Nutrición Hospitalaria

Nutr Hosp. 2015;32(4):1535-1540
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Original/*Pediatría*

Determination of cholesterol in human milk: an alternative to chromatographic methods

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Abstract

Introduction: **human milk (HM) is considered the best option for feeding healthy infants. Cholesterol (CHOL) is important for proper development of the nervous system, and for hormone and vitamin synthesis in growing infants. Breastfeeding and dietary CHOL intake during infancy have been suggested to affect blood lipid levels and the risk of cardiovascular disease in adulthood. Gas chromatography is the technique most widely used to determine CHOL in HM. Chromatographic methods are specific for the determination of CHOL and other sterols present in HM, but are extremely time consuming, and the costs and equipment requirements mean that they are not accessible to all laboratories.**

Aim: **the present study describes the optimization and validation of an enzymatic-spectrophotometric method for CHOL determination in mature HM.**

Method: **determination of CHOL involves fat extraction with chloroform:methanol, hot saponification and extraction of the unsaponifiable fraction with diethyl ether. CHOL was determined by an enzymatic method in which the concentration of the lutidine dye formed is stoichiometric to the amount of CHOL, and is measured by the increase in light absorbance at 405 nm.**

Results: **human milk fat (mg/mL) (27.5 ± 1.3) and CHOL (0.113 ± 0.004) in analyzed HM were within the ranges reported by others authors. Analytical parameters of the proposed method were assessed: The precision values (%) (expressed as the relative standard deviation (RSD)) were: 3.5 and 6.7 for intra- and inter-day, respectively. Accuracy, estimated by recovery assays, was 110 ± 1.6%.**

Conclusion: **the validated enzymatic-spectrophotometric method for determining CHOL in HM constitutes an alternative for fast and simple analysis of CHOL with equipment requirements accessible to any laboratory.**

(*Nutr Hosp*. 2015;32:1535-1540)

DOI:10.3305/nh.2015.32.4.9139

Key words: *Human milk. Cholesterol determination. Enzymatic-spectrophotometric method. Validation.*

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Recibido: 17-IV-2015.
Aceptado: 26-VI-2015.

DETERMINACIÓN DEL COLESTEROL EN LECHE HUMANA: UNA ALTERNATIVA A LOS MÉTODOS CROMATOGRÁFICOS

Resumen

Introducción: **la leche humana (HM) se considera el modo óptimo de alimentación en lactantes sanos. El colesterol (CHOL) es importante para el correcto desarrollo del sistema nervioso y la síntesis de hormonas y vitaminas en el crecimiento del lactante. Se ha constatado que la lactancia materna y la ingesta dietética de CHOL durante la infancia influye en los niveles de lípidos en sangre, así como en el riesgo de enfermedad cardiovascular en la edad adulta. La técnica más utilizada para determinar el CHOL en HM es la cromatografía de gases. Los métodos cromatográficos son específicos para la determinación del CHOL y otros esteroles presentes en la HM, pero el elevado tiempo consumido, los costes y la necesidad de una instrumentación específica hacen que no sea accesible para cualquier laboratorio.**

Objetivo: **el presente estudio describe la optimización y validación de un método enzimático-espectrofotométrico para la determinación del CHOL en HM madura.**

Métodos: **la determinación del CHOL requiere una extracción lipídica con cloroformo:metanol, saponificación en caliente y extracción del insaponificable con dietil éter. El CHOL fue determinado por un método enzimático en el que la concentración de lutidina formada es estequiométrica a la cantidad de CHOL, y se mide a 405 nm.**

Resultados: **la cantidad de grasa (mg/mL) (27,5 ± 1,3) y de CHOL (0,113 ± 0,004) en la HM analizada se halla en el intervalo indicado por otros autores. Se evalúan parámetros analíticos del método propuesto: la precisión (expresada como desviación estándar relativa en %) fue de 3,5 y 6,7 para intra- e interdía, respectivamente. La exactitud, estimada mediante ensayos de recuperación, fue de 110 ± 1,6%.**

Conclusión: **el método enzimático-espectrofotométrico validado para determinar el CHOL en HM constituye una alternativa para el análisis rápido y sencillo de CHOL empleando equipos accesibles para cualquier laboratorio.**

(*Nutr Hosp*. 2015;32:1535-1540)

DOI:10.3305/nh.2015.32.4.9139

Palabras clave: *Leche humana. Determinación de colesterol. Método enzimático-espectrofotométrico. Validación.*

Abbreviations

CHOL: cholesterol.
CVD: cardiovascular disease.
HM: human milk.
HPLC: high-performance liquid chromatography.
LDL-C: low density lipoprotein-cholesterol.
MFGM: milk fat globule membrane.
ppm: parts per million (mg/L).
RSD: relative standard deviation.

