EFFECTS OF SERUM STORAGE ON THE DETERMINATION OF CHOLESTEROL

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Serum samples are generally processed soon after separation. However, in some cases serum is stored before the determination for a more or less long period of time. Storage at positive or negative temperature might have effects on the evaluation of the serum concentration of biochemical variables. This is particularly true when the liberation of the analyte must precede the biochemical analysis, as in the case of cholesterol. In fact, cholesterol is transported by hydrophilic macromolecular complexes and only after their complete breakdown it is released and can be measured[3].

It is possible that serum storage, by altering the stability of lipoproteins, brings about a release of the inner components of the macromolecules, which can be more easily identified by the current methods of determination than in fresh serum.

In this study we have measured the serum concentration of cholesterol and of some other lipoprotein components in fresh and in stored serum, in order to investigate whether storage has any effect on the enzymatic determination of cholesterol.

MATERIALS AND METHODS

The study was carried out on blood samples drawn after an overnight fast from normolipidemic and hyperlipidemic volunteers. Serum was soon separated by centrifuging blood at 1,500 × *g* for 10 min at 4 °C. Serum samples were then subdivided in aliquots. One sample was soon processed and the others were stored at 4 °C and at -20 °C (in one experiment also at -80 °C) into capped vials until determination. Only samples that had to be analyzed were removed. Samples were allowed to thaw at room temperature, then were mixed by vortexing to insure complete mixing.

Key-words: Apoproteins; Cholesterol; Freezing; Lipoproteins; Serum storage; Triglycerides.

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In 10 cases serum lipoproteins have been fractionated in duplicate by a mixed ultracentrifugation and precipitation procedure. High density lipoprotein (HDL) lipids and apoprotein A-I were measured in the supernate after precipitation of very low (VLDL) and low (LDL) density lipoproteins with phosphotungstate/Mg^{++} (Boehringer Mannheim, FRG). VLDL lipids and apoprotein B were determined as the difference between their concentration in whole serum and that in infranate after ultracentrifugation at density of 1.006 at 202,000 × *g* for 16h at 15 °C. LDL lipids and apoprotein B were calculated as the difference between their concentration in infranate after ultracentrifugation at density of 1.006 and that in the phosphotungstate/Mg^{++} supernate.

Cholesterol and triglycerides were determined by the CHOD-PAP and by the DHBS-color methods, respectively (Kit M, Miles Labs Ltd., Stoke Poges, UK), unless otherwise stated. Intraassay and interassay coefficients of variation were 2.15% and 2.57% respectively for cholesterol, and 1.68% and 4.39% respectively for triglycerides. The determination of cholesterol and triglycerides was also made with 3 other colorimetric enzymatic methods marketed by Kone Diagnostics, Espoo, Finland (Kit K), by Laboratoires Biotrol, Paris, France (Kit R), and by Boehringer Mannheim, FRG (Kit B). In 10 samples cholesterol was determined by the dry chemistry Reflotron system (Boehringer Mannheim, FRG), and in 10 different sera by the chemical method of Babson et al.[4].

Apoproteins A-I and B were measured by an immunoturbidimetric method (Orion Diagnostica, Espoo, Finland). Intraassay and interassay coefficients of variation were 2.78% and 6.92% respectively for apoprotein A-I, and 5.67% and 5.51% respectively for apoprotein B. All the enzymatic, chemical and immunoturbidimetric determinations were made in duplicate.

Statistical analysis was performed by Student's *t*-test for paired data.

RESULTS

Table 1 shows the mean values of serum cholesterol as obtained by the CHOD-PAP method (Kit M) in fresh serum and in serum stored at 4 °C for 7 days and at -20 °C for 7 and 14 days. As it can be seen, cholesterol values were significantly higher in serum stored both at positive and negative temperatures than in fresh serum. The prolongation of the freezing beyond one week was not followed by further significant changes.

When serum was stored at 4 °C, cholesterol values progressively increased until the 4th day, then they remained substantially unchanged. Freezing of se-

	n°	cholesterol		
		fresh serum	7 days	14 days
storage at 4 °C	34	183.7 ± 4.66	204.4 ± 5.30*	-
storage at -20 °C	34	221.1 ± 12.20	235.5 ± 11.38*	239.2 ± 11.27*

Tab. 1 - Mean values ± SEM (mg/dl) of cholesterol in two different series of serum samples processed after separation and after storage at 4 °C for 7 days and at -20 °C for 7 and 14 days. *(* $p<0.001$ vs fresh serum).*

