Mass fragmentographic determination of cholecalciferol and 25-hydroxycholecalciferol in human serum

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Summary. The serum extracts were purified by column-, thin layer- and high pressure liquid chromatography. Deuterated cholecalciferol and deuterated 25-hydroxycholecalciferol were used as internal standards. The quantitative analysis was performed using GC-mass fragmentography technique of TMS-ethers.

Many methods have been developed for the determination of vitamin D and its metabolites[1]. Some of them are based on the protein-binding technique[2-4]; others use gas chromatography or a gas chromatography - mass spectrometry technique[1,5-9]. The sensitivity and specificity of proposed assays varies and also depends on other parameters, such as extraction, choice of internal standards and occurrence of biological background in a sample[1,10,11]. The well-developed gas chromatography and simple electron impact fragmentation patterns of trimethylsilyl ethers of cholecalciferol and its metabolites are very well suited to the mass fragmentographic technique. The GC-SIM method presented here could also be applied for specific investigations of vitamin D metabolism in complex biological systems.

Using known procedures[1,12-15], we synthesized the internal standards **1, 2,** and **3** (cholecalciferol-3,4-d_2, cholecalciferol-2,2,3,4,4,6-d_6 and 25-hydroxycholecalciferol-26(27)-d_3 from cholesterol-3,4β-d_2[16], cholesterol-2,2,3,4,4,6-d_6, and 25-hydroxycholesterol-26(27)-d_3[8,17], respectively. Cholesterol-d_6 was prepared from cholest-4-en-3-one which was labelled in D_2O: D_2SO_4: dioxane. The resulting cholest-4-en-3-one-2,2,4,6,6,-d_5 was acetylated and reduced with sodium borodeuteride in MeOD[18].

Assay. 5 ml serum sample was mixed with 200 ng of **1** (or **2**) and 200 ng of **3,** 10 ml of water, 31 ml of methanol and 62 ml of dichloromethane. The sample was extracted for 30 min by shaking, the lower phase collected and the extraction repeated with 5 ml of methanol and 50 ml of dichloromethane. The combined extracts were evaporated to dryness and applied to a small alumina column, washed with n-hexane and finally eluted with ethanol. The ethanolic eluate was further purified on an alumina TLC plate using 3% methanol in dichloromethane as a solvent system. Zones of cholecalciferol and 25-hydroxycholecalciferol were removed from the plate and eluted with ethanol. The eluates were further purified by high-pressure liquid chromatography in order to remove traces of cholesterol and other impurities. The HPLC of cholecalciferol was performed on a 4% silver nitrate-LiChrosorb SI 60 column (0.3×25 cm, 10 µm) with 4% tetrahydrofuran in n-hexane as a solvent system (flow 3.1 ml/min, 68 bar, retention times (R_t): cholecalciferol, 16 min 10 sec; cholesterol, 19 min 15 sec). The 25-hydroxycholecalciferol eluate was chromatographed on a LiChrosorb SI 60 column (0.3×25 cm, 10 µm (Merck)) with 2% MeOH in dichloromethane as a solvent system (flow 0.3 ml/min, 35 bar). The retention times (R_t) were: cholecalciferol, 5 min 47 sec; cholesterol, 6 min 39 sec; 25-hydroxycholecalciferol, 9 min 15 sec.

The samples were collected, evaporated, dried and finally silylated at room temperature with 50 µl of the mixture of N,O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in the ratio 4:1. The samples were analyzed in a GC-MS apparatus equipped with SE-30 capillary column (figures 1 and 2). The pyroform of the TMS-derivative was measured. The peak of the TMS-isopyroform was only about 15% of the TMS-pyroform; however, the ratio of the pyro- to the isopyro-form depends on the nature of the column.

The following masses were monitored: cholecalciferol, (M^+); m/e 456 and 458 or 456 and 462, 25-hydroxycholecalciferol, (M^+-90); m/e 454 and 457.