Introduction

Human milk (HM) is considered the best option for feeding healthy infants, since it provides optimal nutrition from birth. Infants with exclusive breastfeeding show adequate weight gain and suffer fewer diseases in the first year of life[1]. The main energy source for healthy breastfed infants is strongly correlated to milk fat, providing 40-55% of the total energy. The fat is present in HM in the form of milk fat globules surrounded by milk fat globule membrane (MFGM) formed by the mammary alveolar cells. These MFGM in HM are the principal source of cholesterol (CHOL)[2]. Cholesterol is a major constituent in the mammalian body, serving as a functional and structural component of the cell membrane. CHOL is important for proper development of the nervous system, and for hormone and vitamin synthesis in growing infants[3]. Regarding development of the nervous system, CHOL acts as the major architectural component of compact myelin in this growth stage of life[4].

Variable CHOL contents (expressed as mg/100 mL) have been reported in mature HM by different authors: 9-15[2], 3.4-13[3], 6.5-18.4[5], 7.3-14[6], and 9.5-29[7]. With respect to colostrum, the CHOL content is slightly higher according to some authors[8], while others studies[7,9] indicate that during the course of human lactation the CHOL concentration decreases 50% in mature milk. Infant formulas have much lower CHOL contents (0-0.4 mg/100 mL), and this is the reason for the higher serum total CHOL and low density lipoprotein (LDL)-CHOL levels in breastfed infants compared with formula–fed infants[2]. There is growing evidence that atherosclerotic vascular changes, and the risk of coronary heart disease, begin to emerge from childhood. Breastfeeding and dietary CHOL intake during infancy have been suggested to affect blood lipid levels and the risk of cardiovascular disease (CVD) in adulthood. In this sense, a high CHOL intake from breast milk in early life might program CHOL metabolism against diet–induced hypercholesterolemia in later life, reducing the endogenous synthesis of CHOL and CVD prevalence by as much as 5%. However, it has also been suggested that breastfeeding has early but not long-term effects upon the rate of CHOL synthesis[2,10,11].

CHOL determination in HM involves extraction of the lipid fraction, followed by a saponification process with different alkali reagents (potassium or sodium hydroxide in ethanolic or methanolic medium). Fat extraction has been carried out with different solvents such as hexane:2-propanol[5], acetone:petroleum ether[6] or chloroform:methanol[12]. Other studies have performed direct saponification of the HM sample followed by extraction of the unsaponifiable with chloroform[12], hexane[3,6,7,13-15] or heptane[16].

The most widely used technique for determining CHOL in HM is gas chromatography with mass spectrometry or flame ionization detection[5,6,12,15,16]. High-performance liquid chromatography (HPLC) has also been used by Haug and Harzer[14] and Ramalho et al.[7] – the latter authors having validated the technique for application to HM. Recently, CHOL in HM has also been determined by Fourier Transformed infrared spectroscopy[3,6]. However, chromatographic methodologies specific for the determination of CHOL and other sterols present in HM are time consuming, and the costs and equipment requirements mean that they are not accessible to all laboratories. Enzymatic-spectrophotometric methods have also been used for CHOL determination in HM. These methods are nonspecific, because they cannot distinguish between CHOL and other sterols; nevertheless, the analyses hardly ever overestimate the CHOL content, considering that the presence of other sterols is about 20 times lower than the CHOL content in HM. In this regard, Haug and Harzer[14] compared HM CHOL contents determined by HPLC and enzymatic-spectrophotometric methods - no significant differences being observed between the two techniques (19.2 versus 22.8 mg/100 mL HM, respectively).

Enzymatic-spectrophotometric[17,18] or enzymatic-fluorometric[19] methods have been validated and applied to estimate CHOL contents in bovine milk samples. Enzymatic-spectrophotometric methods, based on the descriptions of Gentner and Haasemann[20], for CHOL determination in bovine milk have also been used in HM[8,13,14], though without validation for samples of this kind.

The present study describes the optimization and validation of an enzymatic-spectrophotometric method for CHOL determination in mature HM as an alternative approach for fast and simple analysis of CHOL.

Materials and methods

Samples

A pool of mature HM from four healthy, well-nourished, non-smoking volunteer mothers without caloric restriction was used for this study. The age of the women was 25-35 years, gestation was > 37 weeks, and the infants had normal weight at birth. The study was approved by the ethics committee of the Hospital Universitario Puerta del Hierro (Majadahonda, Madrid, Spain), and all donors gave informed consent to participation in the study.

Methodology

Fat extraction and saponification, with subsequent extraction of the unsaponifiable fraction were carried out before CHOL determination.

