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ASSESSMENT OF SERUM CHOLESTEROL BY TWO METHODS: GAS—LIQUID CHROMATOGRAPHY ON A CAPILLARY COLUMN AND CHEMICAL IONIZATION-MASS FRAGMENTOGRAPHY WITH ISOTOPIC DILUTION OF [3,4-^{13}C]CHOLESTEROL AS INTERNAL STANDARD

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SUMMARY

A gas—liquid chromatographic (GLC) method and an isotopic dilution—mass fragmentographic (ID—MF) procedure using the same capillary chromatographic separation are described for serum cholesterol assay. GLC included silylation and separation on a highly efficient glass capillary column which allowed the separation of cholesterol from cholestanol and the use of epicoprostanol as internal standard. The concentrations were calculated from the areas of the signals and digitalized by a reporting integrator. The reproducibility was 0.5% and the correlation with the ID—MF technique was 0.997. The ID—MF technique was characterized by the use of [3,4-^{13}C]cholesterol as the labelled standard and a chemical ionization mode. The reproducibility was 0.8%.

INTRODUCTION

Isotope dilution—mass fragmentography (ID—MF), developed from the work of Sweely et al. [1] and Hammar et al. [2], is ideally suited as a definitive method in clinical chemistry. As Cali [3] pointed out, "the isotope dilution aspects of the procedure are done on a weight basis and involve straightforward stoichiometric operations, and the mass spectrometric determinations involve only measurement ratios".

Three ID—MF techniques for plasma cholesterol assay have been proposed [4—6]. They all involve gas—liquid chromatography (GLC) on a liquid phase packed column, use either ^{14}C- or deuterium-labelled cholesterol as the internal standard, and mass spectrometry in the electron impact ionization mode.

In order to enhance the performance of the method we have developed a new technique characterized by the following salient features: a capillary column which allows the separation of cholesterol from very similar compounds; use of [3,4-^{13}C]cholesterol, so that ^{13}C, a non-radioactive isotope, does not induce radiolysis, exchange processes or isotopic effects; the use of a chemical ionization mode which leads to a simple fragmentation with a great relative abundance of the high-mass ions.

In the course of improving the chromatographic separation, a new capillary GLC technique was set up and compared with the ID—MF technique.

MATERIALS

Serum. Specimens were collected from adult patients after an overnight fast and stored at - 20° or analyzed within a few hours.

Chemicals. All chemicals were from Merck (Darmstadt, G.F.R.) with the exception of cholesterol (SRM 911a, National Bureau of Standards, Washington D.C., U.S.A.), [3,4-^{13}C]cholesterol (isotopic labelling; C_3, 83%; C_4, 87.5%, from Commissariat à l'Energie Atomique, Saclay, France) 5α-cholestan-3β-ol (cholestanol), epicoprostanol and 5α-cholestane (Sigma, St. Louis, Mo., U.S.A.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (Supelco, Bellefonte, Pa., U.S.A.).

Chromatograph. A Packard-Becker 427 gas chromatograph (Delft, The Netherlands) equipped with a flame ionization detector and an SE-30 (LKB, Bromma, Sweden) capillary column (Type 2101-203, 25 m × 0.22 mm I.D., 120,000 theoretical plates) was used. Chromatograms were recorded on an (HP 3380A) reporting integrator (Hewlett-Packard, Washington D.C., U.S.A.).

Mass spectrometer. A Finnigan 3300 mass spectrometer equipped with the 6100 Interactive Data System (Sunnyvale, Calif., U.S.A.) was used.

METHODS

Gas—liquid chromatography

Saponification and extraction. These were performed according to the reference technique of Abell et al. [7], with slight modification. In a PTFE-lined, screw-capped, 15-ml glass tube were mixed 1 ml of a 0.25 m*M* solution of epicoprostanol in absolute ethanol, 0.1 ml of aqueous potassium hydroxide (6.0 m*M*) and 50 μl of serum. The tube was left for 1 h at 60° and, after cooling, 10 ml of a 0.025 m*M* solution of 5α-cholestane in hexane were added. After mixing, 2 ml of bidistilled water were added and the tube was shaken for 1 min on a Vortex mixer. A 2-ml aliquot of the hexane phase was transferred in a 2-ml glass-tube and evaporated under nitrogen at 60°.

