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Journal of Chromatography B, 657 (1994) 31–36

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Reversed-phase high-performance liquid chromatographic determination of 7α-hydroxy-4-cholesten-3-one in human serum

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(First received November 12th, 1993; revised manuscript received February 24th, 1994)

Abstract

A method for the determination of 7α-hydroxy-4-cholesten-3-one in human serum has been developed. The method is based upon solid-phase (C_{18}) extraction of serum after addition of 7β-hydroxy-4-cholesten-3-one as internal standard. The extract is analysed by reversed-phase HPLC using acetonitrile–water as a mobile phase. The effluent is monitored at 241 nm. The overall recoveries of the method range from 96% to 105%. The coefficient of variation for the within-run precision is 3.2% ($n = 20$, mean = 13.0 ng/ml) and for the between-run precision 3.8% ($n = 32$, mean = 13.3 ng/ml). The limit of detection is 3 ng at a signal-to-noise ratio of 3:1, which corresponds to 1 ng/ml using 3 ml of serum. The median value of 7α-hydroxy-4-cholesten-3-one found in blood donors ($n = 27$) was 8.9 ng/ml (range 2–35 ng/ml).

1. Introduction

Bile acids are the end products of hepatic cholesterol catabolism and they represent a major pathway for excretion of steroids/sterols from the human body [1]. Two primary bile acids, cholic acid and chenodeoxycholic acid, are formed in the liver from cholesterol. The rate limiting step in this biosynthesis is a 7α-hydroxylation of cholesterol [2], catalyzed by the microsomal cholesterol-7α-hydroxylase. The activity of this enzyme is regulated by a feed-back mechanism which is dependent on the entero-hepatic circulation of bile acids [3]. Disturbances in the entero-hepatic circulation of bile acids, either as a result of medical treatment (*e.g.* administration of cholestyramine, ileal resection) or as a consequence of pathological processes (*e.g.* inflammatory bowel disease) thus influence the production of bile acids [3]. In the absence of liver disease a disturbed entero-hepatic circulation usually results in a decreased absorption of compounds such as bile acids (see above) and the result may be a more or less pronounced steatorrhea [4].

Previously the rate of bile acid synthesis has been evaluated by determination of the cholesterol 7α-hydroxylase activity in liver biopsies [5]. Recently it has been reported that determination of the plasma levels of 7α-hydroxy-4-cholesten-3-one correlates well with the cholesterol 7α-hydroxylase activity found in liver biopsies [6]. Thus the plasma levels of 7α-hydroxy-4-choles-

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0378-4347/94/$07.00
SSDI 0378-4347(94)00156-Y

ten-3-one may reflect the rate of bile acid synthesis [7].

The present study describes a reversed-phase HPLC method with a non-radioactive internal standard for the determination of 7α-hydroxy-4-cholesten-3-one in human serum.

2. Experimental

2.1. Instrumentation

The HPLC-system consisted of a Waters 625 LC-system equipped with a Waters 991 photodiode array detector and a Nova-Pak C_{18} steel column, 300 × 3.9 mm I.D., 4 μm particle size (Millipore, Milford, MA, USA). The 991 photodiode array detector software package graphed and calculated the peak areas. Parameter variables were set as follows: flow-rate 1.0 ml/min, wavelength 241 nm and injection volume 50 μl. The mobile phase consisted of acetonitrile–water (98:2, v/v) and was degassed with helium. GC–MS analysis was performed on a GC Delsi Di 200 gas chromatograph coupled to a Nermag R10-10H Quadrupole instrument. The GC column used was a 15 m × 0.25 mm fused-silica capillary column coated with 0.25 μm DB 5, 5% phenyl (J and W Scientific, Folsom, CA, USA). Otherwise the conditions were as described previously [8]. Radioactivity collected under the 25-hydroxy-cholecalciferol peak was determined in a 1211 Minibeta liquid scintillation counter (LKB/Wallac, Sollentuna, Sweden) after addition of 14 ml of a scintillation cocktail (Hionic Fluor) to 3 ml of effluent from the HPLC column.

