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DETERMINATION OF VITAMIN D_3 IN COD-LIVER OIL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The present *British Pharmacopoeia* monograph for cod-liver oil requires a bioassay for the vitamin D_3 content which is both time-consuming and complex. Alternative assays employing chromatographic procedures have been described but all these involve prior saponification of the oil. A selective extraction for vitamin D_3 without the need for saponification is reported in this paper. The extraction utilizes only chromatographic assay using argentation on reversed-phase silica, with vitamin D_2 as the internal standard. Reproducibility of injection gave a coefficient of variation of 0.6%, and repeatability of extraction for six samples gave a coefficient of variation of 6.8%.

INTRODUCTION

The present assay for vitamin D_3 in cod-liver oil in the *British Pharmacopoeia* is a biological procedure depending on the development of rickets in rats and its subsequent correction by administration of the oil and takes about 5 weeks[1,2]. An alternative non-biological procedure, such as one employing high-performance liquid chromatography (HPLC), might be expected to be more accurate, precise, cheaper and more rapid. Chromatographic assays of vitamin D_3 in cod-liver oil and many other non-biological assays of fat-soluble vitamins from complex matrices have involved prior saponification to remove esterified lipids and release any associated vitamin[3–8]. However, under these conditions previtamin is formed from vitamin D_3, and this isomer possesses 34–56% of the biological activity of vitamin D_3 in the rat[9,10]. In an attempt to circumvent this problem, vitamin D_2 which behaves in this way has been used as the internal standard, but if the fish-liver oil contained previtamin D_3 before extraction, saponification would cause re-equilibriation and the original previtamin content would be unknown. Vitamin A from liver tissue has been determined without prior saponification[11] and recently this approach has been used for the analysis of vitamin D* in resins, oils, dry concentrate and multivitamin formu-

* Vitamin D refers to both vitamin D_2 and D_3.

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lations[8,12–15]. The fat-soluble vitamins of cod-liver oil have been identified by HPLC without use of an extraction procedure[16], but vitamin D_3 cannot be assayed using this approach because of its low concentration relative to those of vitamin A and cholesterol (the weight relationship may be 1:100:3000 respectively[6]). Vitamin D_3 and related compounds have been extracted from complex matrices using silica cartridges[15,17] and stucturally related steroids have been resolved by argentation reversed-phase HPLC[18]. This paper reports a method for the assay of vitamin D_3 in cod-liver oil without prior saponification that utilizes such chromatographic separations in sequence to effect a preliminary purification then assay of the vitamin.

EXPERIMENTAL

Apparatus

An LDC minipump was used together with a Cecil 272 spectrophotometer set at 268 nm and a Rheodyne 7125 loop injector.

Materials

LiChroprep Si 60 15–25 μm and SEP-PAK Silica cartridges were purchased from BDH and Waters Assoc. (Milford, MA, U.S.A.), respectively. Stainless-steel columns between 100 and 200 mm × 4.6 or 5.0 mm I.D. were slurry-packed at 300 bar with the normal phases LiChrosorb Si 60 (10 μm; BDH) and Partisil 5 (Whatman), and the reversed phases: Spherisorb S5 ODS (Phase Separations, Queensferry, Great Britain), Hypersil ODS (Shandon Southern, Runcorn, Great Britain) and Nucleosil C_{18} (5 μm; Macherey, Nagel & Co., Düren, G.F.R.).

Hexane (GPR) and ethyl acetate (AnalaR) were purchased from BDH, propan-2-ol from Koch-Light and methanol from James Burrough Ltd. Retinol acetate, D-α-tocopherol acid succinate, vitamin D_2 (ergocalciferol) and 7-dehydrocholesterol were obtained from Sigma (St Louis, MO, U.S.A.) and vitamin D_3 (cholecalciferol) from Aldrich (Milwaukee, WI, U.S.A.). Fish-liver oil preparations were purchased from local pharmacies.

Saponification

This was carried out as described by Bell and Christie[3].

Formation of previtamin D

Portions (1 ml) of each vitamin D_2 and D_3 (10 μg) in methanol–water (95:5) were heated at 73°C and 102–110°C in screw-cap Wheaton vials.

