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Novel LC–MS/MS method for assay of 7α-hydroxy-4-cholesten-3-one in human plasma Evidence for a significant extrahepatic metabolism

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Abstract

A new isotope dilution LC–MS/MS method for assay of 7α-hydroxy-4-cholesten-3-one without need for derivatization is described. This method was used in catheterization experiments on healthy fasting volunteers. The levels of this generally used marker for bile acid synthesis were slightly but significantly higher in the hepatic vein than in the brachial artery. In contrast, the levels of the precursor to 7α-hydroxy-4 cholesten-3-one, 7α-hydroxycholesterol, were the same in the two vessels. It is concluded that there is a net extrahepatic metabolism of 7α-hydroxy-4-cholesten-3-one. The similarity and very high correlation between the levels in the two vessels ($r = 0.97$) are consistent with the contention that 7α-hydroxy-4-cholesten-3-one is a suitable marker for the activity of the hepatic cholesterol 7α-hydroxylase and thus bile acid synthesis.

Keywords: Liquid chromatography–mass spectrometry; Isotope dilution; CYP7A1; Bile acid synthesis

1. Introduction

About 20 years ago it was shown that plasma levels of 7α-hydroxycholesterol reflect the activity of the cholesterol 7α-hydroxylase (CYP7A1) in humans [1]. In view of the fact that 7α-hydroxycholesterol is the product in the CYP7A1 catalyzed reaction, this oxysterol is theoretically the preferred marker for bile acid synthesis in vivo. There are however disadvantages with use of this steroid. 7α-Hydroxycholesterol may be formed from cholesterol in connection with lipid peroxidation, and part of it present in a lipid extract may thus be of non-enzymatic origin and may eventually have been formed during the workup procedure. Accurate and sensitive assay of 7α-hydroxycholesterol requires sophisticated and expensive methods based on isotope dilution-mass spectrometry. The immediate enzymatic product of 7α-hydroxycholesterol is 7α-hydroxy-4-cholesten-3-one, a compound that is not formed by lipid peroxidation and that is easily assayed by HPLC, utilizing the strong UV absorbance of this steroid. A number of studies have accumulated suggesting that plasma levels of 7α-hydroxy-4-cholesten-3-one seem to adequately reflect the activity of CYP7A1 in humans as well as in experimental animals [2–6].

Theoretically a number of other factors than CYP7A1 activity could however be of importance for the plasma levels of this compound. Thus, it is possible that a portion of the 7α-hydroxycholesterol secreted from the liver may be converted into 7α-hydroxy-4-cholesten-3-one extrahepatically. In addition there is a possibility that 7α-hydroxy-4-cholesten-3-one is metabolized extrahepatically.

It was considered to be of interest to determine whether or not there is a net secretion or uptake of 7α-hydroxycholesterol

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and 7α-hydroxy-4-cholesten-3-one in the human liver. Based on catheterization experiments on healthy human volunteers evidence is presented here that hepatic secretion and uptake of 7α-hydroxycholesterol is about the same. The secretion of 7α-hydroxy-4-cholesten-3-one from the liver is however higher than the corresponding uptake, suggesting a low but significant extrahepatic metabolism of this compound.

2. Experimental

2.1. Preparation of $^2H_6$-labelled 7α-hydroxy-4-cholesten-3-one

This compound was prepared from $^2H_6$-labelled 7α-hydroxycholesterol [7] by oxidation with cholesterol oxidase under the same conditions as described previously [8]. The material was purified by preparative thin-layer chromatography using toluene/ethyl acetate (7:3, v/v) as mobile phase.

