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Journal of Chromatography B, 718 (1998) 23–32

JOURNAL OF CHROMATOGRAPHY B

Method for simultaneous measurements of traces of heptadeuterated cholesterol and cholesterol by gas chromatography–mass spectrometry: application in humans

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Received 3 March 1998; received in revised form 23 July 1998; accepted 23 July 1998

Abstract

An assay was developed to quantify deuterated cholesterol (used as a tracer) and cholesterol using gas chromatography–mass spectrometry. Ergosterol and epicoprostanol were used as internal standards. Deuterated cholesterol was quantified by comparing its peak area to that of epicoprostanol and cholesterol to ergosterol. The mean absolute recovery in spiked serum was 99.96%; the precision was in the range 0.16–10.9% and accuracy 90.4–100%; the limit of detection in plasma was $3 \cdot 10^{-5}$ mmol l^{-1}. Using two internal standards, the method described herein seems particularly suitable for application in humans i.e., measuring traces of deuterated cholesterol (range: 0–$6.26 \cdot 10^{-4}$ mmol l^{-1}) along with natural cholesterol (range: 0.065–4.42 mmol l^{-1}) in human plasma and lipid fractions postprandially.

Keywords: Isotopes; Cholesterol

1. Introduction

Dietary cholesterol has been implicated in the etiology of atherogenesis for decades. Nevertheless the study of its postprandial metabolism, based on the safe use of stable isotopes, is more recent in humans [1]. Several methods for plasma cholesterol measurement have already been described. Colorimetric maximal method with Abell–Levy–Brodie–Kendall (ALBK) extract procedure [2] is still the reference method for quantification of plasma cholesterol (Center for Disease Control, Atlanta, GA, USA) [3]. Isotopic dilution gas chromatography–mass spectrometry (GC–MS) method has now been proposed as a definitive method by the American Association of Clinical Chemistry given its higher precision and sensitivity [4–7]. Chemical ionization of molecules is an appropriate method for quantitative application [4,5,8] as well as electron impact ionization [6,7]. In this case, a lot of ions are produced and the most stable ones are chosen, i.e., for cholesterol m/z 458, 368, 329 among which 329 is predominant. While cholesterol separation is performed on a non-polar packed column in a method reported by Cohen et al. [5], more recent methods use non-polar capillary columns such as DB5 [4],

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0378-4347/98/$19.00
PII: S0378-4347(98)00364-8

DB17 [6] or DB1 [7] with reduced retention times and improved separations.

Different authors used either ergosterol, epicoprostanol or 5α-cholestane as secondary standard only in order to confirm reproducibility [9,10]. The very large pool of endogenous cholesterol makes physiological tracer studies difficult because of the large dilution of labeled cholesterol [8]. For a precise analysis in isotopic dilution conditions, the tracer-to-tracee ratio must preferably be close to 1. Therefore, for the best accuracy, two internal standards (ergosterol and epicoprostanol) have now been introduced in the beginning of the protocol in our method, in the same ranges of concentration as plasma cholesterol and heptadeuterated cholesterol, respectively.

We developed such a GC–MS method in order to measure the occurrence of dietary cholesterol in plasma and lipoprotein fractions during the postprandial period. In fact, despite increasing interest in cholesterol matter, few GC–MS studies have paid attention to its dietary counterpart [11–14]. During studies the amount of ingested heptadeuterated cholesterol in humans is low for evident ethic, physiological and economic reasons. Moreover, the step of intestinal absorption reduces almost by half the amount of heptadeuterated cholesterol to be found in plasma [11]. The level of cholesterol from dietary origin is then at about 10^{-4} mmol l^{-1}, whereas total plasma cholesterol is about 5 mmol l^{-1} [13,15]. The applicability of the adapted method described herein to the follow-up of dietary deuterated cholesterol in plasma and chylomicrons for 72 h in human subjects illustrates its suitability for metabolic studies. This method successfully achieves our methodological goals i.e., low sample volume, minimum handling of samples and good precision and accuracy.