2.2. Chemicals

Acetonitrile, *n*-hexane and methanol were of HPLC grade (Riedel-de Haen, Seelzel, Germany). Chloroform and pyridine were of p.a. quality (E. Merck, Darmstadt, Germany). Chlortrimethylsilane and 1,1,1,3,3,3-hexamethyldisilazan were purchased from E. Merck. Bulk C_{18} 55–105 μm (Millipore) was used for solid-phase extraction of serum. 5-Cholesten-3ß,7α-diol was purchased from Steraloids (Wilton, NH, USA). 5-Cholesten-3ß,7ß-diol and cholesterol oxidase (EC 1.1.3.6) were purchased from Sigma (St. Louis, MO, USA). 25-Hydroxy-[26,27-methyl-^{3}H]cholecalciferol, specific activity 6.43 TBq/mmol, was purchased from Amersham International (Amersham, Buckinghamshire, UK). Hionic Fluor scintillation cocktail was purchased from Packard Instrument Chemical Operations (Groningen, Netherlands). Cholesterol was determined with a commercial kit (Randox, Crumlin, UK). Apolipoprotein A_1 and apolipoprotein B were determined with commercial kits (Orion, Espoo, Finland). Authentic 7α-hydroxy-4-cholesten-3-one was a generous gift from Dr. M. Axelson, Karolinska Hospital, Sweden.

2.3. Samples

Blood from 27 blood donors, 22–53 years of age (mean 38 yr), was drawn into a Vacutainer tube with no additive (Monoject, Sherwood medical, Ballymoney, UK). The serum was collected by centrifugation at 1500 *g* for 10 min and stored at −20°C until analysis.

2.4. Synthesis of reference compounds

7α-Hydroxy-4-cholesten-3-one was prepared from 5-cholesten-3β,7α-diol and 7β-hydroxy-4-cholesten-3-one was prepared from 5-cholesten-3β,7β-diol by enzymatic oxidation with cholesterol oxidase according to Aringer and Nordström [9]. 6-Oxygenated derivatives of 4-cholesten-3-one were synthesized as described previously [8].

2.5. Identification of reference compounds

After enzymatic oxidation and work up of 7α-hydroxy-cholesterol and 7β-hydroxy-cholesterol respectively, the organic extracts were dried and the respective residues were further purified by HPLC (see above). Each residue yielded only one metabolite which was collected and converted into a trimethylsilylether derivative [8] prior to analysis by GC–MS (see above).

For routine purposes in the HPLC-procedure a tentative identification and purity check was achieved for 7α-hydroxy-4-cholesten-3-one and 7β-hydroxy-4-cholesten-3-one by spectral analysis on a Waters 991 photodiode array detector (Millipore) at wavelengths in the range 220–260 nm.

2.6. Solid-phase extraction

7α-Hydroxy-4-cholesten-3-one was extracted from human serum essentially according to the solid-phase extraction procedure used for plasma described by Axelson *et al.* [7]. However, 100 ng of our proposed internal standard (7β-hydroxy-4-cholesten-3-one) dissolved in 10 μl of methanol replaced the addition of 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol suggested by these authors. Initially both internal standards were added to the sample in order to compare their efficiency. The subsequent procedure was as described above [7]. The final residue was dissolved in 100 μl of methanol and 50 μl of this mixture was injected onto the HPLC system.

3. Results

3.1. Identification

To confirm the identity of the synthesized reference compounds, 7α-hydroxy-4-cholesten-3-one and 7β-hydroxy-4-cholesten-3-one, GC–MS analysis of the trimethylsilylether derivative of the respective reference compound was performed. The synthesized 7α-hydroxy-4-cholesten-3-one showed identical chromatographic properties and yielded a mass spectrum identical to that of the authentic reference. The trimethylsilylether derivative of the tentative 7β-hydroxy-4-cholesten-3-one yielded the mass spectrum shown in Fig. 1. The spectrum shows a molecular ion at *m/z* 472 and fragment ions typical of 7β-hydroxy-4-cholesten-3-one [9].