2.2. LC–MS/MS analysis of 7α-hydroxy-4-cholesten-3-one

Human serum, 1 or 0.5 mL, was added to a centrifuge tube together with 30 ng of $^2H_6$-labelled 7α-hydroxy-4-cholesten-3-one dissolved in acetonitrile, 15 μL. The steroid was extracted with 4 mL of chloroform/methanol (2:1, v/v). The water phase was re-extracted with 3 mL of chloroform. The combined extracts were pooled and the solvent was evaporated to dryness. The material was dissolved in toluene, 1 mL, and applied to a Sorbent silica cartridge (100 mg), preconditioned with hexane, 3 mL. The cartridge was washed with isopropanol in hexane, 0.5% (v/v) and 7α-hydroxy-4-cholesten-3-one was then eluted with 5 mL of isopropanol in hexane, 50% (v/v). After evaporation of the solvent the residue was dissolved in acetonitrile, 100 μL.

The LC–MS/MS system used was the Waters Quattro micro quadropole system equipped with an APCI probe and a HPLC system (Waters 2695 Alliance). Chromatographic separation was performed with a Discovery RP Amide C16 column (10 mm × 3 mm, 5 um; Supelco) at 40 °C. The mobile phase was 100% acetonitrile and the flow rate was 0.5 mL/min.

The ions used for the tracing of unlabelled 7α-hydroxy-4-cholesten-3-one was *m/z* 401 > 177 and the ion for tracing the $^2H_6$-labelled 7α-hydroxy-4-cholesten-3-one was 407 > 177. The mass spectrometer was operated in the positive mode. Corona current, 3 μA, Cone voltage 25 V, Source temperature 120 °C, Cone gas flow 62 L/h, Desolvation Gas Flow 400 L/h, Collision energy 23 eV.

The quantitations were performed with use of a standard curve, plotting the ratio between the response of 7α-hydroxy-4-cholesten-3-one in the *m/z* 177 tracing generated from the mother ion *m/z* 401 (unlabelled) and the response in the *m/z* 177 tracing generated from the mother ion *m/z* 407 ($^2H_6$-labelled).

In order to establish a normal reference range, serum samples from 26 healthy subjects of both sexes, age 24–32 years, were analyzed. These subjects were normolipidemic, had normal liver tests, and were not on therapy.

2.3. Catheterization experiments

Nine healthy normolipidemic males, aged 21–38 years (mean age 29 years) with normal liver tests were recruited for the study on hepatic uptake of 7α-hydroxy-4-cholesten-3-one. These volunteers were the same as those participating in a recent study on transport of oxysterols over the blood–brain barrier [9]. Following an overnight fast blood samples were taken simultaneously from two catheters which were inserted percutaneously. A Cournand catheter was inserted at the level of the inguinal ligament and the tip was advanced under fluoroscopic control to a right-sided hepatic vein. One catheter was inserted into the brachial artery.

The samples obtained from the above experiments were not possible to use for accurate determination of 7α-hydroxycholesterol, due to lipid peroxidation that gave high levels of 7β-hydroxycholesterol and 7-oxo-cholesterol. In order to study a possible uptake or secretion of 7α-hydroxycholesterol samples from a previous almost identical experiments with another group of six male normolipidemic volunteers with normal liver tests was used [10]. In this case the levels of 7α-hydroxycholesterol had been measured immediately after sample collection.

The plasma levels of 7α-hydroxycholesterol were assayed by isotope dilution-mass spectrometry as described previously [9].

2.4. Ethical aspects

All subjects participating in the two studies were informed on the nature, purpose and possible risks of the study prior to given their voluntary consent to participate. The study protocol was reviewed and approved by the institutional ethics committee.

3. Results

3.1. Validation of method

Fig. 1 shows a typical LC–MS/MS chromatogram of a serum extract of 7α-hydroxy-4-cholesten-3-one to which $^2H_6$-labelled 7α-hydroxy-4-cholesten-3-one had been added as an internal standard. Fig. 2 shows a typical standard curve generated by analysis of standard mixtures of 30 ng $^2H_6$-7α-hydroxy-4-choleten-3-one with varying amounts of unlabelled steroid.

Replicate measurements ($n = 10$) of 7α-hydroxy-4-cholesten-3-one in 1 mL serum samples gave a CV of 1.8% (within-run precision). With 0.5 mL serum a CV of 2.1% was obtained. With 0.2 mL serum a CV above 10% was always obtained.

