phase was collected and stored at –20 °C for posterior analysis.

Extractions with hexane and hexane–isopropanol were carried out according to the methodologies of Baldoni et al. [8] and Sugiyama et al. [9], respectively, with some modifications. Briefly, serum was mixed with hexane or with a mixture of 6.0 ml of hexane and 3.0 ml of isopropanol at a ratio of 1:10 (v/v), agitated manually for 20 s and then centrifuged at $2500 \times g$ for 10 min at 20 °C. The aqueous phase was collected and stored at –20 °C. In the extraction procedure using only hexane, the aqueous phase was collected and subjected to two subsequent identical extraction steps.

For ether extractions, normal human serum was mixed with 100% ether in the ratio of 1:10 (v/v). The mixture was agitated manually for 20 s and centrifuged at $2500 \times g$ for 10 min at 20 °C. This step was repeated three times with the aqueous phase.

2.3. Detergent extraction

This was carried out according to a modification of Bordier's [12] methodology. Briefly, serum was mixed 2:1 (v/v) with previously washed Triton X-114 and

incubated for 10 min at 4 °C, followed by 5 min at 37 °C. The mixture was then centrifuged at 12000 × *g* for 15 min at 4 °C, and the upper layer was collected, mixed again with Triton X-114 at a ratio of 10:1 (v/v), incubated and centrifuged as described above. The upper layer was then collected and stored at –20 °C, for posterior analysis.

2.4. Silica extraction

This was carried out according to a modification of Neoh et al.'s methodology [13]. To 1 ml of serum, diluted 1:2 in 0.05 M borate buffer, pH 8.3, 360 mg of silica were added (Sigma, St. Louis, USA). The mixture was incubated for 2 h at 4 °C, under constant agitation. It was then centrifuged at 2500 × *g* for 10 min at 4 °C. A total of 360 mg of silica was again added to the supernatant and the mixture was incubated for 1–16 h, followed by centrifugation, as described above. The supernatant was stored at –20 °C for posterior analysis.

2.5. Determination of lipid and protein concentrations

Cholesterol and triglyceride levels were determined with enzymatic assays based in the Trinder reaction (Labtest Diagnóstica, Lagoa Santa, Brazil). Albumin concentrations were determined by chromogenic reaction with bromocresol green and total protein concentrations by means of the biuret reaction.

2.6. Calculation of lipid extraction efficacy and protein/lipid purification ratio

The percentage of lipid extraction was calculated by means of the following formula:

$$\begin{aligned} & \text{\% of lipid extraction} \\ & = 100 - \frac{\text{triglycerides or cholesterol serum concentration after lipid extraction} \times 100}{\text{triglycerides or cholesterol serum concentration before lipid extraction}} \end{aligned}$$

The protein/lipid purification ratio indicates the extent to which protein was purified, in relation to lipid, after lipid extraction, i.e., how many folds the total protein concentration increased in relation to the lipid concentration (triglycerides + cholesterol). It was calculated with the help of the following formula:

$$\begin{aligned} & \text{Protein/lipid purification ratio} \\ & = \frac{\text{concentration of protein in extracted serum} \times \text{concentration of lipid in original serum}}{\text{concentration of protein in original serum} \times \text{concentration of lipid in extracted serum}} \end{aligned}$$

3. Results

In order to determine the best conditions for specific lipid extraction with silica, a second absorption with new silica was carried out for different time periods (from 1 to 16 h). A second incubation of the serum with silica for 4 h led to nearly maximal cholesterol