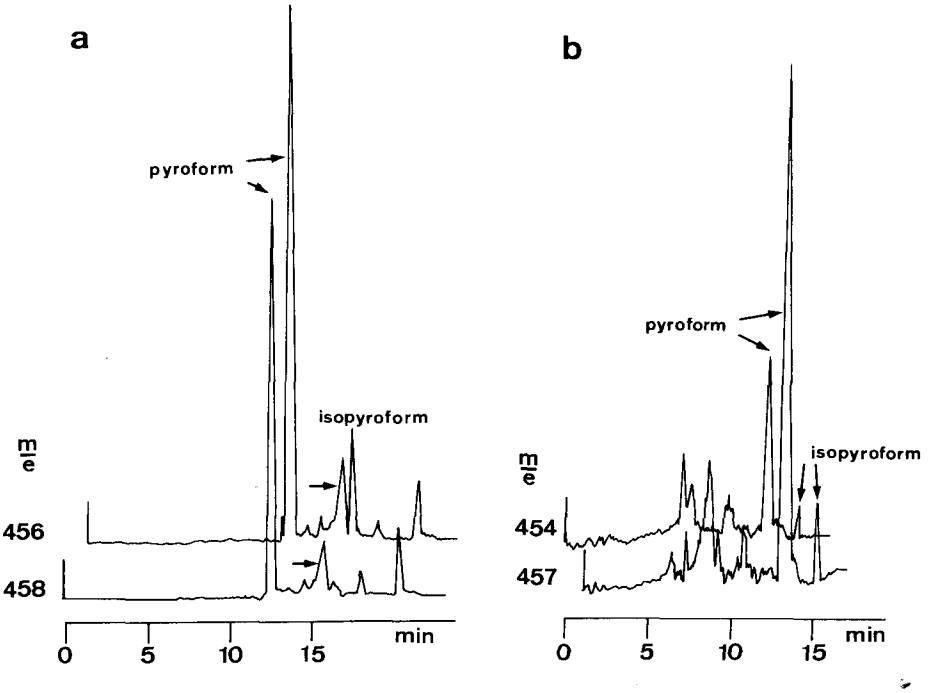


Fig. 1. GC-SIM chromatogram of trimethylsilyl ethers of cholecalciferol (a) and 25-hydroxycholecalciferol (b). GC: Capillary column SE-30 (0.29 mm i.d., 25 m), helium 1.8 ml/min. *a* Temperature program 220 °C–260 °C, 2 °/min, *b* isothermal hold at 250 °C. MS: Micromass 16F MS-apparatus, electron impact mode, 24 eV, 95 µA trap current, ACC Voltage 4 kV, SEV Voltage 3 kV, cycle time 0.3 sec.

