METHODS FOR THE ESTIMATION OF ERGOCALCIFEROL AND DIHYDROTACHYSTEROL IN HUMAN SERUM

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SUMMARY

It was attempted to develop separate methods for the estimation of calciferol and dihydrotachysterol in human serum for hypoparathyroid patients who take, daily, milligram quantities of these substances. The two methods are very similar; they involve extraction from serum with an acetone-alcohol mixture, thin-layer chromatography and gas chromatography. The method for dihydrotachysterol covers the probable range of concentrations in hypoparathyroid patients. Because of a poorer overall percentage extraction, the method for calciferol covers only half the probable range of calciferol concentrations in these people.

INTRODUCTION

The aim of this work was to develop separate methods for the estimation of ergocalciferol and dihydrotachysterol in the sera of hypoparathyroid patients. Thomas et al. measured the mean antirachitic activity of normal adult human serum as 2 i.u. per ml (equivalent to 0.05 μg of calciferol per ml). In hypoparathyroid patients on doses from 50000 to 200000 i.u. (1.25 to 5 mg) of ergocalciferol daily the serum antirachitic activity was 4 to 20 times greater than the normal mean (equivalent to 0.2 to 1 μg of calciferol per ml). In a few people studied by Mawer et al., the total antirachitic activity of serum was approximately evenly distributed between calciferol and 25-hydroxycalciferol. Hence an estimate of the range of ergocalciferol concentrations in hypoparathyroid patients is obtained by halving the concentrations inferred from the work of Thomas et al. This gives an expected range of 0.1 to 0.5 μg of ergocalciferol per ml of serum or 1 to 5 μg per 10 ml of serum. No equivalent data is available for dihydrotachysterol, because the antirachitic activity of dihydrotachysterol is very low (Illig et al.). In the absence of such data, it was assumed that the range for dihydrotachysterol was the same as that calculated for ergocalciferol, but the range could be less because usually the doses of dihydrotachysterol taken are less (by a factor of 2 to 4) than those of ergocalciferol.

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The methods to be described are a combination of a direct method of extraction of serum, thin-layer chromatography and gas chromatography. The development of the gas chromatographic systems and their quantitative aspects have been described elsewhere.

MATERIALS AND METHODS

Chemicals were of "Analar" quality wherever possible. All solvents were re-distilled and dried over anhydrous sodium sulphate. Tritiated cholecalciferol, used to check losses at various stages of the procedures, was prepared by catalysed exchange in tritiated glacial acetic acid at the Radiochemical Centre, Amersham and then purified as published by Thompson et al. Tritium was counted in a Packard Tricarb liquid scintillation counter using the external standard method for the correction of quenching. Radioactive cholecalciferol bound to serum proteins was counted by drying 0.1-0.2 ml of serum on a Millipore filter and placing the filter in 10 ml of counting solution (a solution of 2,5-diphenyl-oxazole, 1,4-bis(4-methyl-5-phenyl-oxazol-2-yl)benzene and naphthalene in toluene). When tritiated cholecalciferol was added to 5 ml of serum in a small volume (10-20 µl) of ethanol, mixed (so that the tritiated cholecalciferol became bound to serum proteins), then counted on Millipore filter as described above, it was found that the counts obtained were 62% of those obtained by adding the same amount of tritiated cholecalciferol directly to the counting solution.

This figure (62%) was used to correct counts obtained with serum samples for comparison with counts obtained when tritiated cholecalciferol was dissolved (and hence fully dispersed) in the counting solution. The tritiated cholecalciferol was stored in alcohol at −18° and the specific activity of purified material fell constantly over two years (see Fig. 1) while, at the same time, decomposition products appeared and some of the tritium exchanged with the solvent. At 1 year from the first purification it was calculated that 54% of the original pure cholecalciferol existed as various decomposition products. The tritiated cholecalciferol was purified just before use by thin-layer chromatography.

Trimethylsilyl ethers were prepared either by reaction with HMDS*, TMCS and pyridine in hexane or by reaction with BSA in hexane. The two methods gave the same results. In the first method up to 0.5 mg of sterol was dissolved in 0.2 ml hexane and 0.2 ml of dry pyridine. 0.3 ml of HMDS and 0.05 ml of TMCS were then added. The mixture was left overnight at room temperature and then evaporated to dryness at 50° by a jet of nitrogen. The residue was dissolved in a mixture of hexane–HMDS = 98:2 and centrifuged. The supernatant was removed and stored in a stoppered tube, in a desiccator, at 4°. For quantitative work the precipitate was washed with a further quantity of hexane–HMDS = 98:2 mixture, centrifuged, and the supernatant added to the first extract. The combined extracts were then made up to a known volume with hexane–HMDS mixture. In the second method up to 1.0 mg of sterol was dissolved in 0.5 ml of hexane and 0.5 ml (or less for smaller weights of sterol) of BSA was added. The mixture was heated in a stoppered test-tube at 50° for 45 min and then the contents of the tube were evaporated at 50° by a jet of nitrogen. The residue was dissolved in a measured quantity of hexane–HMDS = 98:2 mixture and stored as above.