2. Experimental

2.1. Reagent

Heptadeuterated cholesterol (D7-chol) (25,26,26,26,27,27,27-$^{2}H_{7}$; 98% enrichment) was supplied by Eurisotop (St. Aubin, France), ergosterol (Ergo) by Fluka (Buchs, Switzerland), cholesterol (Chol), epicoprostanol (Epi), bistrimethylsilyltrifluoroacetamide (BSTFA), chlorotrimethylsilane (TMCS) by Sigma (St. Quentin-Fallavier, France), ethanol, heptane, by Carlo Erba (Rueil Malmaison, France), Triton X-100 by Bayer Diagnostics (Tournai, Belgium). All chemicals used in this study were of analytical-reagent grade.

2.2. Human subjects and test-meals

Seven adult (aged 20–28 years) male volunteers participated in the study after giving written informed consent to a protocol approved by the Medical Ethics Committee. All were normolipemic and had E3/E3 Apo E genotype.

The three experimental test-meals consisted of commercially available food and contained 14.8% protein, 49.1% lipid (63 mg natural Chol) and 36.1% carbohydrate. In the first test-meal only, 80 mg D7-chol was dissolved in 25 g margarine. The two other test-meals were consumed at h8 and h24, whereas at h14, the subjects ingested a meal without lipids.

2.3. Biological samples

A baseline (h0) fasting blood sample was collected before the first test-meal. Ten blood samples were obtained at h3, h6, h8, h11, h14, h24, h27, h30, h48, h72 after the labeled test-meal. Blood was collected in tubes containing EDTA and a protease inhibitor cocktail was added as reported [16]. Plasma was separated from whole blood by centrifugation (10 min, 1547 *g*, 10°C). On the first test-meal, the chylomicron fraction containing chylomicrons plus large chylomicron remnants was isolated as adapted from methods described previously [17,18] from 1.5 ml plasma layered under 1.5 ml (0.9%) NaCl by ultracentrifugation at 15°C (35 000 *g* for 6 min) in a Beckman (Palo Alto, CA, USA) TLX 100 ultracentrifuge and 100.3 rotor.

2.4. Internal standard (I.S.) selection

Two I.S.s were required because the difference between Chol (mean: 5 mmol l^{-1}) and D7-chol (μmol l^{-1} range) concentrations. Ergo and Epi were chosen as I.S. for Chol and D7-chol, respectively. Epi is not present in blood and natural Ergo only

represents traces as compared to added Ergo as I.S. Moreover, Chol and Ergo on the one hand or D7-chol and Epi on the other hand, have a good chromatographic resolution. All these sterols present a 3α-hydroxyl group and can be derivatized to their trimethylsilyl (TMS) ethers.

2.5. Solutions of standards and internal standards

Chol (386.7 mg), Ergo (198.3 mg), Epi (38.87 mg) and D7-chol (39.4 mg) were dissolved in 100 ml ethanol to give concentrations of 10, 5, 1 and 1 mmol l^{-1}, respectively. These solutions were further diluted to give the required concentrations.

2.6. Hydrolysis reagent

We combined 7.0 ml aqueous 6.4 *M* potassium hydroxide with 0.62 g Triton X-100 dissolved in about 100 ml absolute ethanol and brought the total volume to 125 ml with absolute ethanol.

2.7. Preparation of calibration curves

For Chol analysis in plasma, different standard solutions were prepared to contain 0.1, 0.25, 0.5, 0.75, 1, 3, 5, 7 and 9 mmol l^{-1} Chol along with 5 mmol l^{-1} Ergo (I.S.). For D7-chol analysis, the standard solutions were prepared to contain 0.05, 0.1, 0.5, 1, 5 and 10 μmol l^{-1} D7-chol along with 5 mmol l^{-1} Chol to mimic plasma conditions and 1 μmol l^{-1} Epi (I.S).

For chylomicron fraction analysis, the same solutions were diluted 10-fold with ethanol. Calibration curves were obtained by plotting the peak-area ratios of Chol or D7-chol to respective I.S. versus the amounts of Chol or D7-chol.

2.8. Extraction procedure

Two hundred μl Epi (1 μmol l^{-1} solution) and 900 μl hydrolysis reagent were added to 200 μl plasma in 15-ml glass-stoppered test tubes. The test tubes were placed in a 70°C water-bath for 1 h. After briefly cooling, 200 μl Ergo (5 mmol l^{-1} solution), 2 ml distilled water and 5 ml hexane were added. After Vortex mixing, then centrifugation, 3 ml supernatant were evaporated to dryness under a stream of dry nitrogen. Derivatizing reagent (0.3 ml BSTFA–TMCS, 4:1, v/v) was added and left for 1 h at room temperature. After 0.2 ml hexane addition and mixing, 1 μl of the extract was injected on the GC column. Standards, plasma and chylomicron fraction were treated in the same way.

