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Short communication

Determination of cholesteryl 14-methylhexadecanoate in blood serum by reversed-phase high-performance liquid chromatography

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Abstract

A simple reversed-phase HPLC method has been developed for the determination of cholesteryl 14-methylhexadecanoate (CMH) in the blood serum. Lipids are extracted from 0.1 ml of blood serum and after centrifugation, the extract is chromatographed and individual cholesteryl esters, including CMH are separated and eluted with an acetonitrile–2-propanol mixture. The quantification of cholesteryl 14-methylhexadecanoate is precise and highly reproducible and the analysis may be completed within 35 min. The level of CMH in the blood of cancer patients appears to be a useful marker of malignant tumors.

1. Introduction

Cholesteryl 14-methylhexadecanoate (CMH), a naturally occurring lipid discovered in our laboratory [1], is apparently involved as a cofactor in protein synthesis [2] and bears a close relation to malignant growth. Its concentration in blood serum significantly increases during the induction and growth of experimental tumors [3] in rats and may also be useful as a tumor marker in cancer patients (J. Hradec, manuscript in preparation). A previously described reversed-phase HPLC method [4] was time-consuming because of a long retention time for CMH, and not suitable for routine analyses. The procedure described by Duncan et al. [5] for the separation of some individual cholesteryl esters from free cholesterol and glycerides gave no separation of cholesteryl elaidate from CMH and the other critical pair (CMH and cholesteryl heptadecanoate) was only poorly resolved. Since no alternative techniques have been reported so far, the method described in this paper was developed. It enables the reliable quantification of CMH and of several other cholesteryl esters.

2. Experimental

2.1. Chemicals

Cholesteryl 14-methylhexadecanoate was synthesized in our laboratory as described earlier [6]. All other esters used as standards for the determination of retention times were purchased from Sigma-Aldrich (Prague, Czech Republic).

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If necessary, they were further purified by HPLC as described in this paper. HPLC-grade acetonitrile, 2-propanol, ethanol and diethyl ether were obtained from the same source. Stainless steel columns, 250 × 4 mm I.D., packed with Separon SGX C_{18}, 5 μm were obtained from Tessek (Prague, Czech Republic).

2.2. Apparatus

The HPLC system consisted of a Beckman Model 114M solvent delivery module equipped with a Model 210A sample injection valve as well as of LCO 100 column oven and LCD 2082.1 variable wavelength detector supplemented with the Apex integrating software (all of ECOM, Prague, Czech Republic).

2.3. Procedure

A 100-μl aliquot of blood serum was added to 200 μl of ethanol–diethyl ether (1:2, v/v) containing 10 μg of cholesteryl heptadecanoate as an internal standard and a known quantity of CMH as a standard addition in a disposable microcentrifuge tube and vortex-mixed for 0.5–1 min. After standing at room temperature for 20–30 min the mixture was centrifuged for 5 min at 13 000 *g* in a Beckman microfuge. A portion of the upper layer was carefully aspirated. A 10–20 μl volume of this material was injected onto the chromatograph and the column was eluted with acetonitrile–2-propanol (45:55, v/v) at a flow-rate of 0.50 ml/min. The column was maintained at 32°C in a column oven. The effluent was monitored at 205 nm and 0.04 AUFS. If the integration was started before the cholesteryl stearate eluted, the determination of CMH may be completed within approx. 35 min. In the case of blood serum no cholesteryl esters eluted later than cholesteryl stearate. The limits for CMH detection are approximately 0.3 μg/sample and the response is linear up to 60 μg CMH/sample.

Table 1
Retention times of some cholesteryl esters

Ester	Retention time (min)
Cholesteryl palmitate	22.24
Cholesteryl 14-methylhexadecanoate	24.88
Cholesteryl heptadecanoate (I.S.)	25.97
Cholesteryl stearate	28.24
Cholesteryl oleate	18.20
Cholesteryl elaidate	23.96
Cholesteryl linoleate	15.73
Cholesteryl linolenate	13.83

3. Results and discussion

The ethanol–diethyl ether mixture used for the extraction of serum was very efficient and 3–5 times higher yields of cholesteryl esters were obtained compared with several other extraction methods [5,7] (results not shown). This enabled the use of small volumes of serum for analysis. Analysis of 25 normal and pathological sera confirmed our earlier findings [8] that no measurable quantities of cholesteryl heptadecanoate are present in the serum and hence this ester is suitable as an internal standard. Moreover, it is also advisable to add a known quantity of CMH as an additional standard, in particular to sera containing very low quantities of this ester, to facilitate the detection of CMH.