Kieselgel PF-254 silicagel (Merck Chemical Co.) was used for thin-layer chromatography using both 2-mm and 300-μm thickness. Before use the adsorbant was washed with chloroform–methanol = 1:1, redistilled ethanol and then redistilled petroleum ether. The powder was dried for two days in an oven at 80°, ground in a mortar with a pestle, and then sieved through a 100-mesh sieve to remove lumps of material. This was necessary to reduce the baseline “compound noise” during gas chromatography following the elution of bands from thin-layer chromatography plates and evaporation of the eluate to small volumes; adsorbant treated in this way gave no interference during gas chromatography when the adsorbant was eluted with redistilled organic solvents and the eluate evaporated to a small volume (50 ml evaporated to 5 μl). Plates were prepared, using a spreader in the usual way, by spreading a suspension of the adsorbant in water. For Kieselgel PF-254 the suspension for spreading plates was prepared by shaking 100 g of silicagel with 180 ml of deionised water. Plates of silicagel impregnated with silver nitrate were prepared in the following way:

The silicagel was weighed and then a quantity of crystalline silver nitrate equal to 5% of the weight of the silicagel was weighed out. The silver nitrate was dissolved in the appropriate quantity of de-ionised water to produce the consistency of silicagel suspension suitable for spreading plates and then the plates were spread in the usual way. The operations of putting samples onto plates of silicagel and the development of plates in an appropriate glass tank were carried out in a Perspex nitrogen-box fitted with rubber arm-gloves.

A Beckman GC-4 gas chromatograph was used; it was a dual column analytical instrument fitted with dual flame ionisation detectors. The column packing was 1% OV-17 on silanised Gas Chrom Q and the carrier gas was oxygen-free nitrogen. Further details have been given earlier.

* The abbreviations used in this paper are as follows: BSA = bis (trimethylsilyl) acetamide; HMDS = hexamethyldisilazane; TMCS = trimethylchlorosilane; OV-17 = methylphenyl (50:50) silicone polymer; OAc = acetate derivative; TMSi-ether = trimethylsilyl ether derivative; \( D_4 \) = ergocalciferol; \( D_4 \) = cholecalciferol; DHT2 = dihydro-tachysterol2; DHT3 = dihydro-tachysterol3.

Extraction of calciferol and dihydrotachysterol from serum

The same method was used for both calciferol and dihydrotachysterol. 100 ml of acetone-ethanol = 1:1 were added to 5 ml of serum, the mixture shaken, and then placed in a water bath at 40° for 20 min. After filtering through Whatman No. 1 filter paper, and washing of the precipitated proteins with 20 ml of acetone-ethanol = 1:1, the mixture was evaporated to dryness in a rotary evaporator, at 50° with a flow of nitrogen through the apparatus. The serum lipids so obtained were extracted with 3 x 15 ml of warm ether and the combined extracts were evaporated to dryness at 50° under nitrogen.

Purification of the serum extract containing dihydrotachysterol

The extract was dissolved in a small volume of dichloromethane and this solution was spotted in a line onto a 2-mm thick Kiesel gel PF-254 plate (20 cm x 20 cm). A marker of pure dihydrotachysterol (20 µg) was placed at each side of the plate. The plate was developed with a solvent mixture of dichloromethane-ether-pyridine = 80:20:1 and the marker spots were located by viewing under ultra-violet light. The dihydrotachysterol band was cut out, transferred to a small sintered glass funnel, and eluted with dichloromethane-ethyl acetate = 1:1. The eluate was taken to dryness and the resulting extract was rechromatographed on a 300-µm Kiesel gel PF-254 plate using the same solvent as for the thicker plate.

The dried eluate from the second plate was dissolved in 0.5 ml of hexane and 30 µl of BSA were added. The mixture was heated at 50° for 45 min and then evaporated to dryness by a jet of nitrogen. The residue was then dissolved in 20 µl of dichloromethane and transferred to a glass sample holder in 1-µl aliquots using a Hamilton syringe. After each aliquot the solvent was allowed to evaporate before the next aliquot was added. Also using a Hamilton syringe, a known weight of cholesterol OAc was added to the glass sample holder as a standard measure of detector response. The sample was then ready for gas chromatography.