Assay procedure

Throughout the procedure the samples were protected from direct light. The procedure finally adopted for the extraction and assay of vitamin D_3 from cod-liver oil consisted of three main steps (described in detail below): (1) extraction, in two stages on normal-phase silica; (2) purification, on reversed-phase HPLC; (3) assay, by reversed-phase HPLC in the presence of silver ions (Fig. 1.)

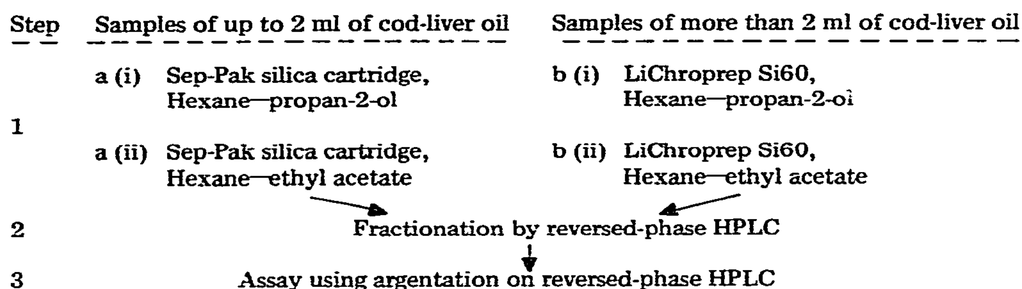

Fig. 1. Flow diagram for the extraction and assay of vitamin D_3 in cod-liver oil.

Step 1. Extraction

(a) Samples of up to 2 ml of cod-liver oil. A sample (2 ml or 2 g) of cod-liver oil was diluted 1:2 into hexane and 200 μl of vitamin D_2 (10 μg ml^{-1} in hexane) were added. A Sep-Pak cartridge was washed with 5 ml of hexane–propan-2-ol (95:5, v/v) followed by 5 ml of hexane using a glass syringe. The cod-liver oil was loaded onto the pak, flushed with 20 ml of hexane and eluted with 4 ml of hexane–propan-2-ol (95:5, v/v), the last 2 ml of which were collected (including the yellow band) and evaporated to dryness under nitrogen; the residue was redissolved in 2 ml of hexane. This solution was loaded onto a fresh cartridge (previously washed with 5 ml of hexane), and eluted with 3 ml of hexane–ethyl acetate (85:15, v/v) then 5 ml of hexane–ethyl acetate (80:20, v/v). The final 5 ml of eluent were collected and evaporated to dryness under nitrogen.

(b) Samples of more than 2 ml of cod-liver oil. LiChroprep (5 g) in 20 ml of hexane–propan-2-ol (95:5, v/v) was slurry-packed into a sintered glass filter (No. 2, 95 mm × 22 mm) under suction and washed with 60 ml of hexane. Cod-liver oil (up to 10 ml and diluted 1:2 into hexane) was loaded onto the column, washed with 60 ml of hexane, and eluted with 40 ml of hexane–propan-2-ol (95:5, v/v); the last 20–30 ml (including the yellow band) were collected. This fraction was evaporated under nitrogen and the residue redissolved in 10 ml of hexane. Silica (5 g) was used for the second preparative column, and the procedure was a scaled-up version of that employing the Sep-Pak cartridge and hexane–ethyl acetate described above.

Step 2. Purification by preparative reversed-phase HPLC

The mobile phase was methanol–water, the proportions of which were adjusted for the column in use (88–95%, v/v methanol). The extract from the normal-phase separations was emulsified in the mobile phase (500 μl) and injected into the reversed-phase column (flow-rate: 2 ml min^{-1}). The large peak preceding vitamin D and that of vitamin D were collected in one fraction, extracted twice into hexane and dried under nitrogen. Any residual UV-absorbing material was washed off at 4 ml min^{-1} with 100% methanol.

Step 3. Assay of vitamin D_3

Both the reversed-phase packing and mobile phase were the same as those used for step 2, except that 2% (w/v) silver nitrate were present in the latter (filtered using 5-

μm glass fibre). The sample was dissolved in the mobile phase and aliquots, not exceeding 220 μl, were injected. Precautions were necessary to avoid accumulation of silver precipitates: the mobile phase was maintained in the dark, stainless steel unions present in the flow cell were sealed with PTFE tape to prevent jamming, the column top meshes were regularly checked and, when dark brown, the column top was replaced.