The elution system used in our experiments provided a satisfactory separation of cholesteryl esters with saturated, as well as unsaturated higher fatty acids provided that their retention time was longer than that of cholesteryl laurate (retention time 15.12 min, not shown) (Fig. 1, Table 1). Esters with shorter retention times, including cholesteryl arachidonate, could not be completely separated from glycerides. For the separation of similar esters, the proportion of acetonitrile–2-propanol should be increased and/or small amounts of water should added to the elution mixture. Increased temperature of the column during the analysis did not only accelerate the analysis but the separation of critical pairs was also greatly improved. Temperatures higher than 32°C accelerated the analysis further but the separation became impaired. A high column efficiency (more than 70 000 theoretical plates/m) was absolutely required for satisfactory results.

Table 2
Reproducibility of the method and recovery of added CMH

No.	CMH added (μg)	CMH recovered			Recovery (%)
		Total	Net	S.D.	
1	none	8.59	–	0.109	–
2	5	13.54	4.94	0.201	98.9
3	10	18.68	10.09	0.210	100.0
4	25	33.45	24.86	0.622	99.4
5	50	59.7	51.22	1.5	102.4

Analyses were performed on the same serum of a blood donor and each group contained 5 samples. Where indicated, CMH was added to the extraction mixture before the addition of the serum and samples were processed further as described in the text. Total CMH is all CMH found in the sample, net CMH is total CMH minus endogenous CMH and represents thus the addition. All values are given in μg of CMH/sample. The results were obtained by analyses performed during three days.

The method described in this paper may be used for the quantitation not only of CMH but also of several other cholesteryl esters. If only CMH is to be determined, only the portion of the chromatogram containing elaidate, CMH and heptadecananoate may be integrated since integration of all peaks in the chromatogram would be time-consuming and is not necessary. For the determination of CMH, the present method gave a very good reproducibility and also the recovery of CMH added to the extraction mixture prior to analysis was satisfactory (Table 2).

The newly developed method described in this paper has so far been used for analyses of CMH in sera of more than 1000 patients with cancer or other diseases, and of healthy individuals. The results showed significant differences between the patients with malignant tumors and other diseases and indicated that the determination of

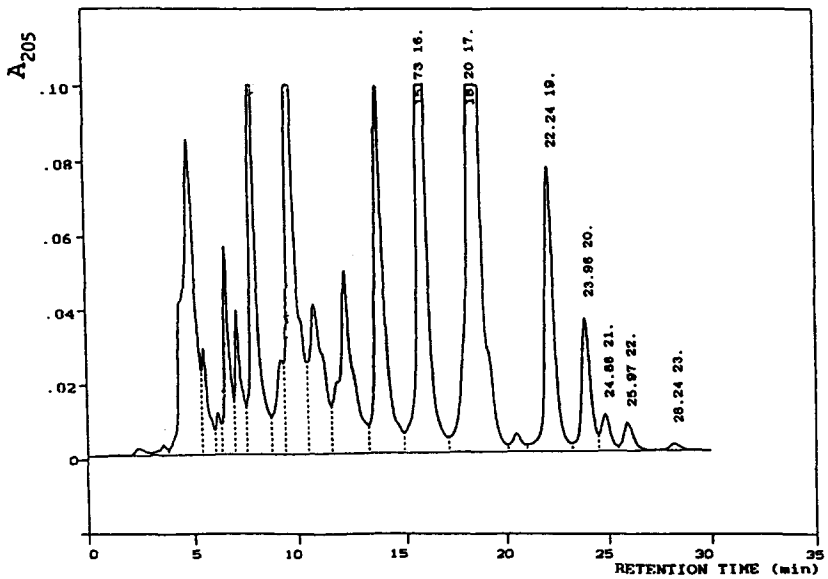

Fig. 1. Separation of cholesteryl esters from normal human serum. To the extract from 100 μl of the serum 3 μg of CMH and 10 μg of cholesteryl heptadecanoate were added. Retention times of some standards of cholesteryl esters are given in Table 1. Experimental details are described in the text. Individual cholesteryl esters were identified by comparison of their retention times with those of standard compounds.

CMH may be a useful marker for malignant diseases (J. Hradec, to be published).

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