In a typical recovery experiment addition of 5 ng 7α-hydroxy-4-cholesten-3-one to 5 samples with a concentration of 16.9 ± 2.1 ng/mL gave a concentration of 22.9 ± 2.0 ng/mL, with a deviation of 4.5% from the expected result.

Replicate measurement of the same serum sample during 5 different days gave a CV of 7.1% (between-run precision).

Assay of serum samples from 26 healthy subjects of both sexes, age 24–32 years, gave a mean level of 18 ng/mL with a range 5–48 ng/mL.

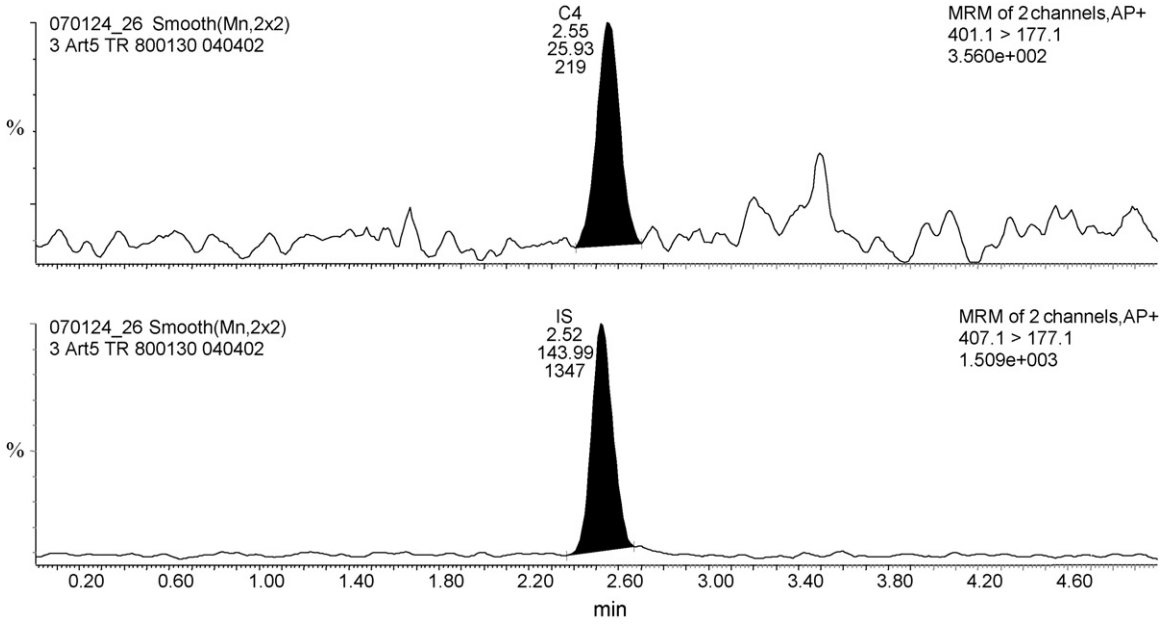

Fig. 1. Ion chromatograms (*m*/*z*) obtained in the analysis of an extract of serum to which $^{2}H_{6}$-labelled 7α-hydroxy-4-cholesten-3-one had been added as internal standard. The lower chromatogram shows the tracing of the *m*/*z* 177 ion generated from the mother ion *m*/*z* 407.1 (corresponding to internal standard) and the upper chromatogram shows the tracing of the ion *m*/*z* 177 generated from the mother ion *m*/*z* 401 (unlabelled steroid).

3.2. Catheterization experiments

Table 1 summarizes the results. The levels of 7α-hydroxy-4-cholesten-3-one were slightly but significantly higher in the hepatic vein than in the artery ($p < 0.05$). There was a high correlation between the levels in the two compartments ($r = 0.97$). Assuming a blood flow through the liver of 0.7 L/min (10), it could be calculated that there is an extrahepatic metabolism of 7α-hydroxy-4-cholesten-3-one corresponding to about 2 mg/24 h.