RESULTS AND DISCUSSION

Vitamins D_2 and D_3 co-elute during normal-phase chromatography on silica and are either partially or wholly resolved by reversed-phase HPLC. The initial approach for the determination of vitamin D_3 in cod-liver oil was to collect the fraction correlating with the D_2 standard by normal-phase HPLC and assay the vitamin by reversed-phase HPLC (Fig. 2). Complexation of the two vitamins with silver ions and subsequent separation on C_{18} bonded silicas as used by Tscherne and Capitano[18] was the assay procedure of choice. It gave the best and most reproducible resolution of a variety of reversed-phase systems investigated employing various combinations of organic modifiers and water as the mobile phase. However, problems arose with repeated injections onto the silica column; the retention of vitamin D varied, the columns were short-lived and frequent blockages developed that were attributed to the high viscosity of the fish-liver oil (after a 1:2 dilution into hexane) and, possibly, particulate matter present in the oil. Other procedures investigated for the isolation of vitamin D were various combinations using precipitation in methanol and digitonin at −18°C and 4°C, respectively, silica and C18 SEP-PAK cartridges, reversed- and normal-phase HPLC.

Method of assay

The final procedure developed involves, in step 1, extraction by two separations (one of which was developed by Lofty *et al.*[15]) on preparative normal-phase

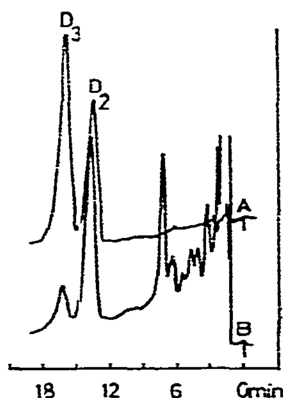

Fig. 2. Chromatogram obtained in step 3 after one normal-phase fractionation in step 1 (Partisil 5; mobile phase, hexane–propan-2-ol (98:2, v/v); flow-rate, 1 ml min^{-1}). A, standards; B, cod-liver oil extract. D_2 and D_3, vitamins D_2 and D_3, respectively. Step 3 conditions: Spherisorb S5 ODS (100 mm × 5 mm I.D.); mobile phase, methanol–water (85:15, v/v) with 2% (w/v) silver nitrate; flow-rate, 1.5 ml min^{-1}.

silicas, in step 2, purification by reversed-phase HPLC and, in step 3, assay on the same reversed-phase packing in the presence of silver ions (Figs. 1 and 3). Resolution of the two standard vitamins in the final assay ranged between 1.4 and 1.9 for the three packings investigated but was lower for the fish-liver oil extracts (1.1 to 1.5; column efficiencies: 1309–4215 theoretical plates per 100 mm for the vitamin D_3 standard). Purification on reversed phase (step 2) yielded characteristic elution profiles for the different preparations with large amounts of UV-absorbing material eluting with 100% methanol, thus demonstrating the necessity for this step (Fig. 4). Isolation of vitamin D_3 from 2 ml of cod-liver oil yielded enough material for duplicate or more determinations. Good separations were also seen with a single Sep-Pak extraction (hexane–ethyl acetate, in step 1a) with subsequent purification in step 2. However, for some extractions, improved baselines were obtained using two Sep-Pak normal-phase separations and, in addition, use of the two normal-phase systems

