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Short Communication

Pigment Interferes with Cholesterol Analysis in Erythrocyte Lipid Extracts: A Procedure for Removal

Wen-Qi Wang and Anders Gustafson†

Department of Cell Biology I, University Hospital of Lund, S-221 85 Lund, Sweden

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A major problem in cholesterol analysis in erythrocyte lipid extracts is interference from co-extracted pigments. The content of such pigments is increased in lipid extraction with solvents containing polar alcohols (e.g., methanol), or water, or in repeated extractions. The pigment, which is believed to be heme,[1] affects cholesterol analysis by both chemical[2,3] and enzymatic methods,[4–6] and furthermore affects lipid separation in chromatography and scintillation counting.[1] Efforts to solve this problem have included extraction of lipids from cell ghosts,[7–9] usage of less polar alcohols in the extraction,[1,7] and resolution of lipids in redistilled chloroform.[1] In addition, a means of correcting for pigment absorbance has been suggested by a formula,[6] or by using a sample blank during cholesterol analysis.[1] Two weaknesses in these procedures are that less polar alcohol-containing solvents fail to extract phospholipids completely,[1] and that small pieces of cell membrane ghosts are easily lost, when removing the first hemolytic supernatant. The formula previously suggested by us[6] to correct pigment absorbance does not work if the amount of pigment is too high (> 0.4 absorbance at 400 nm). The simplest way might be the use of redistilled chloroform,[1] but this solvent has been suggested to be hazardous in man.[10] This problem was solved simply by redissolving the dried lipid extract in an isopropyl alcohol–silica gel mixture.

Materials

Analytical reagent-grade chemicals and solvents were used. Chloroform, methanol, toluene, 1-butanol and ether were obtained from Merck, D-6100 Darmstadt (Germany). Isopropyl alcohol was purchased from Chemicon AB (Malmö, Sweden); hexane from Labscan, Co. Dublin (Ireland), and cyclohexane from Sigma (St. Louis, USA). Enzymatic kits (CHOD–PAP reagent 236 691) for cholesterol analysis were from Boehringer Mannheim GmbH Diagnostica. 5(6)-Cholesten-3β-ol and cholesteryl oleate were used as references and obtained from Sigma (St. Louis, USA).

Experimental and discussion

Erythrocyte extraction. Aliquots of 0.4 ml of washed and packed erythrocytes were extracted with 7.5 ml of methanol–chloroform (2:1 v/v), at 30 min intervals and with methanol added 30 min before chloroform.

Recommended procedure for removal of pigments from erythrocyte lipid extraction. One ml of crude lipid extract supernatant was evaporated under nitrogen to dryness, to which were added 2 ml isopropyl alcohol and 0.05 g silica gel H. After mixing and centrifugation ($30 \times g$ for 2 min), aliquots of 0.2 ml supernatant were used for cholesterol analysis with isopropyl alcohol–CHOD–PAP reagent (236 691).[6]

Increasing the amount of silica gel (0.03 to 0.20 g) promoted the capacity of the isopropyl alcohol–silica gel system in removal of the pigment (absorbance > 2.0 per ml extract supernatant, measured at 400 nm) without affecting the cholesterol concentration in the isopropyl alcohol. Similar results were obtained with the use of silica gel DG and silica gel 60 H.

Whenever the cholesterol content was lower in the supernatant of the isopropyl alcohol–silica gel mixture, more supernatant was evaporated and then redissolved in 0.2 ml isopropyl alcohol for cholesterol analysis. Alternatively, more lipid extract can be evaporated and redissolved in the isopropyl alcohol–silica gel mixture. A prewash of the silica gel is unnecessary.

Development of the recommended procedure. The recommended procedure addresses the problem of whether the extracted pigment from erythrocytes has been eliminated

† To whom correspondence should be addressed.

Table 1. Changes in pigment absorbance and cholesterol concentration in supernatants of solvents after addition of silica gel ($n=6$, mean$\pm$SD).[a]

Solvent	Pigment absorbance	After silica gel addition	
		Pigment absorbance	Cholesterol (μg|0.2 ml)
Cyclohexane	0.113(10)	−0.018(1)	0.11(6)
Hexane	0.122(12)	−0.019(1)	0.12(5)
Toluene	0.108(3)	−0.014(1)	4.19(13)
Chloroform	0.131(14)	−0.018(1)	7.85(11)
Isopropyl alcohol	0.078(15)	0.017(4)	7.89(8)
Butanol	0.178(3)	0.008(2)	7.89(5)
Methanol	0.292(57)	0.103(4)	7.84(12)
Methanol–chloroform 1:1	0.287(45)	0.109(5)	7.90(11)
Methanol–chloroform–water 1:1:0.1	0.286(3)	0.141(4)	7.95(7)
Methanol–chloroform–water 1:1:0.2	0.291(7)	0.183(1)	7.96(11)
Methanol–chloroform–water 1:1:0.35	0.308(14)	0.225(5)	8.14(4)
Control[b]	0.291(8)		7.91(5)

[a] To dried erythrocyte lipid extract were added 2 ml of solvents and then 0.05 g of silica gel H. The pigment absorbance in the supernatant was measured against methanol at 400 nm. The cholesterol concentration was determined with the kit method.[6] Pigment absorbance was substracted. [b] Control: dried erythrocyte extracts were redissolved in 2 ml of methanol–chloroform (1:1) without the addition of silica gel.

in the silica gel without affecting the cholesterol concentration. We found (Table 1) that the solubility of pigment in solvents or solvent mixtures is related to the polarity of the solvents in the order polar solvents (methanol-containing solvents) > medium polar solvents (butanol, isopropyl alcohol) > non-polar solvents (hexane, cyclohexane, toluene, chloroform), as well as to the relative amount of water in the solvent mixture. Addition of silica gel, however, reduced the pigment content in all these solvents. It appeared that silica gel binding is more pronounced in non-polar and non-water-containing solvents than in polar and water-containing solvents. For unknown reasons, silica gel removed relatively more extracted erythrocyte pigment than the heme standard.

Cholesterol concentration was also reduced by the silica gel addition in most solvents, but not in isopropyl alcohol, chloroform or butanol. This phenomenon was reproduced using the cholesterol standard (up to 250 μg/2 ml).

The difference in the capacity of silica gel for binding pigments and cholesterol makes it possible to remove the pigment with silica gel without significantly affecting the cholesterol concentration. Isopropyl alcohol is recommended since the cholesterol content in the solvent can be directly quantitated using our previously recommended enzymatic kit. It is also superior to butanol since it is easier to evaporate off, when needed. Chloroform may be used, although we observed that the standard cholesterol concentration was 4.2% ($p<0.01$, $n=8$) lower than that in the control.

According to our previous findings,[6] a pigment absorbance of less than 0.1 at 400 nm does not affect cholesterol analysis at 500 nm. With the recommended isopropyl alcohol–silica gel system, the content of pigment in the crude erythrocyte extract was reduced to less than 0.020 absorbance at 400 nm, from the combined effects of pigment binding in the silica gel, dilution with isopropyl alcohol, and reduced solubility of pigment in isopropyl alcohol.

Direct addition of silica gel to an isolated erythrocyte extract supernatant failed to remove most of the pigment. According to the data in Table 1, this may be due to the reduced capacity of silica gel to bind pigment in water-containing solvents.

Summary

Removal of pigments from erythrocyte lipid extracts was achieved simply by adding isopropyl alcohol (2 ml) and silica gel H (0.05 g) to an aliquot of dried extract. This procedure is superior to other available methods because of its simplicity and high efficiency.

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