The levels of 7α-hydroxycholesterol were not significantly different in the two vessels (Table 2), suggesting that the secretion of the steroid from the liver is about the same as the uptake.

4. Discussion

4.1. Methodological considerations

Theoretically, isotope dilution-mass spectrometry should be the most accurate technique for assay of 7α-hydroxy-4-cholesten-3-one. About 20 years ago we developed such a technique based on use of a deuterium labelled 7α-hydroxy-4-cholesten-3-one as internal standard, preparative thin-layer chromatography and combined gas chromatography–mass spectrometry after conversion of the steroid into a trimethylsilyl ether derivative [11]. This method is however considerably more time-consuming than later developed relatively simple HPLC methods utilizing UV-detection and an internal

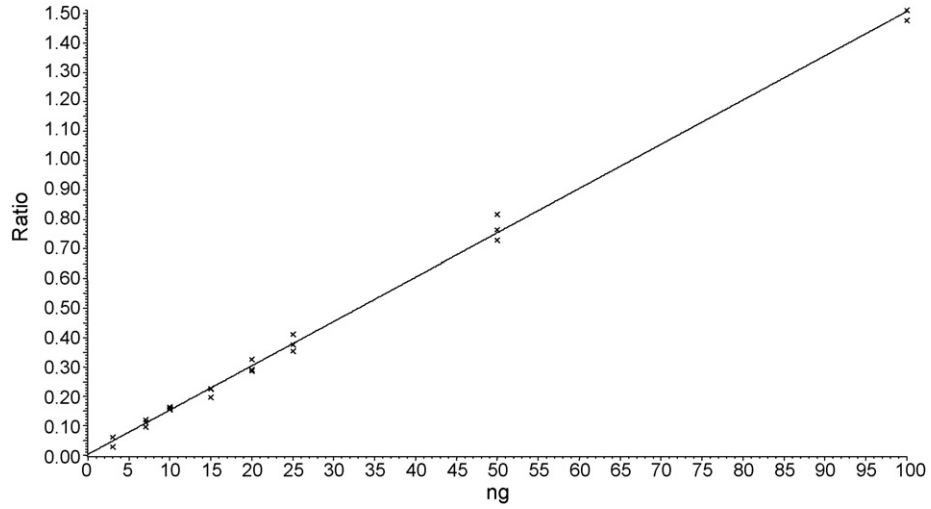

Fig. 2. Standard curve generated by LC–MS/MS analyses of 30 ng of $^{2}H_{6}$-labelled 7α-hydroxy-4-cholesten-3-one together with varying amounts of unlabelled 7α-hydroxy-4-cholestern-3-one.

Table 1
Catheterization experiments measuring levels of 7α-hydroxy-4-cholesten-3-one (ng/mL) in the brachial artery and the hepatic vein of fasting healthy volunteers

Subject	Artery	Hepatic vein	Arterio-venous difference
1.	5.7	6.2	−0.5
2.	26.8	31.9	−5.1
3.	8.4	9.3	−0.9
4.	14.2	17.2	−3.0
5.	4.6	7.4	−2.8
6.	15.3	13.4	1.8
7.	11.1	15.9	−4.8
8.	12.0	15.8	−3.8
9.	1.6	2.5	−0.9
Mean			−2.2
S.E.M.			0.8

standard chemically different from 7α-hydroxy-4-cholesten-3-one [2–6]. Very recently a highly sensitive method for assay of 7α-hydroxy-4-cholesten-3-one was reported utilizing LC–MS/MS coupled in electrospray ionization (ESI) mode and use of deuterium labelled 7α-hydroxy-4-cholesten-3-one as internal standard [12]. In this method 7α-hydroxy-4-cholesten-3-one was extracted from human serum by a salting-out procedure, derivatized into the corresponding picolinoyl ester (7α-picolinate) and then purified by a dispensable C18-cartridge prior to the LC–MS/MS step. Use of the picolinate derivative increases the sensitivity of the assay but makes it more complicated than the present method of assay. The reproducibility of the present LC–MS/MS method was found to be similar to that of the previously reported method and recovery experiments established the accuracy of the method. In a direct comparison the results obtained with our LC–APCI–MS/MS method was in complete agreement with those obtained with a HPLC method [6]. The levels obtained in healthy volunteers obtained in the present study were in accord with previously published data [2].