Fig. 2 shows a diode-array detector spectrum analysis of 7α- and 7β-hydroxy-4-cholesten-3-one. Fig. 3a shows the elution profile of 7α-hydroxy-4-cholesten-3-one and 7β-hydroxy-4-cholesten-3-one in a 50-μl sample extracted from 2 ml of a human serum. Analysis of the UV spectrum of the two indicated compounds yielded spectra that were indistinguishable from those presented in Fig. 2. Optimal resolution was obtained using isocratic elution with a mobile phase consisting of acetonitrile–water (98:2, v/v). Fig. 3b shows the same serum without addition of 7β-hydroxy-4-cholesten-3-one. No peak from any endogenous compound has so far appeared at the retention time of 7β-hydroxy-4-cholesten-3-one.

3.2. Linearity

Known amounts of 7α-hydroxy-4-cholesten-3-one and 7β-hydroxy-4-cholesten-3-one (5–1000ng) were injected onto the HPLC in order to determine the accuracy of the detector. Linearity was obtained for each of the two

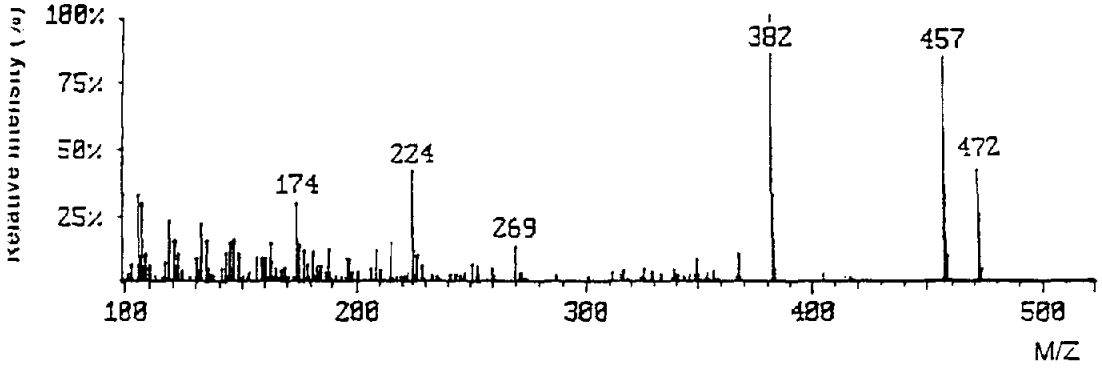

Fig. 1. GC–MS analysis of the trimethylsilylether derivative of 7β-hydroxy-4-cholesten-3-one.