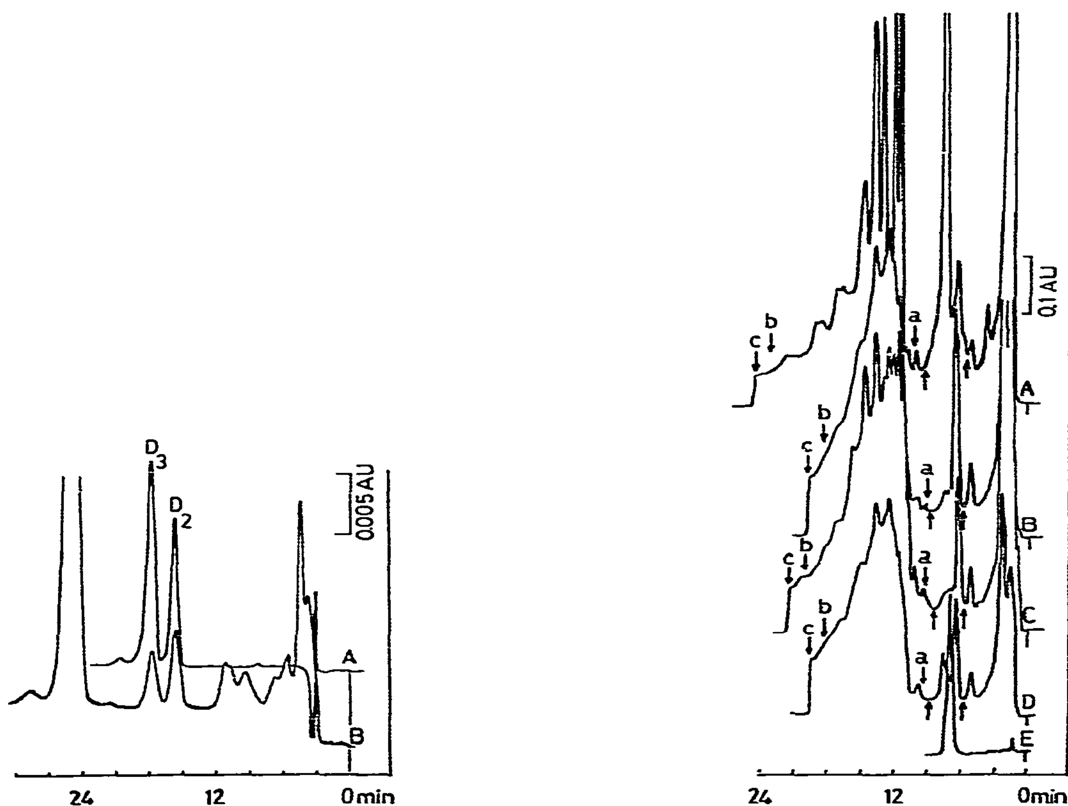

Fig. 3. Chromatograms obtained after the extraction procedure. A, vitamin D standards; B, cod-liver oil extracted without prior saponification. Support, Hypersil ODS (100 mm × 4.6 mm I.D.); mobile phase, methanol–water (95:5, v/v) with 2% (w/v) silver nitrate; flow-rate, 1 ml min^{-1}.

Fig. 4. Chromatograms given by extracts of different fish-liver oil preparations in step 2. A, halibut; B, C and D, different cod-liver oil preparations; E, vitamin D_2 and D_3 standards. Support, Hypersil ODS (100 mm × 4.6 mm I.D.); mobile phase, methanol–water (95:5, v/v); flow-rate, 2 ml min^{-1}, ↑↔↑, collected fraction; a, 100% methanol 4 ml min^{-1}; b, 95% methanol 4 ml min^{-1}; c, 95% methanol 2 ml min^{-1}.

might exclude interfering components present in supplemented formulations or alternative preparations, such as other fish-liver oils, and perhaps give a broadly applicable method. The procedure using two preparative silica separations and purification by reversed-phase HPLC without prior saponification took less than half a day for 2 ml (steps 1a and 2) and up to a whole day for 10 ml of fish-liver oil (steps 1b and 2).

Purity of the vitamin D peaks

Extraction of 10 ml of cod-liver oil in the absence of the internal standard demonstrated that no peak co-eluted with vitamin D_2 (Fig. 5,I). However, partial interference was evident for a saponified sample that was purified using the same extraction regime (steps 1b and 2, Fig. 5,II). The purity of the D_3 peak from extracts that were prepared with and without saponification in the absence of the internal standard were investigated by repeated assays and monitored at different wavelengths. The UV-absorption profiles were not significantly different from those of the standard D_3. Vitamin D_3 fractions from the assay (step 3) were also extracted into hexane. Half the extract was examined by normal-phase HPLC and the remainder by step 2 (Fig. 6). No significant contamination was evident since the extra peaks were also present in the control fractions that were collected immediately before and after the D_3 peak during the assays.