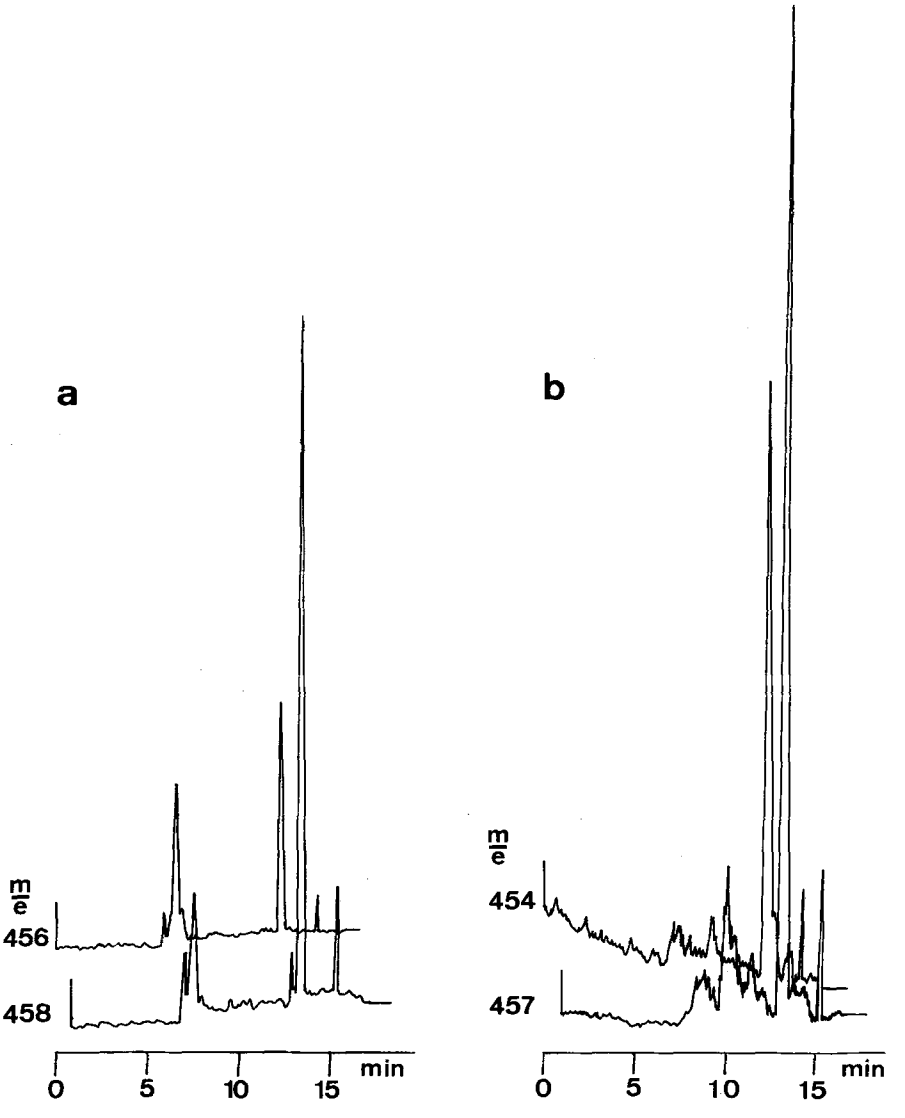
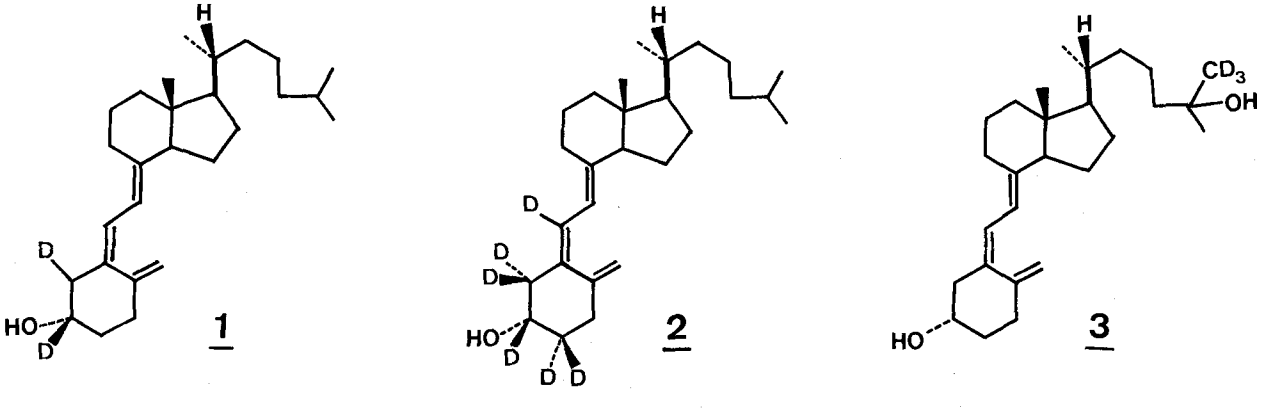


Fig. 2. GC-SIM chromatogram of silylated serum samples after column-, thin layer- and high-pressure liquid chromatography: *a* (cholecalciferol fraction) HPLC performed on 4% silver nitrate-silica gel column, *b* (25-hydroxy-cholecalciferol fraction) HPLC performed on silica gel. Details in figure 1.



GC-SIM assay of cholecalciferol and 25-hydroxycholecalciferol in serum was possible only after careful purification of serum extracts; therefore, special attention was paid to this problem. Crude serum extracts after filtration on LH-20 Sephadex[8] were useless because of high biological background and extremely large excess of cholesterol.

Application of column- and thin layer chromatography on alumina was also not sufficient in the assaying of 25-hydroxycholecalciferol (figure 3) because of at least 1 peak present next to the TMS-pyroform of 25-hydroxycholecalciferol.