Fat extraction and unsaponifiable fraction: Lipids were extracted according to the procedure of Alemany-Costa et al.[21] Briefly, 2 mL of HM was taken, and 40 mL of a chloroform:methanol mixture (1:1, v/v) (Merck & Co., Inc., Whitehouse Station, NJ, USA) containing 0.05% of butylhydroxytoluene (Sigma Chemical Co., St. Louis., MO, USA) was added, followed by homogenization (Polytron PT 2000, Kinematica AC, Switzerland) during three min at 250 W. After adding 20 mL of chloroform and mixing again with the Polytron, the sample was filtered through a Buchner funnel without vacuum. Twenty mL of a 1 M potassium chloride solution (Merck & Co., Inc., Whitehouse Station, NJ, USA) were added to the filtrate and kept at 4ºC overnight. Then, after phase separation, the chloroform phase was concentrated in a rotary evaporator (bath at 40ºC) and taken to dryness under a nitrogen stream. Saponification of the lipid fraction was likewise performed according to Alemany-Costa et al.[21]. Briefly, 3 mL of 1N potassium hydroxide (Poch, S.A., Sowinskiego, Poland) in methanol was added to the lipid fraction, and a hot saponification step at 65ºC during one hour was performed. The unsaponifiable fraction was extracted with 20 mL diethyl ether (Merck & Co., Inc., Whitehouse Station, NJ, USA) and filtered (Whatman no. 1.90 mm, Maidstone, England). Then, it was taken to dryness under a nitrogen stream and dissolved with 2 mL isopropanol (Merck & Co., Inc., Whitehouse Station, NJ, USA).

CHOL determination: The determination of CHOL was performed using an enzymatic method (Boehringer Mannheim / R-Biopharm AG, No. 10139050035, Darmstadt, Germany) in which CHOL is oxidized by CHOL oxidase to cholestenone. In the presence of catalase, the hydrogen peroxide produced by this reaction oxidizes methanol to formaldehyde. The latter in turn reacts with acetyl acetone, forming a yellow lutidine dye in the presence of ammonium ions. The concentration of the lutidine dye formed is stoichiometric to the amount of CHOL, and is measured by the increase in light absorbance at 405 nm.

Analytical parameters

Precision: Intra-day precision was evaluated by the analysis of four replicates on the same day, while inter-day precision was evaluated by 8 replicates on two different days. The results are expressed as the relative standard deviation (%RSD).

Accuracy: In order to evaluate the accuracy of method recovery, assays from HM samples were done. To check the accuracy of the enzymatic spectrophotometric method, determinations of different quantities of CHOL standard solution were made.

Statistical analysis

In order to evaluate differences in function of type of fat extraction, a one-way analysis of variance (ANOVA) was applied to the results obtained, followed by Tukey's post hoc test. A significance level of $p<0.05$ was adopted for all comparisons. The Statgraphics® Centurion XVI statistical package (Startpoint Technologies Inc., USA) was used throughout.

Results and discussion

Optimization of lipid extraction

The sample/solvents ratio and use of filtration with or without vacuum or by centrifugation were optimized.

Two different volumes of the extractant solvents for lipid extraction were assayed in application to 4 mL of HM: a) 80 mL of chloroform:methanol (1:1) (v/v) + 40 mL of chloroform; and b) 40 mL of chloroform:methanol (1:1) (v/v) + 20 mL of chloroform with vacuum filtration. The values obtained are shown in table I. No

Table I
Human milk: optimization of fat extraction

Human milk (mL)	*Solvent volume (mL) (C:M + C)*	*Filtration or centrifugation*	*Fat content**	
			mg	*mg/mL*
4	40 + 20	Vacuum	110.9±12.8	27.7±3.2[a]
	80 + 40	Vacuum	110.0±15.3	27.5±3.8[a]
2	40 + 20	Vacuum	47.7±2.3	23.8±1.2[y]
		Without vacuum	55.0±2.6	27.5±1.3[z]
		Centrifugation	44.0±1.0	22.0±0.5[y]

C= chloroform; M= methanol. *Values are expressed as mean±standard deviation of three replicates. Different superscript letters denote significant differences ($p<0.05$) between different solvent volume applied to 4 mL of human milk (a) or between filtration or centrifugation applied to 2 mL of human milk and fixed solvent volume (y-z).

significant differences ($p<0.05$) in fat content according to the extractant volumes used were observed. Fat contents (~27 mg/mL) were within the range reported by other authors (16-64 mg/mL of mature HM[6,12,22]).

Given the difficulty of collecting HM for scientific studies from mothers who are simultaneously feeding their babies, fat extraction with half of the HM volume (2 mL) was carried out, employing 40+20 of solvent volume and applying vacuum filtration. The fat contents obtained (see Table I) were lower (23.8 mg/mL), though precision improved (RSD = 5%). Filtration without vacuum and centrifugation (at 3500 rpm, 0°C, during 10 min) was also evaluated (Table I). The best balance between precision and fat content corresponded to filtration without vacuum (27.5 mg/mL, RSD = 4.7%). Therefore, the finally selected conditions were 2 mL of HM and fat extraction with 40+20 of solvent volumes, without vacuum application in the filtration – this protocol being easier and faster than the other options.