Purification of the serum extract containing ergocalciferol

The purification of the extract containing calciferol was carried out in the same way as for the extract containing dihydrotachysterol, except that the silicagel plates (both 20-mm and 300-µm thickness) were made with 5% silver nitrate-impregnated silicagel. Also a marker of 80 µg of calciferol was needed for the 2-mm thick plate in order to be visualised under ultra-violet light; for the 300 µm thick plate this could be reduced to 20 µg. The preparation of the trimethylsilyl ethers and the sample for gas chromatography were exactly the same.

Gas chromatography of the extracts

The conditions for gas chromatography were exactly the same for the extract containing calciferol as for the extract containing dihydrotachysterol. The development of these systems has been described earlier. The conditions for gas chromatography were designed to separate pyrocholecalciferol-TMSi-ether, pyroergocalciferol-TMSi-ether, cholesterol-TMSi-ether and cholesterol-OAc. The same gas chromatography conditions separated dihydrotachysterol-TMSi-ether, dihydrotachysterol-TMSi-ether and cholesterol-OAc. Cholesterol-TMSi-ether was not separated from dihydrotachysterol-TMSi-ether and cholesterol must be separated from the dihydro-
tachysterols before formation of the TMSi-ethers and gas chromatography. The column (10 ft. x 2 mm i.d.) was packed with 1\% OV-17 on 100–120 mesh silanised Gas Chrom Q. The temperature of the column was 210° and the carrier gas was nitrogen at 60 ml/min. The glass sample tube was introduced into the gas chromatograph using a solid sampler.*

RESULTS

Extraction

The extraction procedure was relatively quick and studies with tritiated cholecalciferol showed that 87–96\% of the radioactivity added to 5 ml of serum (total weight of cholecalciferol was 4 µg) was extracted, and 65–70\% of this ran at the \( R_F \) of vitamin D on a Kieselgel PF-254 plate using chloroform–ether–pyridine = 80:20:1 as developing solvent. It was also shown that the method extracted 90–100\% of the counts in the serum of a chick which had been injected subcutaneously with tritiated cholecalciferol three days earlier. For the same weight of cholecalciferol (4 µg) the extraction procedure involving alkaline saponification and extraction into benzene gave a recovery of 60\% of the counts and, after chromatography, about 30\% of the counts were attributed to cholecalciferol.

Purification of the extract containing dihydrotachysterol

After the development of the 2-mm silicagel plates and cutting out of the dihydrotachysterol band, the plates could then be sprayed with concentrated sulphuric acid and heated to carbonise the remaining lipids on the plate. An example is shown in Fig. 2. It can be seen that dihydrotachysterol (\( R_F = 0.56 \)) was clearly separated from free cholesterol (\( R_F = 0.36 \)). The band with a higher \( R_F \) than dihydrotachysterol was assumed to be neutral lipids (including cholesterol esters). It can be seen from Fig. 2 that some of the neutral lipid band was taken with the dihydrotachysterol band in this experiment. When the dihydrotachysterol was eluted and then re-chromatographed on a 300 µm PF-254 plate, using the same solvent mixture, then a clear separation was obtained between the dihydrotachysterol and the small amount of neutral lipid from the first plate. Thus, in two thin-layer chromatography steps, it was possible to separate dihydrotachysterol from most of the serum lipids. The dihydrotachysterol purified in this way was then reacted with BSA and put into the gas chromatograph.

Purification of the extract containing calciferol

After the development of the 2-mm silicagel plates and cutting out of the calciferol band, the plates could then be sprayed with concentrated sulphuric acid, and heated to carbonise the remaining lipids on the plate. Three examples are shown in Fig. 3. It can be seen that there were three main bands with higher \( R_F \) values than calciferol (\( R_F = 0.18–0.24 \)) and several other bands of lower \( R_F \) than calciferol. The band immediately above the calciferol was free cholesterol (\( R_F = 0.32–0.39 \)) and was separated from calciferol. The two upper bands were probably neutral lipids, including cholesterol esters, which were separated by the silver nitrate-impregnated silicagel into two bands, the lower one containing the esters with unsaturated fatty acids. It can be seen from this diagram that most of the serum lipids were separated from

calciferol in this step, but a further thin-layer (300-μm thickness) chromatographic separation was carried out before conversion to the trimethylsilyl ethers and gas chromatography.