Silylation. A 0.2-ml volume of a freshly prepared mixture of BSTFA and TMCS (4:1, v/v) was added to the dry residue. After 1 h at 60°, 0.8 ml of hexane was added and 1 μl of the cooled mixture was injected into the chromatograph with an all-glass solid injector. The temperature of the injector and

detector was 290°, that of the column was programmed from 250° to 285° at 1°/min. The carrier gas was nitrogen and the flow-rate 3 ml/min. The peak area ratios epicoprostanol:cholestane and cholesterol:epicoprostanol were calculated. The reproducibility of the extraction yield was checked by the epicoprostanol:cholestane ratio, the serum cholesterol concentration was calculated by comparison of the cholesterol:epicoprostanol ratios obtained with the serum and a 5 m*M* standard cholesterol solution.

Isotope dilution—mass fragmentography

The chromatographic step was similar but cholestane was omitted and epicoprostanol replaced by 1 ml of a 0.25 m*M* solution of [3,4-^{13}C¼cholesterol dissolved in absolute ethanol.

The SE-30 capillary column was coupled directly to the ion source of the mass spectrometer by an all-glass connection without any separator. The chemical ionization mode was used with methane as the reagent gas at a pressure of 0.9 Torr. Relative intensities of the *m/e* 443.5 and *m/e* 445.5 ions were recorded. These fragments resulted from the loss of a methyl group from the molecular ion of the trimethylsilyl ether of [^{12}C]cholesterol and [3,4-^{13}C]-cholesterol, respectively. The 443.5:445.5 ratio was calculated for: (i) serum and the 5 m*M* cholesterol solution, each with the added amount of [3,4-^{13}C]cholesterol; (ii) serum treated without the addition of the labelled cholesterol; and (iii) the [3,4-^{13}C]cholesterol solution alone. The concentration of the serum cholesterol was calculated using the following equation in which, in order to avoid interference of background variation, all terms were expressed as ratios of the areas of the signals 443.5:445.5.

$$x = \frac{a - d}{1 - a/c} \cdot \frac{1 - b/c}{b - c} \cdot k$$

where x = concentration of serum cholesterol, k = concentration of the cholesterol standard solution, a = 443.5:445.5 for serum plus [3,4-^{13}C]cholesterol, b = 443.5:445.5 for the standard solution plus [3,4-^{13}C]cholesterol, c = 443.5:445.5 for serum without labelled cholesterol, and d = 443.5:445.5 for the [3,4-^{13}C]cholesterol solution alone.

RESULTS AND DISCUSSION

Gas—liquid chromatography

A typical chromatogram is shown in Fig. 1. The use of a highly efficient capillary column resulted in the separation of the trimethylsilyl ether of cholesterol from silylated derivatives of very similar compounds. Epicoprostanol is a sterol very closely related to cholesterol, having a hydroxyl group at the same position, a similar solubility in organic solvents, and similar reactivity towards silylating agents. Thus, since it is well separated, it could be used as an ideal secondary standard, treated from the very beginning of the assay in the same way as cholesterol. Conversely, 5α-cholestane, used as a secondary standard in all the previous techniques, except for that of Hindriks et al. [8], was added to the extraction solvent. Not being derivatized, in our method it only confirmed the reproducibility of the extraction yield of silylated epico-

prostanol. The high resolution of the chromatographic procedure gave a complete separation of cholestanol from cholesterol (Fig. 2). This sterol is present in serum, usually at a low concentration of about 25 μM [9], but it is increased in one type of hyperlipidemia, cerebrotendinous xanthomatosis [9].