2.9. GC–MS conditions

GC–MS was carried out using selective ion monitoring (SIM) mode on a Hewlett-Packard 5890 serie II gas chromatograph fitted with a Hewlett-Packard 7673 automatic sampler and interfaced to a Hewlett-Packard 5972 A mass spectrometer. The temperatures of transfer line, ion source, quadrupole and electron multiplier were 280, 195, 155 and 110°C, respectively.

The HP-5MS column (30 m×0.25 mm I.D., 0.25 μm film thickness) coated with cross-linked 5% phenyl methyl silicone was used. A splitless injection mode was selected with a solvent delay time of 5 min. The initial temperature of the column was held at 250°C for 1 min, programmed at 30°C/min to 280°C. Injection port was set at 280°C. Helium was used as carrier gas with a flow-rate of 1.0 ml/min. Ions of *m/z* 329, 336, 363 and 370, obtained by electronic impact, were monitored for quantification of Chol, D7-chol, Ergo and Epi, respectively, as the most stable and most abundant ions.

2.10. D7-chol quantification

D7-chol was always quantified in presence of Chol in order to mimic plasma conditions.

Ion *m/z* 336 was chosen to represent D7-chol as the most stable and most abundant ion present in its mass spectrum. However, Chol fragmentation also gives ion *m/z* 336: this ion is in negligible quantity in relation to Chol, but can influence D7-chol quantification because Chol and D7-chol are not resolved. We have determined the 336/329 ratio from Chol calibration curve in order to determine a correction factor that could be used for calculation of actual D7-chol concentration.

For D7-chol quantification, the time zero (h0) sample (collected before D7-chol intake) was used as a blank matrix sample.

2.11. Validation criteria

2.11.1. Linearity

Working standards were prepared as described above. Nine reference samples were used for calibration curve of Chol (0.1 to 9 mmol l^{-1}) and six for calibration curve of D7-chol (0.05 to 10 μmol l^{-1}). I.S.s were included at constant concentration of 5 mmol l^{-1} for Ergo and 1 μmol l^{-1} for Epi in order to obtain an area ratio close to one with Chol and D7-chol, respectively. Each determination was done six-fold. The calibration factors were calculated according to least-squares linear regression.

2.11.2. Precision and accuracy

Precision and accuracy were determined for both inter- and intra-day variability. These measurements were made by GC–MS analyses of Chol and D7-chol standards at nine and six concentrations, respectively, on six consecutive days (inter-day variation or reproducibility).

Seventeen quality control samples for Chol (5 mmol l^{-1}) and 10 for D7-chol (1 μmol l^{-1}) were analysed during the same day (intra-day variation or repeatability).

2.11.3. Analytical recovery

For D7-chol, analytical recovery was determined by comparing peak area ratios of plasma spiked extracts to those obtained on standard solutions at the same concentration. The assay was made for five concentrations and each was analysed in triplicate.

2.11.4. Limit of detection (LOD) and limit of quantification (LOQ)

The signal given at 13.10 min for m/z 336 with a signal-to-noise ratio of 3 was determined as LOD (Fig. 2). The signal given at 13.10 min for m/z 336 with a signal-to-noise ratio of 10 was determined as LOQ [19].

3. Results and discussion

Mass spectra and formulas of Chol, Ergo, D7-chol and Epi are shown in Fig. 1.