Because the previtamin might be present in the cod-liver oil, the formation and elution positions of this isomer in steps 2 and 3 were investigated. In the former system previtamin D_2 preceded vitamin D_2 as a shoulder and, likewise, previtamin D_3 eluted before D_3. In the latter system the elution positions were reversed with the isomers emerging after their respective vitamins such that previtamin D_2 co-eluted with vitamin D_3 (Table I). Consequently, the D_3 previtamin if present should be carried over with the step 2 fraction and subsequently be detected during the assay.

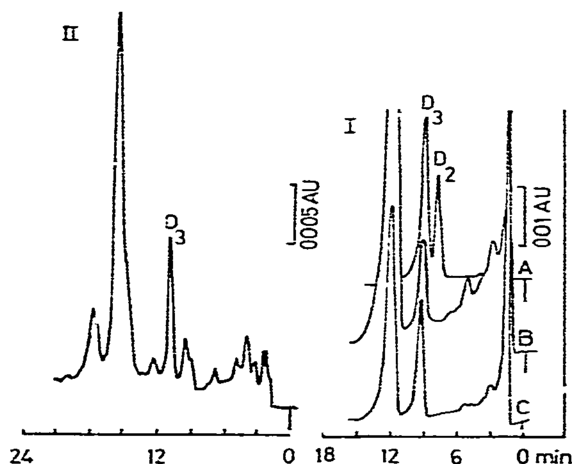

Fig. 5. Chromatograms of cod-liver oil extracts prepared without the internal standard. (I) A, vitamin D standards; B and C, extracts from different cod-liver oil preparations; support, as for Fig. 2; mobile phase, methanol–water (88:12, v/v) with 2% (w/v) silver nitrate; flow-rate 1.5 ml min^{-1}. (II) Saponified cod-liver oil; support and mobile phase, as for Fig. 3; flow-rate, 1.5 ml min^{-1}.

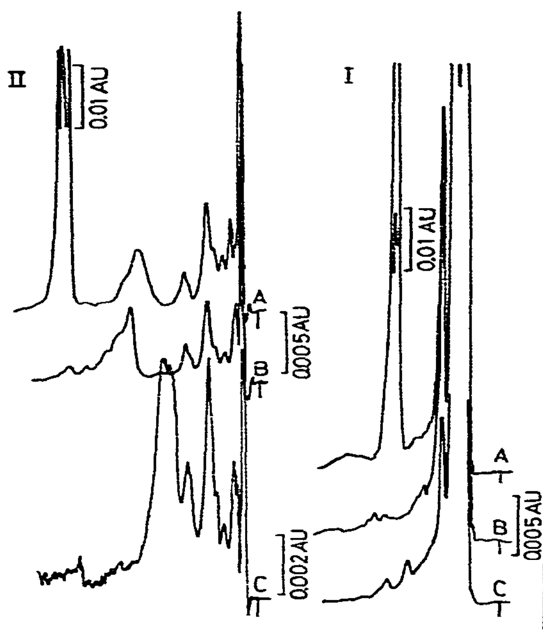

Fig. 6. Composition of vitamin D_3 peaks for cod-liver oil collected during assay (step 3). A, vitamin D_3 peak; C, blank immediately before and B, blank immediately after the vitamin D_3 peak. (I) Support, LiChrosorb Si 60 (200 mm × 4.6 mm I.D.); mobile phase, hexane–propan-2-ol (98.5:1.5, v/v); flow-rate, 1.5 ml min^{-1}. (II) Support, Hypersil ODS (100 mm × 4.6 mm I.D.); mobile phase, methanol–water (92:8), flow-rate, 1.5 ml min^{-1}.