Analytical parameters

Precision:

Results corresponding to intra- and inter-day precision are shown in table II. The intra- and inter-day precision values obtained are consistent with the acceptable RSD percentages obtained from the Horwitz function and from the Association of Official Analytical Chemists Peer Verified Methods program[23], which indicate that for an analyte content of 100 ppm, the acceptable %RSD range is 5.3-8.

The CHOL content in the analyzed HM (Table II) was within the range indicated in the literature (0.03-0.29 mg of CHOL/mL HM)[2,3,5-7].

Table III summarizes different validated methods applied to determinate CHOL in HM or milk. On comparing inter- and intra-day precision with other authors from enzymatic assays applied to determine CHOL in milk (Table III), our values (3.5% and 6.7% for intra- and inter-day precision, respectively) agree with those of Larsen.[19], who reported an intra-plate precision of 2.7% and an inter-plate precision of 7.5% for total CHOL, and are higher than the intra-day precision reported by Saldanha et al. (0.82%)[17]. However, our values are lower than those found by Viturro et al. (intra- and inter-assay 4.8% and 9.1%, respectively)[18].

Accuracy:

CHOL standard solutions: On assaying different CHOL amounts between 0.015-0.100 mg per assay (Table II), a good correlation was obtained between the theoretical and experimental CHOL amounts (r=0.998).

Table II
Cholesterol determination: precision and accuracy results

PRECISION (%RSD)			
Human milk	*Cholesterol (mg/mL)*	*Intra-day (n=4)*	*Inter-day (n=8)*
	0.113±0.004	3.54	6.72

ACCURACY (mg cholesterol in assay)			
Cholesterol standard solution (n=3)	*Theoretical*	*Experimental*	*Recovery (%)*
	0.015	0.016±0.004	109.7±23.1
	0.020	0.025±0.002	117.5±3.5
	0.030	0.031±0.000	103.0±0.0
	0.040	0.044±0.001	110.0±3.5
	0.050	0.050±0.004	105.0±1.4
	0.075	0.076±0.006	101.3±7.5
	0.100	0.105±0.007	105.0±7.1

	Present	*Added*	*Found*	*Recovery (%)*
Human milk (n=7)	0.022	0.050	0.076	110
	0.021		0.077	112
	0.020		0.076	110
	0.021±0.001		0.075	108
			0.075±0.001	110±1.6

Results expressed as mean± standard deviation.

Table III
Cholesterol determination: validated methods

Sample	*Method*	*Cholesterol (mg/mL)*	*Precision RSD (%)*	*Recovery (%)*	*References*
Human milk	Enzymatic/Colorimetric	0.113±0.004	3.5-6.7	110	This study
Human milk	HPLC-DAD	0.129±4.06	<3	>96	Ramalho et al. 2011
Whole milk	Enzymatic/UV	0.100±0.0009	0.8	97	Saldanha et al. 2004
	HPLC-UV	0.100±0.0009			
Bovine milk	Enzymatic/Colorimetric	0.263±0.119	4.8-9.1	98-106	Viturro et al. 2009
Milk	Enzymatic/FL	0.136	2.7-7.5	–	Larsen et al. 2012

DAD: Diode array detector; FL: Fluorimetric; HPLC: high performance liquid chromatography; UV: ultraviolet.

Recovery assays: Three non-spiked aliquots of HM and four aliquots spiked with 0.050 mg of CHOL in the assay were analyzed. Percentage recovery was calculated as: (CHOL spiked aliquots - CHOL in non-spiked aliquots) × 100)/(spiked CHOL). The recovery values obtained (110±1.63%) are close to the accepted percentage recovery values depending on the analyte level involved (Table II), indicated by González and Herrador[23] from the Association of Official Analytical Chemists Peer Verified Methods program, which states that for an analyte content of 100 ppm, the acceptable percentage recovery range is 90-107%. The percentage recovery values were similar or slightly higher than those obtained by other authors from milk using enzymatic methods (97%[17] and 98-106%[18]).

Conclusions

The present optimized and validated method for CHOL determination is an analytical option that is less expensive, faster, and can be applied to a larger number of HM samples in which CHOL is the main sterol and the levels of other sterols are about 20 times lower than those of CHOL. Furthermore, the method does not require chromatographic instrumentation – a fact that facilitates its use in all types of laboratories.

Acknowledgments

Thanks are due to Hero España, S.A. for financial support of this study.

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