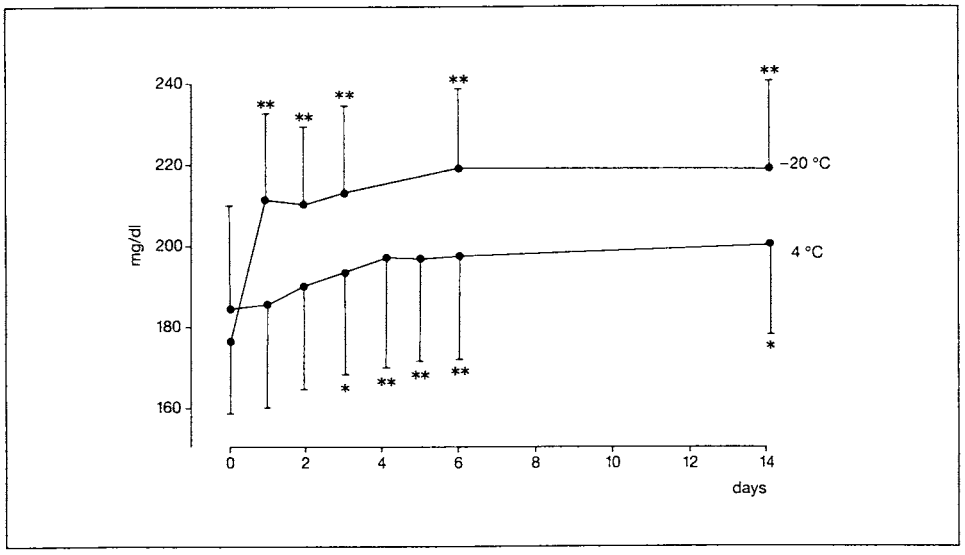

Fig. 1 - Changes of cholesterol during serum storage at 4 °C and at -20 °C. Means of 5 cases. Vertical bars represent the standard error of the mean. *(* p<0.01; ** p<0.001).*

rum brought about a significant increase of cholesterol after 1 day without further changes during the following 13 days (fig. 1). The rise of cholesterol values could be also observed after only 2h of freezing the serum samples both at -20 °C and -80 °C (cholesterol values in fresh serum: 185.8 ± 14.34, after 2h at -20 °C: 202.0 ± 9.90, $p<0.05$, after 2h at -80 °C: 203.8 ± 18.60, $p<0.05$). Both free and esterified cholesterol underwent an increase during the storage at positive or negative temperature (tab. 2).

To exclude the possibility that changes following the storage were due to some peculiarity of the commercial kit, different serum samples were analyzed by three other kits and the dry chemistry Reflotron system immediately after separation, 2h and 7 and 14 days after freezing. The results are summarized in tab. 3. In all the cases serum cholesterol resulted, on the average, higher in frozen than in fresh serum. Cholesterol determined by the Reflotron system in serum stored at 4 °C for 7 days was higher than in fresh serum (214.3 ± 15.78 mg/dl in stored serum and 210.0 ± 15.26 mg/dl in fresh serum, $p<0.05$).

	cholesterol		
	total	free	esterified
fresh serum	209.1 ± 14.37	42.7 ± 3.11	166.4 ± 14.11
storage at 4 °C for 7 days	221.4 ± 14.70**	47.7 ± 2.96**	173.6 ± 14.85**
storage at -20 °C for 7 days	223.3 ± 14.04**	51.2 ± 2.83**	172.1 ± 14.62*

Tab. 2 - Mean values ± SEM (mg/dl) of free and esterified cholesterol in 20 serum samples processed after separation and after storage at 4 °C and at -20 °C for 7 days. *(* p<0.05; ** p<0.001 vs fresh serum).*

	n°	fresh serum	frozen serum		
			2h	7 days	14 days
Kit B	15	244.9 ± 16.96	288.4 ± 19.42**	287.4 ± 19.42**	281.1 ± 19.75**
Kit K	15	276.9 ± 20.19	303.0 ± 19.26**	288.3 ± 20.62**	294.2 ± 19.88**
Kit R	15	257.5 ± 20.11	288.3 ± 22.16**	303.9 ± 23.09**	295.9 ± 23.28**
Reflotron system	10	210.0 ± 15.26	234.7 ± 11.14*	214.3 ± 15.78*	217.4 ± 15.51*

Tab. 3 - Mean values ± SEM (mg/dl) of cholesterol in serum samples processed after separation and after storage at -20 °C with different methods. *(* $p<0.05$; ** $p<0.001$ vs fresh serum).*

fresh serum	stored serum		
	at 4 °C for 7 days	at -20 °C for 7 days	at -20 °C for 14 days
245.2 ± 12.71	242.8 ± 12.96	241.9 ± 13.01	233.6 ± 13.40

Tab. 4 - Mean values ± SEM (mg/dl) of cholesterol determined by the chemical method in fresh and stored serum.