3.1. Specificity and D7-chol quantification

Chol and its I.S. (Ergo) present a good resolution, $R_s = 3.90$, as well as D7-chol and Epi, $R_s = 4.57$ (Fig. 2). Given natural cholesterol fragmentation generates some ion m/z 336 (with a very low abundance), we applied a correction factor to allow actual measurement of ion m/z 336 generated from D7-chol only: the evaluated value was $1.4 \cdot 10^{-4}$ representing the mean area ratio m/z 336/329 for Chol in the range of concentrations used. The abundance of m/z 336 due to Chol fragmentation can thus be calculated in relation to its concentration:

$$\text{Chol abundance } m/z\ 336_{\text{calculated}} = \text{abundance } m/z\ 329 \times 1.4 \cdot 10^{-4}$$

The actual abundance for m/z 336 issued from D7-chol fragmentation is:

$$\text{Actual D7-chol abundance } m/z\ 336 = \text{total abundance } 336_{\text{detected}} - \text{Chol abundance } m/z\ 336_{\text{calculated}}$$

3.2. Linearity

To determine linearity (Fig. 3), the data were fitted to a line by the equation $y = ax + b$ where y = Chol area/Ergo area or D7-chol area/Epi area, b = intercept and a = slope.

For Chol, over the range 0.1–9 mmol l^{-1}, a linear fit was used satisfactorily with a mean r^2 of 0.998: area ratio m/z 329/363 = Chol concentration + 0.013.

For D7-chol, over the range 0.05–10 μmol l^{-1}, the mean r^2 was 0.999 and the equation was: area ratio at m/z 336/370 = 0.998 × D7-chol concentration + 0.017.

3.3. Precision and accuracy

The intra-day precision (repeatability) established in the same tray on 17 assays for Chol (5 mmol l^{-1}) and 10 assays for D7-chol (1 μmol l^{-1}) gave a good precision with C.V. = 2.03 and 4.84%, respectively (Table 1).

The inter-day precision (reproducibility) was clearly satisfactory with C.V.s between 1.48 and