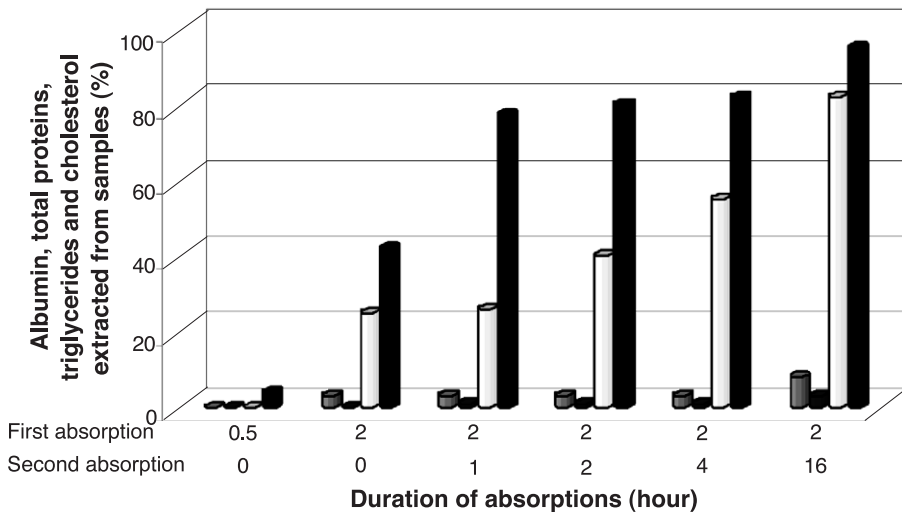


Fig. 1. Extraction of albumin (▨), total proteins (▩), triglycerides (□) and cholesterol (■) from serum by absorption with silica. After a first absorption with silica, the serum was further extracted by a second absorption with new silica, for the time periods indicated in the x-axis. The data represented by the first set of four columns, resulting from 0.5-h incubation with silica, were produced according to the original Neoh and collaborators' methodology.

extraction, whereas triglyceride extraction still improved with a second 16-h incubation (Fig. 1). Using the 16-h incubation, 94% of cholesterol and 71% of triglycerides were removed (Table 1 and Fig. 2A).

Table 1

Concentrations of total protein, albumin, triglycerides and cholesterol in human serum before and after extraction of lipids

Extraction method ^a	Concentration of (% of original amount)				
	Total protein	Albumin	Triglycerides	Cholesterol	Proteins/lipid purification ratio ^b
None	6.4 ± 0.2 ^c (100.0)	3.8 ± 0.2 ^c (100.0)	100 ± 8.0 ^d (100.0)	231 ± 58 ^d (100.0)	1.0
Chloroform–methanol	0.3 ± 0.1 (7.8)	0.4 ± 0.1 (10.5)	13 ± 1.7 (13.0)	3 ± 1.0 (1.0)	1.4
Hexane	5.7 ± 0.4 (89.1)	3.3 ± 0.1 (86.8)	82 ± 10.2 (82.0)	184 ± 36 (80.0)	1.1
Triton X-114	6.3 ± 0.2 (98.4)	3.7 ± 0.1 (97.4)	19 ± 4.0 (19.0)	76 ± 8.3 (32.9)	3.4
Ether	5.9 ± 0.5 (92.2)	3.5 ± 0.1 (92.1)	96 ± 10.0 (96.0)	157 ± 41.9 (68.0)	1.2
Hexane–isopropanol	0.5 ± 0.1 (7.8)	0.2 ± 0.1 (5.3)	44 ± 5.9 (44.0)	19 ± 1.2 (8.2)	0.4
Silica	6.3 ± 0.2 (98.4)	3.6 ± 0.1 (94.7)	28 ± 2.5 (28.0)	17 ± 1.2 (7.4)	7.2

^a The extraction methods were based on the use of the material listed in the first column, as described in Section 2.

^b This ratio shows how many folds the total protein concentration varied in relation to the lipid concentration (triglycerides + cholesterol); 1 = no variation.

^c Mean of three independent determinations ± standard deviation of the mean, expressed as g/100 ml.

^d Mean of three independent determinations ± standard deviation of the mean, expressed as g/l.