Gas chromatography of the dihydrotachysterol extract

Known weights of DHT₃ (2 μg) and DHT₄ (1 μg) were added to a test sample of 5 ml of human serum. A further 5 ml of serum, without additions, served as the control. After extraction, purification and formation of the trimethylsilyl ethers the sample was transferred to a glass sample tube and 0.45 μg of cholesterol-OAc was added to the test sample tube. The chromatograms obtained from the test and control serum extracts are shown in Fig. 4. It is seen that the three main peaks were DHT₃-TMSi-ether, DHT₄-TMSi-ether and cholesterol-OAc. No detectable amount of cholesterol-TMSi-ether was present. From the standard response graphs the weights of DHT₃ and DHT₄ in the gas chromatography sample were calculated as 0.44 μg for DHT₃ and 0.215 μg for DHT₄. Assuming that DHT₄ was the "unknown" in the serum and that a known amount of DHT₃ was added, the weight of DHT₃ in the original sample was calculated as

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0.44 \times \frac{1.0}{0.215} = 2.05 \, \mu g
\]

This calculation assumes that the ratio of weights in the gas chromatography sample is

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Fig. 4. Gas chromatography of test, and control, serum extracts; 2 µg of dihydrotachysterol2 and 1 µg of dihydrotachysterol3 were added to 5 ml of serum (the test serum). 0.45 µg of cholesterol acetate was added to the sample tube containing the test serum extract before gas chromatography.

Column: 10 ft. x 2 mm i.d. 1% OV-17 on silanised Gas Chrom Q (100–120 mesh). Temperatures: inlet 240°; column 210°; detector line 260°; detector 290°; Carrier gas: nitrogen at 60 ml/min

Final attenuation: 1 x 10^4 (5 x 10^-4 amps f.s.d.) Sampling: solid sampling. 1 = DHT2-TMSi ether; 2 = DHT3-TMSi ether; 3 = cholesterol-OAc.

Fig. 5. Gas chromatography of test, and control, serum extracts; 5 µg of vitamin D2 and 5 µg of vitamin D3 were added to 5 ml of serum (the test serum). 0.5 µg of cholesterol acetate was added to the sample tube containing the test serum extract. The conditions for gas chromatography are the same as those given in the legend to Fig. 4. 1 = pyrocholecalciferol-TMSi ether; 2 = pyroergocalciferol-TMSi ether; 3 = cholesterol-TMSi ether; 4 = cholesterol-OAc.

the same as that in the original sample. It is seen that the calculated weight of DHT2 is close to the amount added to serum, and it can be calculated that the overall % extraction was 21.5% from the amount of DHT2 found. In two other experiments in which 3 µg each of DHT2 and DHT3 were added to 5 ml of serum, the % extractions were added to 5 ml of serum, the % extractions were 28% and 24% and the calculated weights of DHT2 were 3.2 µg and 2.7 µg respectively. In these experiments DHT3 was used as the internal standard for DHT2.

Gas chromatography of the calciferol extract

For this method D2 was used as the internal standard for D2. 5 µg of D2 and 5 µg of D3 were added to 5 ml of serum, which was then extracted and purified. After formation of the TMSi-ethers, the extract was transferred to a glass sample tube and 0.5 µg of cholesterol acetate was added before gas chromatography. The resulting gas chromatogram is shown in Fig. 5. Small peaks due to the TMSi-ethers of D2 and D3 can be seen, while the extract from the control serum (which had no additions)

shows no interfering peaks. Some cholesterol-TMSi-ether occurs in the sample but is separated from the calciferol-TMSi-ether. From the response graphs obtained with pure compounds it was calculated that the overall recovery was 3.6%, but it was still possible to obtain an estimate (5.6 µg) of the D₃ in 5 ml of serum. As before, it was assumed that the ratio of weights in the gas chromatography sample was the same as in the original sample.

In a second experiment, when 4 µg of D₃ and D₆ were added to 5 ml of serum, the % extraction was 4.7% and the weight of D₃ was calculated to be 4.4 µg.

Using tritiated D₃, a study was made of the various stages to see if the loss of calciferol occurred at any particular step. It was found that, for the weights of calciferol used in these experiments (3 to 5 µg), calciferol could be lost at all stages of the procedure, but there was no one step where the large loss occurred. As might be expected, the largest losses occurred during extraction and adsorption chromatography, and, together, these processes constituted almost the whole procedure.

**Discussion**

The main problems in the estimation of dihydrotachysterol₂ or ergocalciferol in serum were:

(i) that a relatively large amount of other lipids (including cholesterol) are inevitably extracted from serum with the seco-sterols

(ii) that in many chromatographic systems cholesterol and calciferol migrate at rates which are not very different so that these systems are unsuitable for samples which contain a small amount of calciferol and a large amount of cholesterol, and

(iii) that dihydrotachysterol and calciferol are unstable molecules and the method must allow for losses during extraction and purification.