The method described allowed the assay of both vitamins in ng levels, even when only 2 ml of serum were used. Linearity for this assay was observed between 5 and 300 ng

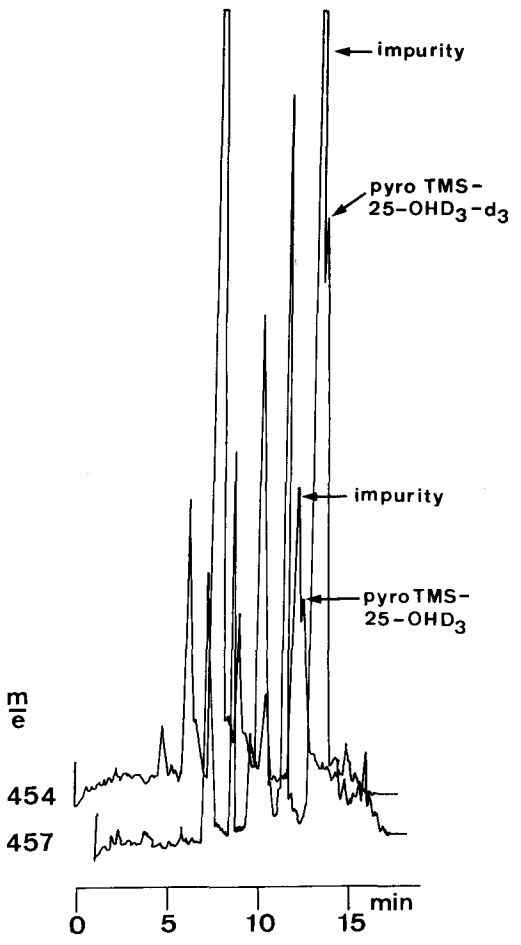


Fig. 3. GC-SIM chromatogram of silylated serum sample; fraction: 25-hydroxycholecalciferol. The sample was purified by column- and thin layer chromatography on alumina. Details in figure 1.

after the addition of cholecalciferol and 25-hydroxycholecalciferol to serum samples. Similar assay for 1,25-dihydroxycholecalciferol in serum is under investigation.

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19 The authors thank Dr. A. Fürst (F.Hoffmann-La Roche, Basel) for a sample of 3β-acetoxy-27-norcholest-5-en-25-one.

The reported occurrence of L-canavanine in soya bean, *Glycine max*[1]

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Summary. The reported occurrence of L-canavanine in soya bean, *Glycine max* could not be verified by enzyme treatment of the extracted non-protein amino acids of the seed.

Recently, Fischer et al.[3] reported the putative occurrence of L-canavanine in the seed of the soya bean, *Glycine max*. This secondary plant metabolite is a principal nitrogen-storing non-protein amino acid of certain legumes[4]. Fischer et al.[3] made the interesting observation that when ground meat was adulterated with soya bean meal, automated amino acid analysis of the meat sample extract produced a ninhydrin-positive peak believed to be canavanine. This finding, if confirmed, would be important since canavanine is a highly toxic non-protein amino acid and soya bean serves as an increasingly important source of human dietary protein. Moreover, canavanine possesses potent insecticidal properties[4], and, if present, could be utilized in experimental studies of toxic natural product-herbivore interaction. Several extensive plant surveys have established that canavanine's distribution is limited to the Papilionoideae (Faboideae), a major subfamily of the Leguminosae[5-7], where it has provided insight into the phylogenetic relationship of certain legumes[8,9]. The efforts of Tschiersch[7], Bell et al.[9], and Lackey[10] all failed to produce positive evidence for seed canavanine in *G. max*. Several ppm of canavanine have been reported in the garden onion, *Allium cepa*[11] and in the fungus of commerce, *Agaricus campestris*[12]. However, these reports have either been challenged[13] or have not been corroborated by independent methods for establishing and verifying minute levels of canavanine. In each instance, canavanine's purported occurrence was predicated solely upon isolation of a ninhydrin-positive substance having the column retention time of canavanine.

Materials and methods. The soya bean sample consisted of an approximately equal mixture of the following varieties: Woodworth, Forrest, Bonus, Dare, York, Williams, Corsoy, Essex Hood 75, and Mack. The soya bean meal (350 g), prepared as described previously[14], was extracted,