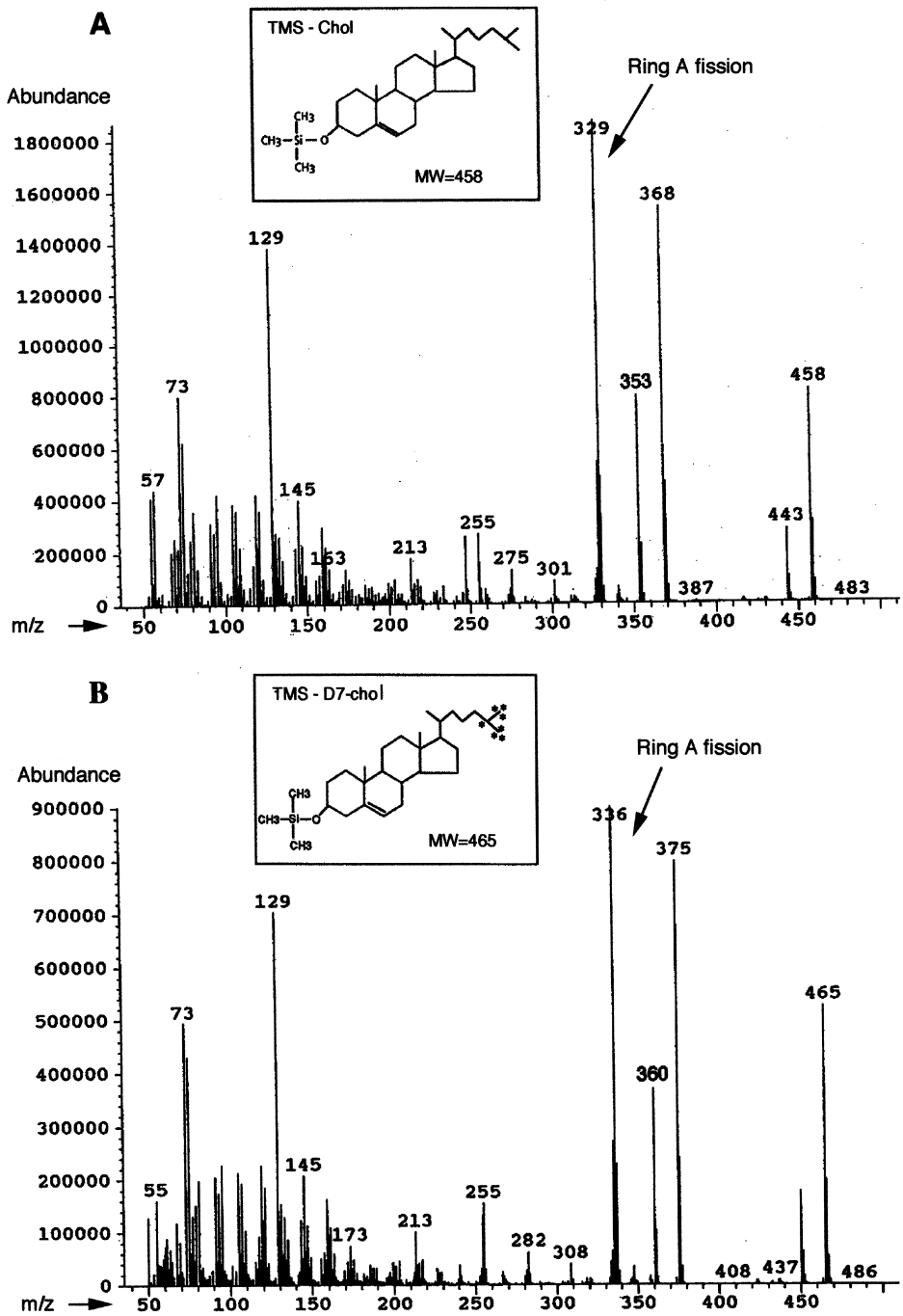

Fig. 1. Mass spectra and formulas. Panel A: mass spectrum and formula of trimethylsilylether-cholesterol (TMS-Chol). Panel B: mass spectrum and formula of trimethylsilylether-heptadeuterated cholesterol (TMS-D7-chol). Panel C: mass spectrum and formula of trimethylsilylether-ergosterol (TMS-Ergo; I.S.). Panel D: mass spectrum and formula of trimethylsilylether-epicoprostanol (TMS-Epi; I.S.). The most abundant and stable ion provided by fragmentation of each derivatized molecule of interest was selected for further quantification using SIM; Chol: 329 u, D7-chol: 336 u, Ergo: 363 u and Epi: 370 u.