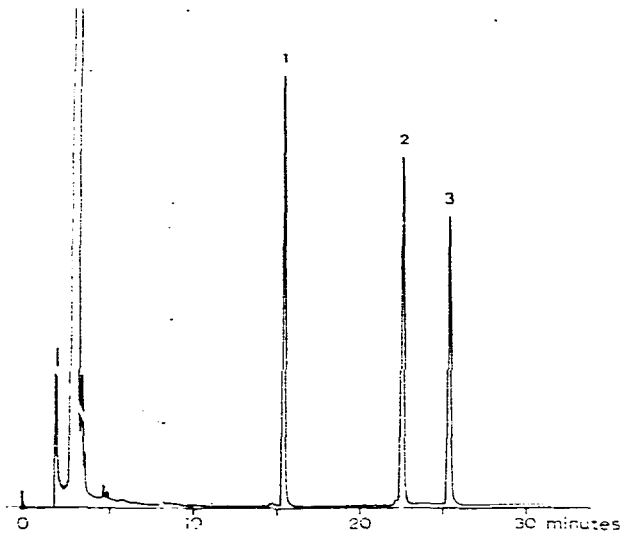

Fig. 1. GLC of serum. Cholesterol (3) concentration was 4.87 mM. No cholestanol was detected in this sample. Cholestane (1) and epicoprostanol (2) were added as described in the text.

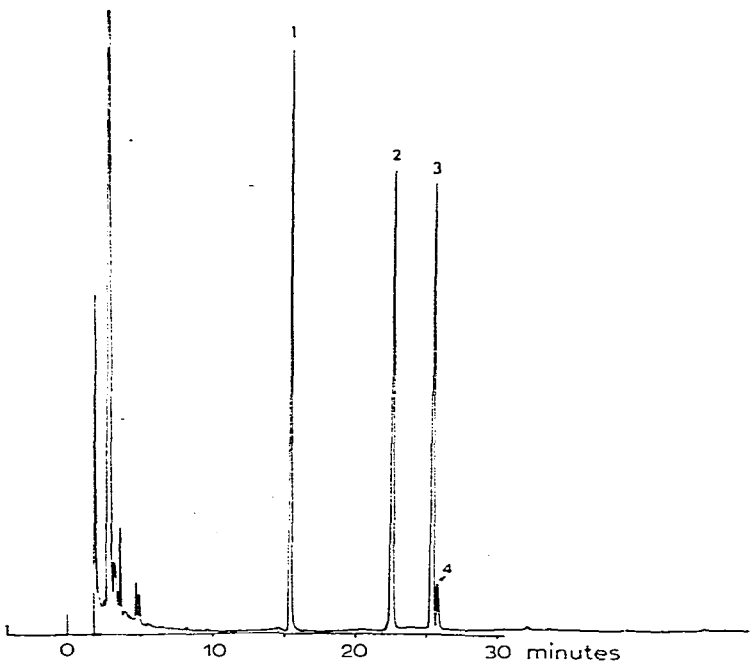

Fig. 2. GLC of a solution of 5 mM cholesterol (3) and 0.4 mM cholestanol (4) solution, (1) and (2) as in Fig. 1.

Thus it is a source of error in packed-column chromatographic assays which cannot separate it from cholesterol [10].

Contrary to usual practice, the cholesterol concentration was calculated from the areas of the signals, measured by a reporting-integrator, and not from their heights. This mode of calculation and the use of a glass capillary column appreciably improved the reproducibility, as can be observed by the following examples, The coefficient of variation (n=28) was 0.8% for the capillary column, and 1.6% for the packed column with measurement of peak height, but only 0.5% with the combined capillary column-reporting integrator.

The procedure was linear up to 18 mM and the limit of detection was 2.5 μM, a concentration which corresponds to an injection of 0.125 pmole of cholesterol. The accuracy of the GLC method was evaluated by comparison with the ID—MF method. The correlation coefficient (n=35) between the two was 0.997. This high accuracy resulted from the specificity of the chromatographic separation, the use of a standard reference material as primary standard and the use of a secondary standard, epicoprostanol, very similar to cholesterol.