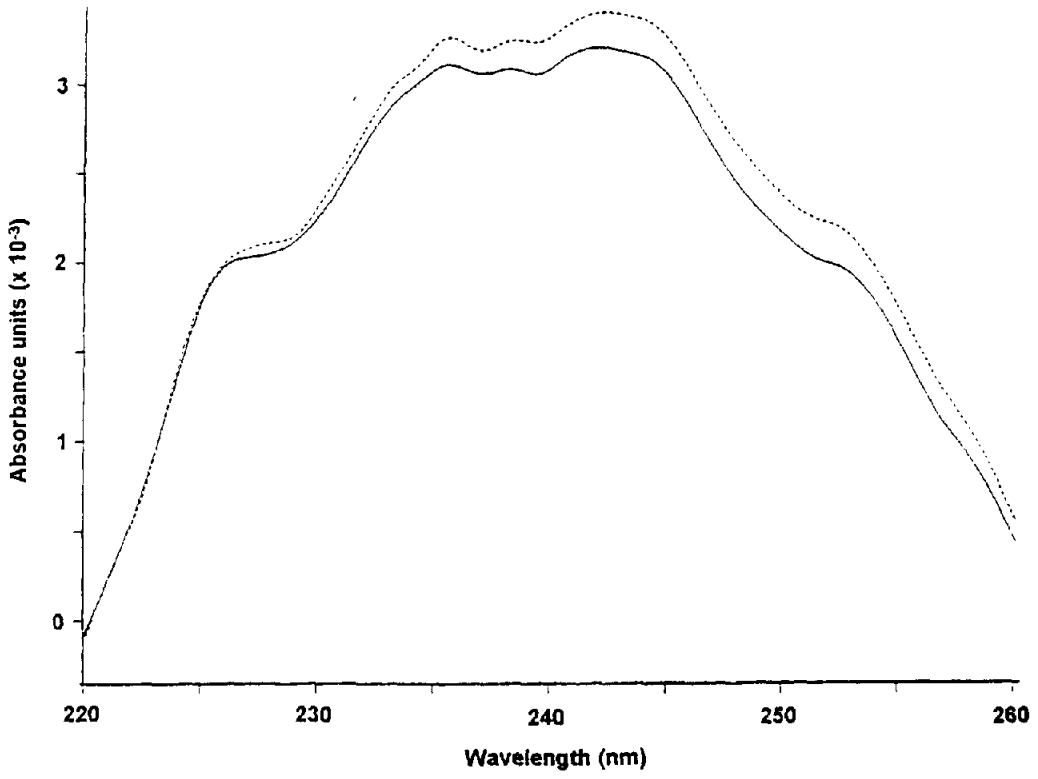

Fig. 2. Diode-array detector spectrum of 7α-hydroxy-4-cholesten-3-one (——) and 7β-hydroxy-4-cholesten-3-one (- - - -).

sterols. Regression data for the two sterols were as follows: $y = 0.00008326x + 0.00050679$, $r = 0.9998$, $n = 14$ for 7α-hydroxy-4-cholesten-3-one and $y = 0.00008467x + 0.00049614$, $r = 0.9998$, $n = 14$ for 7β-hydroxy-4-cholesten-3-one.

3.3. Recovery

The overall recovery of the procedure (solid-phase extraction of serum followed by HPLC analysis) as based upon addition of known amounts of 7α-hydroxy-4-cholesten-3-one to patient serum samples, with 7β-hydroxy-4-cholesten-3-one used as internal standard to correct for losses and variations in injection volumes, was 100 ± 3% (mean ± S.D.). The individual results are shown in Table 1. Extraction yields of 7α-hydroxy-4-cholesten-3-one were similar to those of 7β-hydroxy-4-cholesten-3-one. Extraction yields of 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol were some 30% higher compered to the two sterols.

During the search for a suitable internal standard it was found that 100 ng of 6α-, or 6β-hydroxy-4-cholesten-3-one or 4-cholesten-3,6-dione dissolved in saline were recovered with a yield of 98–103% ($n = 5$) after the extraction procedure. When 100 ng of these compounds were added to serum instead of saline the yields decreased to 48–51%.

3.4. Precision

The coefficient of variation for within-run precision, calculated from 20 duplicate determinations of the same serum, was 3.2% (mean = 13.0 ng/ml). Between-run precision, evaluated by analysis of 32 serum samples on 16 different days, was 3.8% (mean = 13.3 ng/ml).