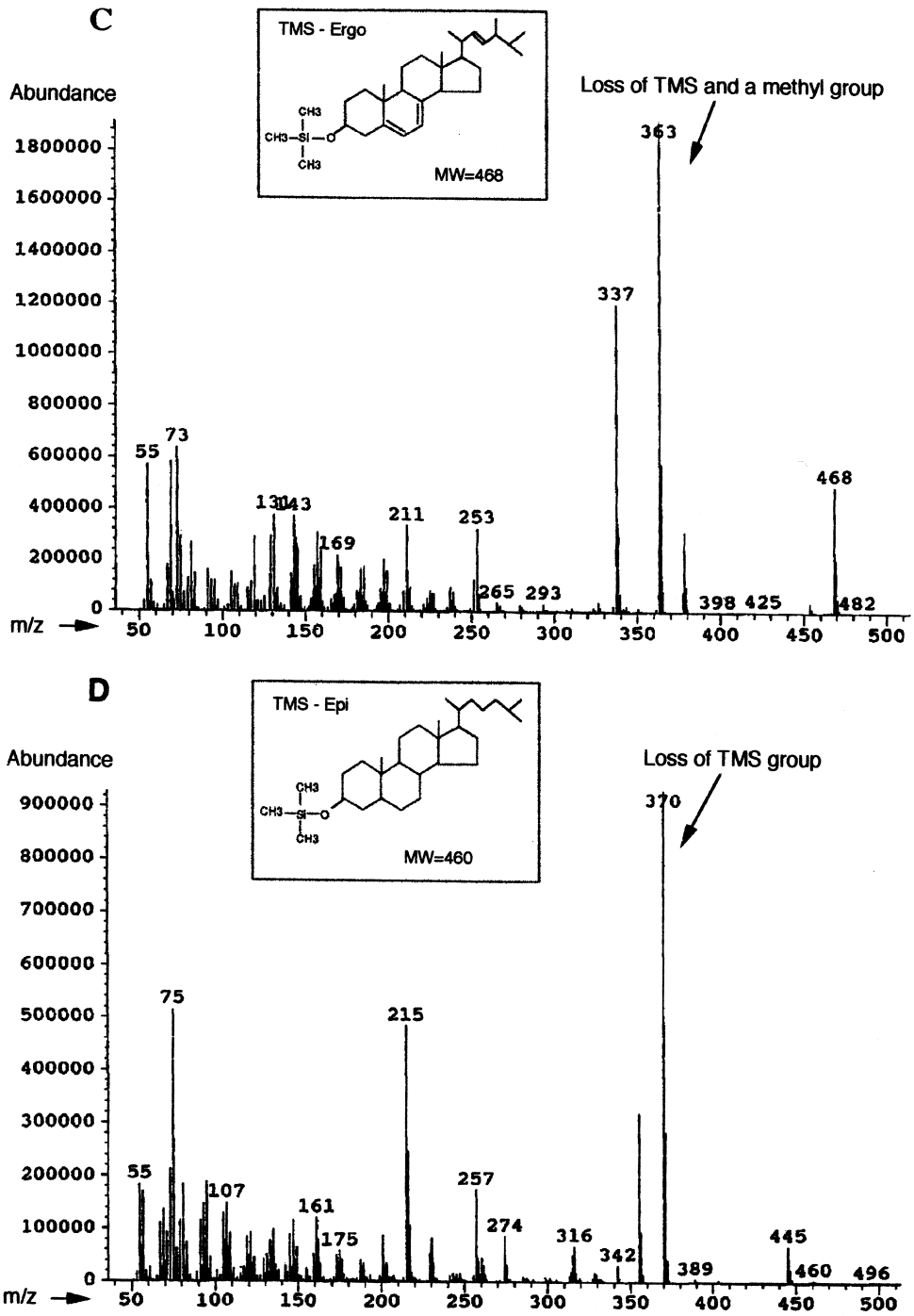

Fig. 1. (*continued*)

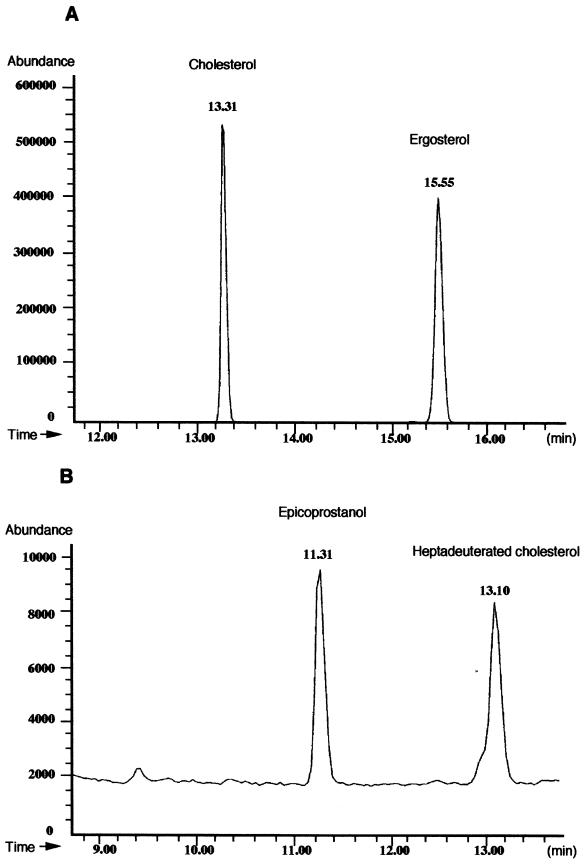

Fig. 2. Total ion chromatogram of TMS molecules. Panel A: Chol and Ergo (5 mmol l^{-1} each). Panel B: D7-chol and Epi (0.1 mmol l^{-1} each). One μl of the extract was injected on the GC column. Chol and D7-chol were resolved from their I.Ss.