In 10 serum samples cholesterol has been determined by the chemical method of BABSON et al.[4] before and after storage at 4 °C for 7 days and at -20 °C for 7 and 14 days. In no case storage resulted in an increase of cholesterol values (tab. 4).

As in the case of cholesterol, triglycerides determined by 4 different commercial kits resulted to be higher in serum stored at positive and negative temperatures than in fresh serum (tab. 5). Also apoproteins A-I and B were significantly higher in stored than in fresh serum (tab. 5).

	n°	fresh serum	stored serum			
			at 4 °C for 7 days	at -20 °C for 2h	at -20 °C for 7 days	at -20 °C for 14 days
apoproteins	20					
A-I		107.3 ± 4.14	121.2 ± 3.98*	-	122.1 ± 4.56**	128.0 ± 5.71**
B		86.5 ± 6.67	96.4 ± 4.51*	-	96.7 ± 3.94*	97.9 ± 3.90*
triglycerides						
Kit M	15	187.2 ± 24.04	-	224.5 ± 20.02*	-	-
	34	72.6 ± 7.48	92.5 ± 8.48*	-	95.5 ± 8.74*	95.1 ± 8.99*
Kit B	15	192.5 ± 22.76	-	224.9 ± 26.45*	213.5 ± 24.47**	212.5 ± 23.03**
Kit K	15	194.1 ± 20.78	-	223.3 ± 26.65*	218.1 ± 21.78**	221.6 ± 23.99**
Kit R	15	194.5 ± 21.71	-	220.1 ± 24.25**	217.9 ± 24.32**	219.8 ± 23.54**

Tab. 5 - Mean values ± SEM (mg/dl) of apoproteins A-I and B and of triglycerides (determined by 4 different commercial kits) in serum samples processed after separation and after storage. *(* $p<0.01$; ** $p<0.001$ vs fresh serum).*

	fresh serum	frozen serum	p
VLDL			
cholesterol	31.5 ± 7.67	40.3 ± 7.98	<0.01
triglycerides	103.0 ± 29.20	106.9 ± 28.79	<0.005
apoprotein B	31.2 ± 7.57	33.3 ± 8.56	ns
LDL			
cholesterol	111.9 ± 29.14	119.7 ± 27.55	<0.02
triglycerides	40.3 ± 9.62	46.5 ± 9.50	<0.005
apoprotein B	78.8 ± 8.68	85.2 ± 9.44	<0.01
HDL			
cholesterol	55.4 ± 3.06	57.9 ± 2.92	<0.05
triglycerides	24.0 ± 4.79	24.1 ± 4.39	ns
apoprotein A-I	108.3 ± 7.27	121.0 ± 8.92	<0.005

Tab. 6 - Mean values ± SEM (mg/dl) of lipoprotein lipids and apoproteins in 10 serum samples before and after 7 days of storage at -20 °C.

In 10 cases the main lipoprotein fractions were separated immediately after sampling, as described in the above section. Lipoprotein cholesterol, triglycerides and apoproteins were measured soon after fractionation and in lipoprotein fractions stored at -20 °C for 7 days. As it can be seen from tab. 6, in all the lipoprotein classes both lipids and apoproteins resulted to be higher in frozen than in fresh lipoprotein fractions.

DISCUSSION

Our data show that the fully enzymatic cholesterol determination results in higher values in stored than in fresh serum. The effects of storage are accelerated when the sample is frozen. In fact, freezing at -20 °C or at -80 °C determines an increase in cholesterol values only after 2h and the prolongation of the storage is not followed by further significant changes. Storage at positive temperature (4 °C) brings about a slower increase in cholesterol values than freezing. A significant rise in cholesterol level was in fact observed only after 3 days of storage.

Several factors may contribute to variations of results of enzymatic analysis. These include variations in the preparation of reagents and standards and technical skill. To minimize their effect, determinations in fresh and in stored serum were done with the same kit and by the same operators using the same instrumentation. However, after storage the changes in cholesterol as well as those in triglycerides and apoproteins were greater than predictable on the basis of the interassay imprecision of enzymatic and immunoturbidimetric methods in our operative conditions.

Other factors such as evaporation, bacterial growth[14], autooxidation[13] and serum lipolytic activities[10] may have influenced our results. Although serum samples were kept under conditions whereby such factors were eliminated or minimized, the possibility of their role in increasing cholesterol value after storage cannot be ruled out. However, the fact that a similar increase in cho-

lesterol has been observed in serum frozen for 2h or stored at positive and negative temperatures for a more prolonged period of time, strongly suggests that storage *per se*, rather than the above mentioned factors, may be responsible for the changes of the analytical response.