The direct extraction of serum with an alcohol and acetone mixture was quick and gave superior results to alkaline hydrolysis plus extraction when a few micrograms of tritiated cholecalciferol were added to serum.

The separation of microgram amounts of calciferol from the large amounts of other serum lipids was achieved by the adsorption chromatography on layers of silver nitrate-impregnated silicagel. The first stage employed 2-mm thick layers and the second 300-µm thick layers. In these two stages, the separation was almost complete. In one trial, a small amount of cholesterol remained with the calciferol but this did not interfere in the gas chromatography stage. Cholecalciferol and ergocalciferol were not separated during adsorption chromatography, but were separated (as the pyrocalciferol-TMSi-ethers) from each other during gas chromatography. Thus the "internal standard" technique (used by Murray et al. with the isovitamins D) using cholecalciferol as an internal standard for ergocalciferol, could be used. The assumption in this technique is that the ratio of weights of ergo- and cholecalciferol in the sample introduced into the gas chromatograph is the same ratio as in the original serum sample. In the experiments reported in this paper, it appeared that this assumption was correct and the method gave the expected results to within 12%.

The results obtained with calciferol method showed that, at a concentration of 5 µg of D₄ per 5 ml of serum, an overall recovery of 4 to 5% was obtained. However, in two trials, estimates of the ergocalciferol concentration were obtained which were within 12% of the expected figure. It can be calculated that the most probable
weight of naturally occurring calciferol in the 5 ml of serum is 0.13 \( \mu g \), but, since the ratio of weights of \( D_2 \) and \( D_3 \) is not known, this figure has not been taken into account for the calculation of overall \% recovery.

This method can be extended to 5 \( \mu g \) of calciferol in a greater volume of serum (10 to 15 ml) by using two 2-mm thick plates in the first thin-layer chromatographic stage. However, only a slightly smaller weight than 5 \( \mu g \) of calciferol could be used unless the overall \% recovery, or the sensitivity of detection, could be improved. The limit of the method is probably about 3 \( \mu g \) per 10 ml of serum. This limit is about the middle of the expected range for hypoparathyroid patients taking ergocalciferol daily.

The method for the estimation of \( DHT_2 \) was very similar to that for \( D_2 \) except that no silver nitrate was added to the silicagel. In three trials, after the two thin-layer chromatography stages, no cholesterol was found in the purified dihydrotachysterol. This was fortunate, because \( DHT_2 \)-TMSi-ether is not well separated from cholesterol-TMSi-ether in the gas chromatographic system used. \( DHT_3 \) was used as an internal standard for \( DHT_2 \) and the correct values (to within 10\%) for the weight of \( DHT_2 \) added to serum were obtained.

The results obtained with the DHT method showed that, at a concentration of 2 to 3 \( \mu g \) of \( DHT_2 \) added to 5 ml of human serum, an overall recovery of 20 to 30\% was obtained. In three trials, estimates of the \( DHT_2 \) concentration were obtained which were within 10\% of the expected figures. The method can be extended to 10 to 15 ml of serum by using two 20 cm x 20 cm plates in the first (2-mm thick) thin-layer chromatography stage. Also, however, for the same overall \% recovery the weight of \( DHT_2 \) could be reduced to 0.5 \( \mu g \). Thus, the limit of the method is about 0.5 \( \mu g \) per 10 ml of serum. This covers the probable range of concentrations in the sera of hypoparathyroid patients taking \( DHT_2 \) daily.

The methods could be made more sensitive by increasing the overall \% recovery or by increasing the sensitivity of detection. The greatest interest is in improving the sensitivity of the calciferol method and in increasing the overall \% recovery in this method. The purification procedures used were fairly rapid and the thin-layer chromatography operations could be carried out under nitrogen. The studies with generally labelled tritiated cholecalciferol suggested a continuous loss of calciferol for the methods used and for the weights (3-5 \( \mu g \)) of calciferol used. A study of the breakdown of calciferol dried out on silicagel in air gave an estimate of the breakdown of calciferol as 0.05 to 0.1 \( \mu g \) per hour; it is not known, however, if this estimate is relevant to calciferol moving on a thin-layer plate or to other steps in the method. The overall \% recovery of calciferol in this work is lower than that reported in the work of de Vries et al. These workers used a colorimetric method of estimation following column chromatography in a darkened room and obtained overall \% recovery figures (from serum) of about 90\% for 2.5 \( \mu g \) of calciferol per 20 ml of serum. It is not known why these two sets of results do not correlate.

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