Isotope dilution—mass fragmentography

The linearity of the technique was tested up to 18 mM and the coefficient of variation (n=25) was 0.8%. Compared with the relevant publications, this technique relies on several improvements. The chromatographic separation was carried out in a glass capillary column, the efficiency of which has already been indicated. The total resolution of cholestanol from cholesterol was an absolute requirement since cholestanol and [3,4-^{13}C]cholesterol (or cholesterol labelled by one ^{14}C atom) have the same molecular weight and a very similar mass-fragmentation pattern.

Mass fragmentography was conducted in the chemical ionization mode. Whereas the electron impact ionization mode, which leads to multiple fragments, is particularly interesting for structure elucidation studies, chemical ionization is more suitable for fragmentographic methods since it produces preponderantly few high-mass ions. Three main fragments (F) appear on the mass spectrum of cholesterol with methane as the ionizing reagent gas: M—H.

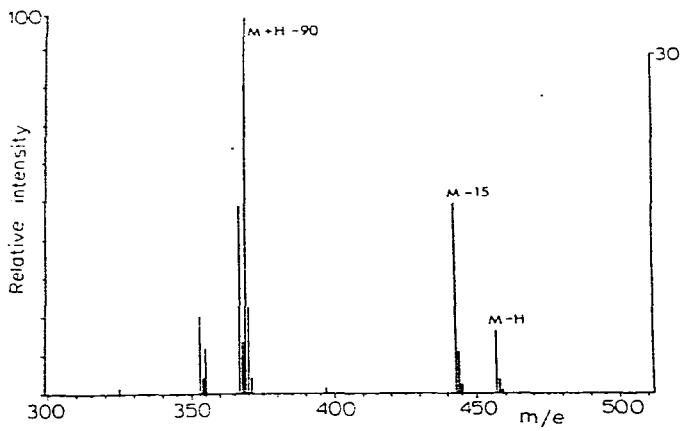

Fig. 3. Mass spectrum of trimethylsilyl ether of unlabelled cholesterol (m/e 458). Chemical ionization mode (for experimental details, see Materials and methods).

M-15 and M+H-90 (Fig. 3). The M-15 fragment, *m/e* 443.5 for cholesterol and *m/e* 445.5 for [3,4-^{13}C]cholesterol, was chosen because of the absence of a doublet arising from the two fragmentation paths F+H and F-H. In the case of M+H-90 the isotopic ion (M+2) which gives (M+2)-H-90, will bring a mass contribution to the fragment M+H-90. The stable isotope ^{13}C was preferred to ^{14}C since, as it is a non-radioactive isotope and does not induce radiolysis of the standard nor contaminate the mass spectrometer. Added to the fact that [3,4-^{13}C]cholesterol is at present 25 times less expensive by weight than [^{14}C]cholesterol, this allowed the use of a concentration of internal labelled standard within the concentration range of unlabelled serum cholesterol. Thus, the errors due to the use of different amplifications on each channel were avoided and the sensitivity was increased. In the case of deuterium-labelled compounds, the lability of the C—H bond will be reflected by an exchange of the label. Such risks are fully avoided by the use of ^{13}C-tagged compounds.

CONCLUSIONS

Although theoretically ideal as a definitive method, the ID—MF method has suffered from technical limitations [11]. The innovations presented, i.e., separation on a capillary column, use of ^{13}C as the labelling isotope and chemical ionization, were factors for improvement of the accuracy and reproducibility of ID—MF serum cholesterol assay. The internal standard [^{13}C]cholesterol could be used in an amount similar to that of serum cholesterol, a highly desirable situation for accurate ID—MF analysis.

The capillary GLC technique described, which has comparable reproducibility and accuracy, has the great advantage of being much more easily set up. It should be useful either as a reference method in some clinical laboratories or for specific investigations such as cholesterol assay in lipid metabolic disorders.

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