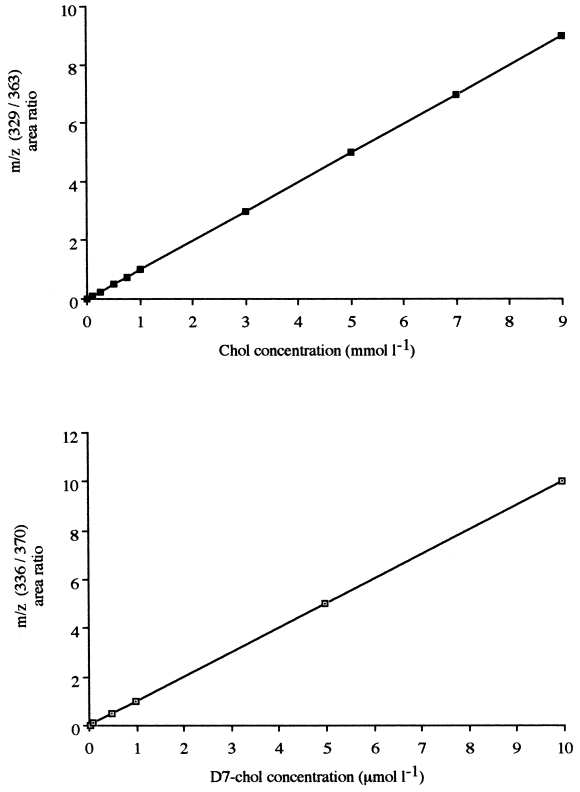

Fig. 3. Linearity of standard curves of Chol (upper panel) and D7-chol (lower panel). Nine (0.1–9 mmol l^{-1}) and six (0.05–10 μmol l^{-1}) concentrations were analyzed for Chol and D7-chol in the presence of Ergo (5 mmol l^{-1}) and Epi (1 μmol l^{-1}), respectively. Area ratios of selected ions (m/z 329/363 and m/z 336/370) were plotted against concentrations. Each point is the mean of six assays.

6.54% for Chol and between 0.163 and 4.88% for D7-chol (Table 2).

These suitable C.V.s were obtained for D7-chol/Chol ratios in the range 1/500–1/50 000. They are comparable to the C.V.s obtained (mean: 3.2%) during similar studies [8] with tracer dilution up to 1/2700. Nevertheless, these C.V.s are higher than those obtained (≤1%) during assays of cholesterol using isotopic dilution with a tracer/Chol ratio of 1 [4–7]. With the very high dilutions (about 1/100 000) attained in the LOD–LOQ range, a C.V. of 10.9% was obtained herein that is still within the values established by IUPAC for limits [20].

Figures for accuracy obtained for Chol (mean: 98.9%) and D7-chol (mean: 99.7%) from six days calibration curves are satisfactory while that obtained (90.4%) at LOD level is still acceptable given the very high dilution (1/100 000 = D7-chol/Chol).

3.4. Analytical recovery

The average extraction recovery of D7-chol in spiked human plasmas was 99.96% (range 95.1–104.3%) over the validated range 0.5 to 5 μmol l^{-1}. These data are given in details in Table 3. The data obtained with spiked plasma are comparable to those obtained with standards thus showing the absence of interferences from the matrix effect of plasma. Thus this method shows a good accuracy with plasma samples.

Table 1
Repeatability of Chol and D7-chol quantification: intra-day precision

Compounds	Theoretical concentrations	*n*	Concentrations found (mean±S.D.)	C.V. (%)	Accuracy (%)
Chol	5 mmol l^{-1}	17	4.97±0.101	2.03	99.3
D7-chol	1 μmol l^{-1}	10	0.95±0.005	4.84	95.0

Table 2
Reproducibility of Chol and D7-chol calibration curves during six consecutive days: inter-day precision

Compounds	Theoretical concentrations	Concentrations measured (mean±S.D.)	C.V. (%)	Accuracy (%)
Chol	0.1	0.094±0.00165	6.54	94
(mmol l^{-1})	0.25	0.242±0.0105	4.35	96.8
	0.5	0.496±0.0289	5.84	99.2
	0.75	0.759±0.0351	4.62	101.2
	1	0.965±0.0366	3.79	96.5
	3	3.08±0.192	6.24	102.6
	5	5.05±0.113	2.24	101
	7	6.95±0.225	3.24	99.3
	9	8.98±0.133	1.48	99.8
D7-chol	0.05	0.0452±0.00493	10.9	90.4
(μmol l^{-1})	0.1	0.0994±0.00485	4.88	99.4
	0.5	0.5±0.0234	4.67	100
	1	1.0±0.0148	1.48	100
	5	4.95±0.0805	1.63	99
	10	9.99±0.0163	0.163	99.9

3.5. LOD and LOQ

The LOD for D7-chol analysis was $3 \cdot 10^{-5}$ mmol l^{-1} but the precision of concentration determination is poor (>10% [20]) at this very low concentration, due to background noise interference. A 10^{-4} mmol l^{-1} concentration was the lowest concentration this method could reproducibly quantitate D7-chol, with a satisfactory precision: C.V.=4.88% (Table 2). This concentration is the LOQ.