The separate vitamins, on heat treatment at 73°C and 107–110°C reached equilibrium after 2.5–3.5 h and no longer than 41 min, respectively. The equilibrium ratios of vitamin to previtamin were *ca.* 81:19 (73°C) and 72:28 (102–110°C) for both vitamins D_2 and D_3 (using a correction factor for the difference in absorptivity between the isomers[12]) and were similar to those determined in other solvents[12,13]. If previtamin

TABLE I

CAPACITY RATIOS (k') RELATIVE TO VITAMIN D_3

	*Step 2**	*Step 3***
Retinyl acetate	0.367	0.602
Previtamin D_2	0.807	1.017
Previtamin D_3	0.896	1.150
Vitamin D_2	0.942	0.847
Vitamin D_3	1.000	1.000
Ergosterol	1.458	0.759
Provitamin D_3	1.458	0.847
Tocopherol acetate	2.275	4.152
Major peak from cod-liver oil extract	0.837	1.418

* Step 2: stationary phase, Hypersil ODS; mobile phase, methanol–water (95:5).
** Step 3: as for step 2 with 2% (w/v) silver nitrate in the mobile phase.

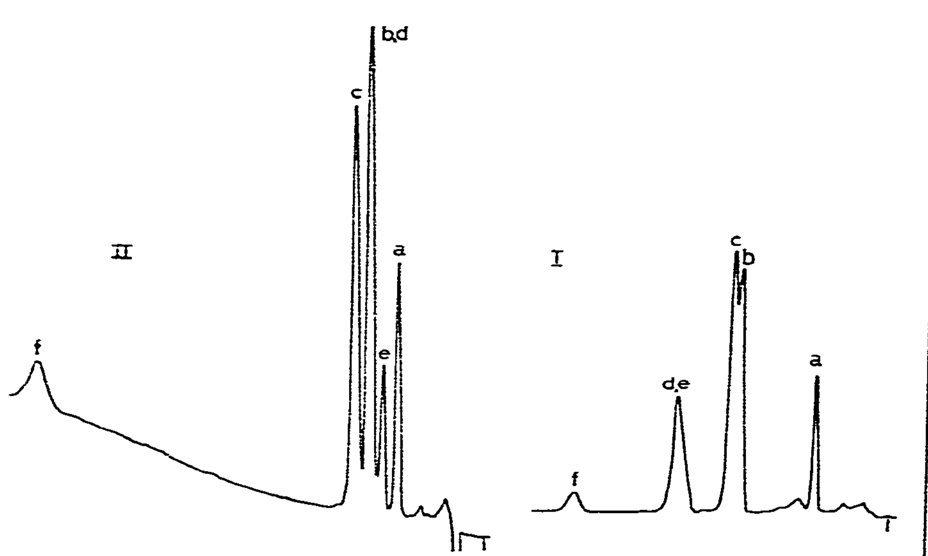

Fig. 7. Elution positions of fat-soluble vitamins and related compounds. Peaks: a = retinol acetate; b = vitamin D_2; c = vitamin D_3; d = provitamin D_3; e = ergosterol; f = tocopherol acetate. (I) support and mobile phase, as for Fig. 3 without silver nitrate. (II) Support and mobile phase, as for Fig. 3. Flow-rate for I and II, 1 ml min^{-1} and 1.5 ml min^{-1}, respectively.

D_3 were to be detected, the lower biological activity of this isomer must be included in the potency measurement, the determination of which has been discussed in the literature[12,13].

The elution positions of the respective provitamins and vitamins A and E were investigated in steps 2 and 3 (Table I and Fig. 7). None should be carried over from step 2 and cause interference with the determination. Vitamin E also was separated and this assay might also be applicable to multivitamin preparations.

Reproducibility and linearity of response

Vitamin D_2 was a suitable internal standard for samples prepared without saponification because of its elution characteristics on reversed-phase chromatography in the absence and presence of silver ions and because no UV-absorbing component co-eluted with vitamin D_2 in the argentation system (step 3, Fig. 5,I). However, a sample blank or alternative internal standard would be necessary for the saponified fractions since a UV-absorbing peak interfered with vitamin D_2 in the assay (step 3, Fig. 5,II). The coefficient of variation of five or more replicate injections for a 10-ml cod-liver oil sample prepared with and without saponification was 3.3% and 0.62% (peak height measurements) respectively. The variation in peak height for fish-liver oil samples (10 ml) that had been spiked with vitamin D_3 after extraction was linear for a concentration range of 0–300 ng per injection. Since interference with the internal standard was evident for the saponified samples and the extraction without prior saponification was faster, only the latter approach was investigated further.