Enzymatic cholesterol assay methods involve several steps before the final colorimetric reaction[6]. Among these, cleavage of cholesterol esters by cholesterol esterase seems to play a key role[2,7,17]. It seems unlikely that storage affects enzymatic cholesterol determination by promoting the hydrolysis of cholesterol esters since an increase of both free and esterified cholesterol occurred. Moreover, cholesterol had a similar increase in stored serum when the enzymatic determination was performed with different cholesterol esterase preparations and in particular with a preparation, Kit B, which is claimed to yield a complete hydrolysis of cholesterol esters[15,17].

The parallel increase in cholesterol, triglycerides and apoproteins A-I and B in stored serum strongly suggests that the effects of storage are likely to be due to a modification of lipoprotein complexes. On the other hand, it is well known that storage and in particular freezing is associated with alterations in electrophoretic mobility and ultracentrifugal properties[9] of serum lipoproteins.

Apoproteins A-I and B show higher immunoreactivity in frozen than in fresh serum, possibly as a consequence of exposure of epitopes after freezing[8,12]. Similarly, cholesterol may increase because storage and freezing-thawing determine alterations in molecular stability of lipoproteins with release of lipids, so that the enzyme-substrate reaction is promoted. The fact that when cholesterol was determined by the chemical method of BABSON et al.[4] no significant difference in fresh and stored serum was observed is in favour of this possibility. In fact, the first step of the method involves the treatment of serum with lipid solvents (ethyl acetate and ethanol) which allow a complete solubilization of the cholesterol content of lipoproteins. Accordingly, ALLAIN et al.[2] and COOPER et al.[7] reported an underestimation of the cholesterol determined by an enzymatic method with respect to cholesterol determined by the chemical method of ABELL et al.[1]. BACHORIK et al.[5] found a slight decrease in the cholesterol concentration of stored (–70 °C) as compared to fresh heparin-$MnCl_2$ supernatant. Cholesterol was determined, according to the LRC Program[11], in isopropanol extracts of the serum samples. In different conditions, serum storage does not appear in general to affect cholesterol determination when it is made on serum extracts[16,18-20].

The fact that cholesterol was significantly higher in frozen than in fresh lipoprotein fractions suggests that the effects of the storage are similar in all the lipoprotein particles. This is not an unexpected finding. Although lipoprotein particles show different physical and chemical characteristics, they share a common structure which is represented by a surface rich in apoproteins and polar lipids and a core rich in cholesterol esters and triglycerides[3]. Thus, it is conceivable that freezing and in general storage can alter the structure of the macromolecular complexes, irrespectively of their dimension and composition, favouring the liberation of the inner components.

The rise of cholesterol in whole serum after storage then represents the result of an alteration of all the lipoprotein particles, in accordance with the electrophoretic and ultracentrifugal abnormalities described in stored lipoproteins[9].

In conclusion, the results of our study suggest that with the current enzymatic methods part of the lipoprotein cholesterol is not measured in fresh serum, while it is in frozen serum or serum stored at 4 °C for at least 3 days. Cholesterol measured in fresh serum seems then to be underestimated; however, this underestimation might have little clinical meaning providing that the reference values are obtained on fresh serum. At any rate, caution must be used before interpreting the clinical value of the serum concentration of cholesterol and of its changes when it is not known whether cholesterol has been measured in fresh or stored serum.

SUMMARY

Cholesterol determined by 4 different enzymatic commercial kits and by the dry chemistry Reflotron system was higher in serum stored at 4 °C and at –20 °C than in fresh serum. The effects of storage seem to be temperature-dependent. In fact, cholesterol values significantly increased only after 2h of freezing. The prolongation of freezing up to 2 weeks was not followed by further significant changes. In serum stored at 4 °C the increase in cholesterol was slower than in frozen serum. Both free and esterified cholesterol underwent an increase after storage. When cholesterol was determined by a chemical method (sulfuric acid-ferric chloride) after extraction with ethyl acetate and ethanol, no difference was observed in fresh and stored serum. Cholesterol, triglycerides and apoproteins A-I and B underwent parallel changes after storage both in whole serum and fractionated lipoproteins. Our findings strongly suggest that in serum stored at positive or negative temperature there is an alteration of the lipoprotein molecules which allows an easier availability of cholesterol for the enzyme-substrate reaction than in fresh serum. Current enzymatic methods underestimate (about 10%) cholesterol when the analysis is performed on fresh serum.

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