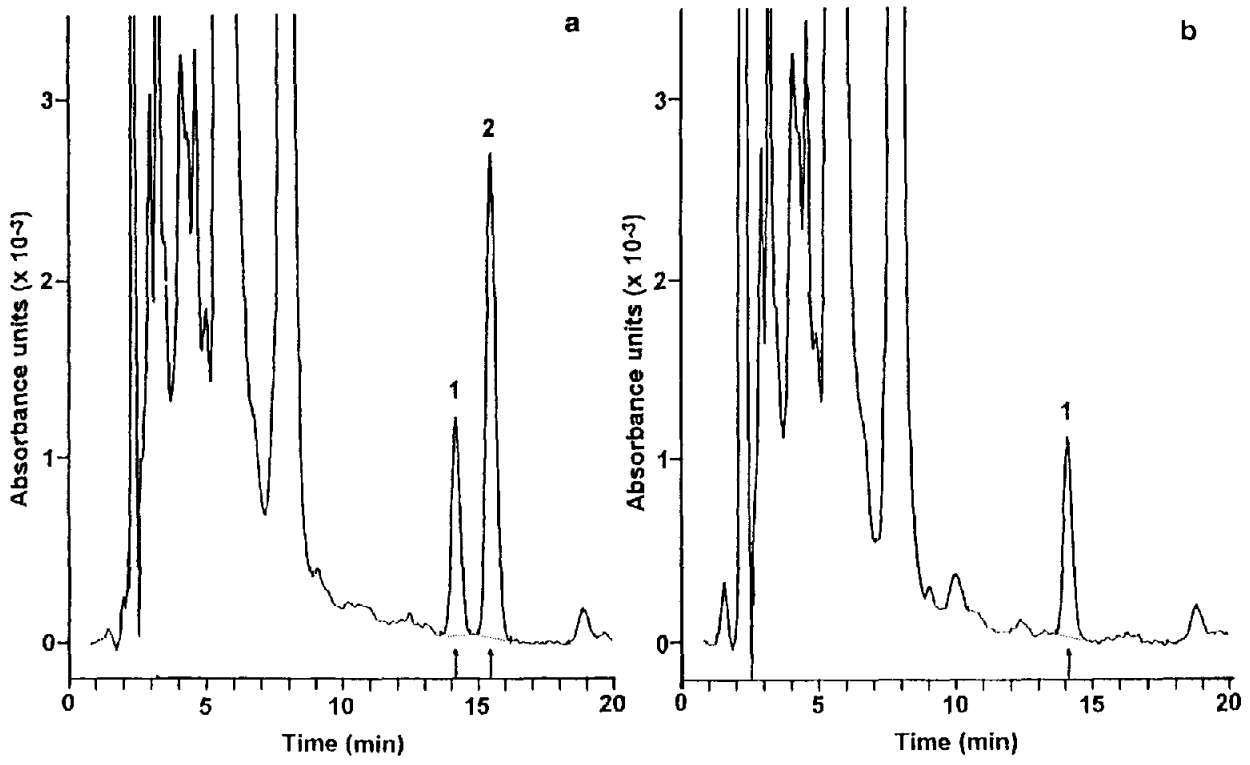

Fig. 3. (a) HPLC analysis of the elution profile of 7α-hydroxy-4-cholesten-3-one (peak 1) and the internal standard 7β-hydroxy-4-cholesten-3-one (peak 2) extracted from 2 ml of a human serum. (b) Same conditions as in (a), but without addition of the internal standard 7β-hydroxy-4-cholesten-3-one. No endogenous peak was found at the retention time of 7β-hydroxy-4-cholesten-3-one.

3.5. Limit of detection

Using 3 ml of serum the limit of detection was 3 ng at a signal-to-noise ratio of 3:1, corresponding to 1 ng/ml.

3.6. Analysis of serum

Fig. 4 shows the individual serum concentrations of 7α-hydroxy-4-cholesten-3-one found in the blood donors when the two different internal standards were used. With 7β-hydroxy-4-cholesten-3-one as internal standard the median value was 8.9 ng/ml (range 2–35 ng/ml). With 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol as internal standard the median value was 7.2 ng/ml (range 1–25 ng/ml). The correlation coefficient was 0.985.

4. Discussion

The present study describes a simplified method for the determination of 7α-hydroxy-4-cholesten-3-one in human serum. The major advantage of our method is the use of an internal standard that can be quantitated in the same way as the substance of interest. The internal standard used, 7β-hydroxy-4-cholesten-3-one, is not commercially available, but may easily be synthesized from 7β-hydroxy-cholesterol by the action of cholesterol oxidase (EC 1.1.3.6). Although our tentative identification does not include NMR data or melting point determination, we believe that the specificity of the enzymatic oxidation [9], together with compatible chromatographic properties [9] and a mass spectrum in agreement with that of 7β-hydroxy-4-choles-

Table 1
Recovery of 7α-hydroxy-4-cholesten-3-one added to 2 ml of a human serum sample

Added[a] (ng)	Observed (ng)	Expected (ng)	Recovery (%)
0	46 ± 2[b]		
20	66	66	100
40	86	86	100
60	107	106	101
100	145	146	99
200	254	246	103
300	332	346	96
400	445	446	100
600	680	646	105
1000	1007	1046	96
2000	2090	2046	102

[a]Different amounts (20–2000 ng) of 7α-hydroxy-4-cholesten-3-one dissolved in 50 μl of methanol were added to 2 ml of a human serum sample which were subsequently extracted as described in Experimental.
[b]Mean value ± S.D. ($n = 18$).