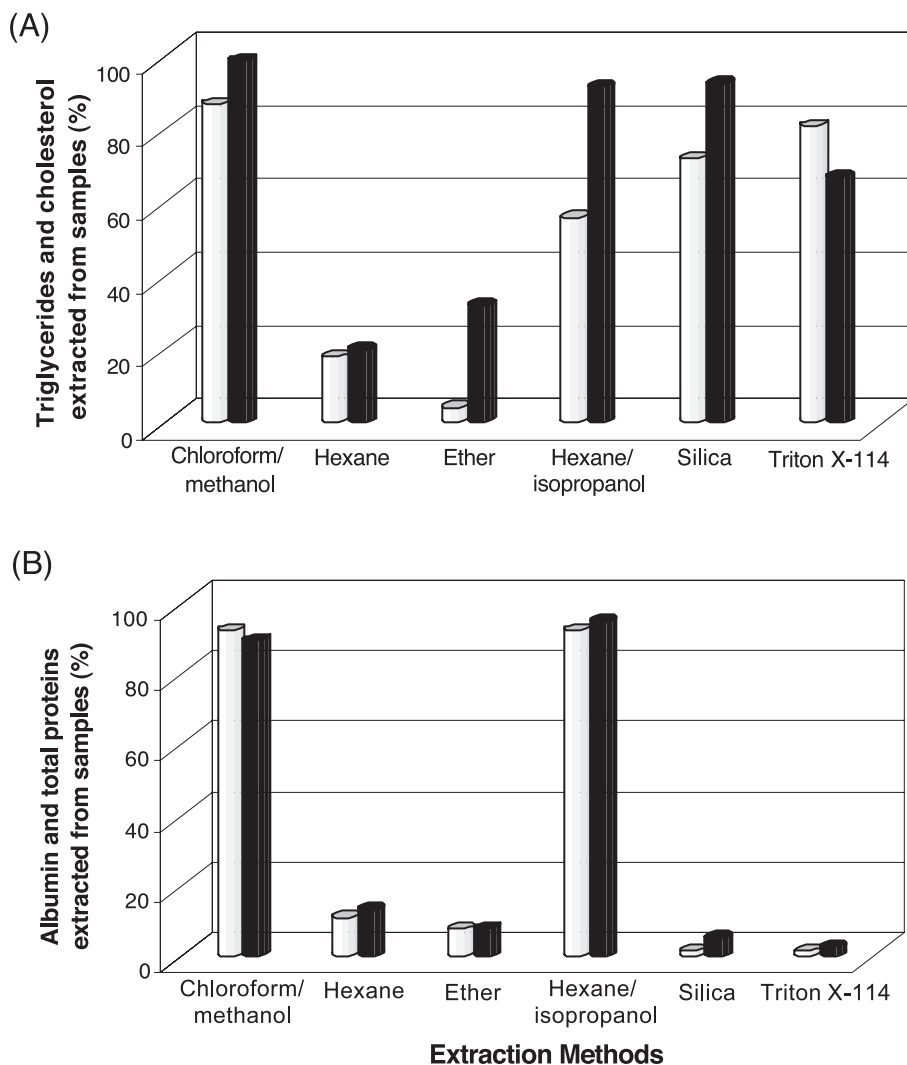


Fig. 2. Proportion of cholesterol, triglycerides, albumin and total proteins, removed from human serum by six different methods. The methods were based on extraction/absorption with the substances indicated in the x-axis, as described in Section 2. The data are representative of three experiments. The extraction with silica was done by two successive 2- and 16-h incubations with new silica. (□) % of removed triglycerides (A) or total proteins (B); (■) % of removed cholesterol (A) or albumin (B).

Chloroform–methanol extracted 90% of the triglycerides and 99% of the cholesterol, hexane–isopropanol removed 53% of the triglycerides and 94% of the cholesterol, ether removed 1.9% of the triglycerides and 31% of the cholesterol, and hexane removed 2% of triglycerides and 25% of the cholesterol (Table 1 and Fig. 2A). Triton X-114 removed 73% of triglycerides and 90% of the cholesterol (Table 1 and Fig. 2A).

The different methods varied in terms of protein-precipitation activity. Chloroform–methanol and hexane–isopropanol precipitated the majority of the serum protein, whereas the serum proteins were preserved when the other methods were deployed (Table 1 and Fig. 2B).

4. Discussion

Usually, complex mixtures of lipids are fractionated according to polarity or to solubility in different solvents [5]. Neutral lipids have low polarity and therefore are best extracted by solvent with hydrophobic characteristics, such as chloroform and ether [2]. Yet, membrane amphipatic lipids are efficiently extracted by more polar solvents, like ethanol and methanol [2]. These, in addition to reducing hydrophobic interactions, interfere with intra- and inter-molecular hydrogen bridges [3].

Most serum lipids behaved as polarized lipids, in that they were readily extracted by chloroform–methanol and hexane–isopropanol mixtures. However, these solvents are not selective: they also precipitated a great amount of total proteins and albumin. On the other hand, extraction with ether and hexane precipitated very little proteins but did not remove much of the lipids.

The extraction method using silica, described by Neoh et al. [13], which involves a 30-min absorption step, did not efficiently remove lipids from human serum. This was only attained when the silica concentration was raised ten times, in two consecutive absorption steps lasting 2 and 16 h. It is possible that the lipid amounts in the samples used by Neoh et al. [13] were smaller than those usually found in human serum.

The use of Triton X-114 also resulted in efficient serum lipid extraction. Lowering the temperature of the serum and Triton X-114 mixture causes the formation of insoluble micelles, containing the detergent and hydrophobic/amphiphilic molecules. The small amount of precipitated serum proteins with the insolubilized Triton X-114 derives from the fact that amphiphilic proteins appear in small amounts in human serum [12].

Contrasting with the extractions with organic solvents, the modified silica-based and the Triton X-114 precipitation methods removed most lipids and preserved most of the protein. Indeed, the protein enrichment in the serum treated with silica or Triton X-114, relative to the lipid content, was 7.2 and 3.4 folds, respectively, whereas the protein enrichment obtained with the use of the organic solvents ranged from only 0.4 to 1.4 folds. The two former methods, therefore, should be preferred over the methods employing organic solvents for lipid extraction, with protein preservation, from mammal serum.

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