The present method has merits in relation to the presently used HPLC methods, but as judged from our comparative studies the latter have probably a sufficient accuracy for most clinical work. If a very high sensitivity is required, e.g. in connection with studies on mice, the alternative isotope dilution LC–MS/MS method utilizing picolinate derivatives [12] should be preferred.

Table 2
Catheterization experiments measuring levels of 7α-hydroxycholesterol (ng/mL) in the brachial artery and the hepatic vein of fasting healthy volunteers

Subject	Artery	Hepatic vein	Arterio-venous difference
1.	60	62	2
2.	26	26	0
3.	42	42	0
4.	63	61	2
5.	15	13	2
6.	24	29	−5
Mean			0.2
S.E.M.			1.1

4.2. Liver uptake of 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one

In view of the fact that 3β-hydroxysteroid dehydrogenase is present in a number of extrahepatic tissues (pancreas, kidney, skin, heart, skeletal muscle) [13], we tested the possibility that part of the 7α-hydroxycholesterol secreted from the liver may be oxidized into 7α-hydroxy-4-cholesten-3-one extrahepatically. If this is the case, higher levels of 7α-hydroxycholesterol would be expected in the artery than in the hepatic vein. The levels of 7α-hydroxycholesterol were however identical in the two vessels. In accordance with the contention that there is no extrahepatic formation of 7α-hydroxy-4-cholesten-3-one, there was no increase in the levels of this oxysterol in the artery. On the contrary there were significantly higher levels of this oxysterol in the vein, consistent with some extrahepatic metabolism of 7α-hydroxy-4-cholesten-3-one.

7α-Hydroxy-3-oxo-4-cholestenoic acid is present in the circulation and may be formed from 7α-hydroxy-4-cholesten-3-one by action of the sterol 27-hydroxylase (CYP27). The latter enzyme is known to have a broad extrahepatic distribution [14]. Another possibility is that 7α-hydroxy-3-oxo-4-cholestanoic acid is formed extrahepatically from 27-hydroxycholesterol by the combined action of 3β-hydroxysteroid dehydrogenase and oxysterol 7α-hydroxylase (CYP7B1). Also the latter two enzymes are known to be present in extrahepatic cells [9,13,15].

In a previous attempt to clarify the possible precursor–product relationships between different oxysterols and 7α-hydroxy-3-oxo-4-cholestenoic acid, we measured the deuterium enrichment in different oxysterols and in cholestenoic acids after infusion of 10 g of $^{2}H_{6}$-cholesterol to a healthy volunteer [16]. The labelling pattern in 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one was identical, suggesting a precursor–product relationship. The labelling pattern in 7α-hydroxy-3-oxo-4-cholestenoic acid was similar but not identical to that of 7α-hydroxy-4-cholesten-3-one, suggesting that the latter oxysterol is one but not the only precursor. The results of the present work are consistent with the possibility that part of the 7α-hydroxy-3-oxo-4-cholestenoic acid present in the circulation originates from extrahepatic 7α-hydroxy-4-cholesten-3-one.

In any case it is evident that the extrahepatic metabolism of 7α-hydroxy-4-cholesten-3-one is small and likely to be relatively constant under most conditions. The results of the present study suggest that the rate of production of 7α-hydroxy-4-cholesten-3-one in the liver is most important for the level in the circulation, consistent with use of the latter level as a marker for cholesterol 7α-hydroxylase activity.

Acknowledgements

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