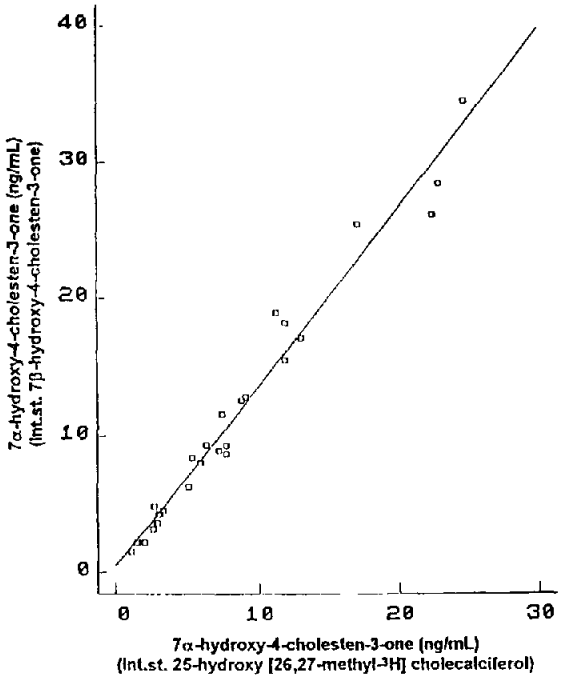

Fig. 4. Comparison between the serum levels of 7α-hydroxy-4-cholesten-3-one obtained with 7β-hydroxy-4-cholesten-3-one or 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol as an internal standard.

ten-3-one [9], justify this assumption. From our data, it may be concluded that the choice of an internal standard is limited by the protein binding of the substance. 7α-Hydroxy-4-cholesten-3-one seems to be bound by protein(s) which are not lipoproteins since cholesterol, which is almost exclusively located in the lipoproteins, is not retained on our solid-phase extraction columns. Thus more than 99% of the cholesterol and the apolipoprotein A_1 and -B content of serum was found in the first two wash fractions of the extraction procedure ($n = 5$, data not shown). Furthermore, 6-oxygenated derivatives of 4-cholesten-3-one, despite their excellent chromatographic behaviour in the absence of serum, were not found to be adequate in this respect. Our chosen standard however, behaved as 7α-hydroxy-4-cholesten-3-one in this respect. The protein binding may also explain the 30% discrepancy between our results and those reported in a previous study [7], since the internal standard (25-hydroxy[26,27-methyl-^{3}H]cholecalciferol) used in that method is known to be bound by a vitamin-D-binding protein [10]. Preliminary results in our laboratory indicate that 7α- and 7β-hydroxy-4-cholesten-3-one are not bound by the vitamin-D-binding protein. However, these results need further confirmation.

References

[1] H. Danielsson and J. Sjövall, *Annu. Rev. Biochem.*, 44 (1975) 233–253.
[2] E. Anderse'n, *Scand. J. Gastroenterol.*, 14 (1979) 657–662.
[3] A.F. Hofmann, *Clin. Gastroenterol.*, 6 (1977) 3–24.
[4] S.M. Grundy, E.H. Ahrens and G. Salen, *J. Lab. Clin. Med.*, 78 (1971) 94–121.
[5] G. Nicolau, S. Shefer, G. Salen and E.H. Mosbach, *J. Lipid Res.*, 15 (1974) 146–151.
[6] M. Axelson, I. Björkhem, E. Reihne'r and K. Einarsson, *FEBS* Lett., 284 (1991) 216–218.
[7] M. Axelson, A. Aly and J. Sjövall, *FEBS Lett.*, 239 (1988) 324–328.
[8] C.G. Eriksson, P. Eneroth and L. Nordström, *J. Steroid Biochem.*, 22 (1985) 649–655.
[9] L. Aringer and L. Nordström, *Biomed. Mass Spectrometry*, 8 (1981) 183–203.
[10] M. Imawari, K. Kida and S. Goodman, *J. Clin. Invest.*, 58 (1976) 514–523.