4. Application

The method described herein was applied to quantify traces of heptadeuterated dietary Chol (D7-chol) after administration to volunteers of a single-dose (80 mg in the first test-meal). Plasma Chol level did not show significant variation during the 72 h studied (Fig. 4, upper panel). Conversely, the time-course of dietary D7-chol occurrence in serum was slow and progressive. D7-chol plateaued about 48 h

Table 3
Recovery of D7-chol in spiked plasma

D7-chol amount added (μmol l^{-1})	D7-chol amount determined (μmol l^{-1})	Recovery (*n*=3) (%)
0.5	0.519	103.9
1	0.951	95.1
2.5	2.47	98.9
4	4.17	104.3
5	4.88	97.6

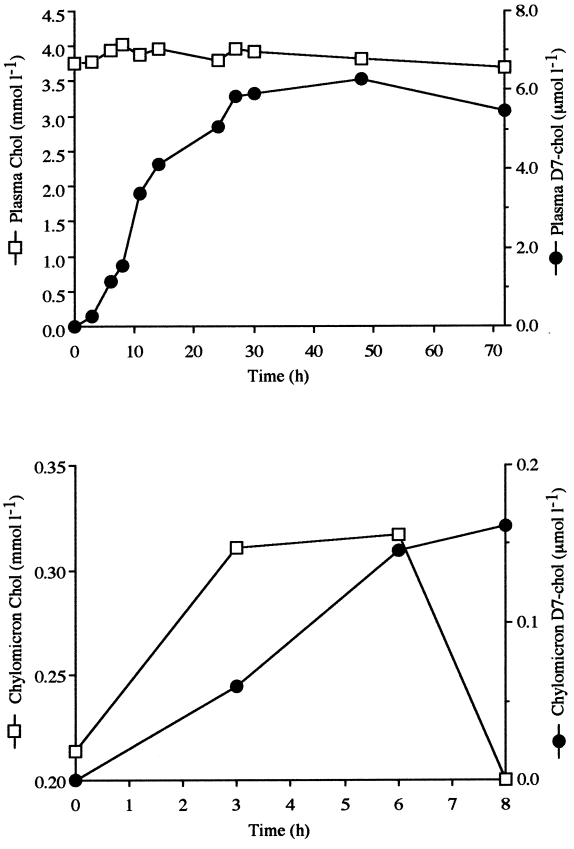

Fig. 4. Time courses of change in Chol and D7-chol concentrations in human plasma and chylomicrons. Seven healthy male volunteers were given a meal containing 80 mg D7-chol (h0) and plasma and chylomicrons were analyzed. Chol concentration (mmol l^{-1}) was calculated from m/z 329/363 area ratio. D7-chol concentration (μmol l^{-1}) was calculated from m/z 336/370 area ratio. Upper panel: plasma Chol and D7-chol concentrations for 72 h. Lower panel: chylomicron Chol and D7-chol for 8 h after labelled test-meal.

after the first meal and then decreased very slowly (Fig. 4, upper panel). This is in line with data of previous works [11–13]. The chylomicron fraction directly represents the source of dietary lipids in plasma after intestinal absorption but has a low level of cholesterol. Thus, to check the suitability of the method, we aimed to determine the Chol and D7-chol abundance in chylomicrons postprandially. After the labeled test-meal, we observed a typical rise in chylomicron Chol during the postprandial period with a maximum after 3 h and return to baseline after 8 h. At the same time, a progressive rise in dietary D7-chol was observed in chylomicron fraction during the postprandial period (Fig. 4, lower panel). These data confirm those obtained during a previous short-term study [13] thus providing a proof of applicability of this newly adapted analytical method.

5. Conclusions

We have developed a reliable, simple and fast method for simultaneous quantification of D7-chol (as traces) and Chol in human plasma and lipoproteins, using GC–MS. The method offers high sensitivity and good accuracy and precision of results and should prove very useful for kinetic studies of cholesterol in humans after given stable isotope as a tracer. The reliability of this method is due to the use of two internal standards at appropriate concentrations. This method could be extrapolated to other natural metabolites.

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