Repeatability of the extraction procedure for six aliquots of 2 ml of fish-liver oil (15 ml was diluted 1:2 into hexane and 4-ml samples were taken) gave a coefficient

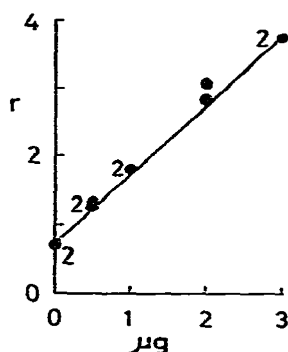

Fig. 8. Linearity of the vitamin D_3 to vitamin D_2 ratio with the content of spiked cod-liver oil samples. r = ratio of vitamin D_3 to vitamin D_2; μg = amount of vitamin D_3 added per ml cod-liver oil before extraction.

of variation of 6.8%, the five best samples of which gave a value of 4.0%. A further four 2-ml samples were spiked with vitamin D_3 (0.5–3.0 $\mu g\ ml^{-1}$ cod-liver oil) before extraction and the variation in peak height obtained was linear (Fig. 8). Addition of the internal standard to 2 ml of cod-liver oil with two subsequent Sep-Pak extractions (step 1a) and one purification only during step 2 gave yields of up to 14% of vitamin D_2 and permitted duplicate or more injections in step 3. This yield was increased to 30% by repeated fractionations using step 2. Consequently, despite the poor recoveries of the internal standard, the yield of both vitamins remained in a fixed ratio that depended on the initial concentration of each.

Six different preparations of fish-liver oil were assayed twice by this procedure (steps 1a, 2 and 3) and good agreement was obtained between the two sets of results for five of the samples (Table II), one of which (preparation 5, Table II) was a malt extract supplemented with cod-liver oil; the coefficient of variation of 15% for this preparation was attributed to the very small peaks that were obtained. The other cod-liver oil preparation which contained orange extract (preparation 6, Table II) gave

TABLE II

ESTIMATES OF THE VITAMIN D_3 CONTENT IN FISH-LIVER OIL BY HPLC

I and II–single extractions. I-1, halibut oil diluted 1:10 into hexane before extraction; I-2 and II-2, cod oil diluted 1:4 and 1:2 into hexane respectively before extraction; I-6, slightly different initial extraction used to that of II-6. C.V. = coefficient of variation; ns = not stated.

Preparation	*Areas relative to vitamin D_2*				*$\bar{x}$ total*	*C.V. (%)*	*I.U. D_3 per ml of oil*	*Labelled I.U. (ml^{-1})*
	I		*II*					
1 Halibut	2.243*	1.823	17.179		1.771	4.0%	708.4	ns
2 Cod	1.115	1.132	2.119	1.999	1.077	5.6%	172.3	200
3 Cod	0.827	0.865	0.845	0.808	0.836	2.4%	33.5	40
4 Cod	1.516	1.512	1.417	1.421	1.467	3.4%	58.7	ns
5 Malt + Cod	0.189	0.224	0.171		0.195	15.4%	4.1	ns
6 Cod + Orange	0.364	0.454	0.645	0.600	0.516	25.2%	20.6	ns

* Value not included in the mean since the attenuation of the recorder was altered during elution.

poor agreement. This was attributed to a slight alteration in the extraction procedure which was made in an attempt to increase the yield. The two supplemented preparations (5 and 6, Table II) required additional extractions but still gave very low yields when assayed by this procedure; further investigations to maximize these yields are necessary. The inclusion of salts might reduce the solubility of the vitamin D in the aqueous fraction and improve the yield from all the preparations. The estimated contents of two of the fish-liver oil preparations were 84% and 86% of the labelled contents. Percentages such as these may reflect the wide limits of error of the bioassay, or could indicate that a proportion of vitamin D_3 remained in association with another component during the isolation procedure, or that deterioration of the vitamin occurred during storage.

This procedure avoids saponification with its accompanying problems and is both rapid and reproducible for fish-liver oil preparations. The assay (step 3) might also be useful for the assay of fat-soluble vitamins in multivitamin preparations since it resolved the three vitamins investigated.

ACKNOWLEDGEMENT

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