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Analytical Methods Section

Determination of cholecalciferol in meat and fat from livestock fed normal and excessive quantities of vitamin D

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A method for the measurement of cholecalciferol in meats and fats is described involving alkaline digestion, clean-up on alumina and two-dimensional HPLC on silica and reversed-phase columns. The method has been used to measure levels in meat and fat ranging from normal (0·2 μg per 100 g) to excessive (200 μg per 100 g). It was developed for the examination of edible tissues from overdosed farm animals, in which the analysis of fat is recommended. An extension of the procedure is described for the measurement of 25-hydroxycholecalciferol; however, residues in the form of this metabolite are concluded to be of lesser practical significance.

Although an absorbance detector at 254 nm is recommended in the procedure, the identification of cholecalciferol can be enhanced by use of a sensitive photodiode array detector during HPLC.

INTRODUCTION

Vitamin D is added to a few foods in Canada to protect vulnerable groups, which may be deprived of other dietary sources and insufficiently exposed to sunlight, from deficiency. Although prevention of rickets remains a primary concern, fears have been aroused in recent decades concerning deleterious effects of high intakes (Seelig, 1970; NTIS, 1978; Davies, 1989). The fortification of foods (milk, margarine and infant formula) is therefore monitored in Canada to ensure that neither inadequate nor excessive quantities are added, and analytical methods have been developed for this purpose (Thompson *et al.*, 1977, 1982).

The fortification of animal feeds is potentially another route for entry of abnormal quantities of nutrients into human foods. Kummerow *et al.* (1976) bioassayed edible tissues from farm animals fed generous quantities of vitamin D and cautioned that such practices could be harmful. While the significance to public health of moderately increased intakes is controversial, occasionally more serious errors in fortification of humans foods and animal feeds have been encountered that involve miscalculations leading to the use of 10 or 100 times the intended amount of vitamin D. In the investigation of one such incident in which several hundred pigs were fed an incorrectly formulated feed supplement, a method of analysis was required to quantify the levels of vitamin D in edible tissues.

A rugged method, based on procedures used routinely in Canada for the examination of fortified milk (Thompson *et al.*, 1982), is described for the measurement of vitamin D in meats and fats from farm animals, such as pigs and poultry, that are sometimes overdosed with vitamin D.

MATERIALS AND METHODS

Apparatus

Boiling flasks (500 ml), heated on mantles and equipped with water condensers, were used for alkaline digestion. Digests were extracted in 250 ml separatory funnels with Teflon stopcocks, the holes in which were redrilled to an internal diameter of 2·5 mm to reduce the risk of blockage. Nitrile Latex gloves and protective glasses were worn during the extraction procedure because of the use of alkali. Glass chromatography columns (1 cm i.d.) with Teflon stopcocks were used for sample clean-up with alumina.

Two HPLC systems used routinely included automated and manual injectors, pumps, 15 cm × 4·5 mm column of silica (Apex 3 μm silica, Jones Chromatography, Columbus, OH), radial compression module with a Resolve 8C18 5 μm reversed-phase cartridge, absorbance detectors with filters at 254 nm and a computer (Waters Chromatography Division, Millipore Corp., Milford, MA, Models 712, 510, 440 and 840). A Waters Photodiode Array Detector (Model 990) and Differential Refractometer (Model 410) were used in development of the procedure.

Reagents

Ethanolic pyrogallol consisted of 1% pyrogallol in 95% alcohol. Extraction solvent was a mixture of HPLC

grade hexane and dichloromethane (85 + 15) prepared fresh daily. The clean-up column was prepared with alumina (neutral, Brockman Activity I, 80–200 mesh, Fisher Scientific Co., Fair Lawn, NJ). HPLC grade solvents (hexane, heptane, cyclohexane, 2-propanol and methanol), with low absorbance at 250 nm, were used in the clean-up and HPLC procedures.

Standards

Cholecalciferol, ergocalciferol (Sigma Chemical Co, St Louis, MO) and 25-hydroxycholecalciferol (gift from UpJohn Company, Kalamazoo, MI) were dissolved in methanol or hexane and the concentrations were calculated from the absorbance at 265 nm (Thompson *et al.*, 1982).

Digestion in alkali

A weighed amount of sample, up to 15 g total but containing 1 g or less of fat, was placed in a digestion flask with enough water to bring the total weight to 25 g. Ethanolic pyrogallol (63 ml) and potassium hydroxide pellets (10 g) were added with a piece of broken pot to prevent bumping. The contents were refluxed for 30 min, with swirling at intervals to ensure mixing.

Extraction

After cooling, the contents of the digestion flask were transferred to a 250 ml separatory funnel, rinsing with water (62 ml) and ethanol (5 ml). The digest was extracted with extraction solvent (3 × 75 ml). The pooled extracts were washed gently twice with water (50 ml) and then vigorously with 80% ethanol (50 ml). The solvent was evaporated with a flash evaporator and the residue was dissolved in hexane (2 ml).

Preliminary purification on columns of alumina

Columns of alumina were used only once and were prepared as follows. The activity of a portion of alumina was weakened by gently grinding fresh powder (4 g) in a mortar with water (0·12 ml, i.e. 3%) under a layer of hexane. The alumina was transferred as a slurry to a glass column, plugged just above the stopcock with glass wool, to form a column of adsorbent 5 cm high. The column was washed with hexane (10 ml) containing 1% 2-propanol.

The sample, dissolved in hexane (2–5 ml), was added with a Pasteur pipette. Cholecalciferol and ergocalciferol were eluted with 30 ml of 1% 2-propanol in hexane; when required, 25-hydroxycholecalciferols were eluted next with 5% 2-propanol in hexane (30 ml).

Since the performance of the alumina column is affected by moisture, the alumina must be tested before use. If the calibrated adsorbent is subsequently stored in a tightly sealed container, the clean-up of vitamins is reproducible for several weeks. In a test, a solution containing 25 μg of cholecalciferol (and, if of interest, 25-hydroxycholecalciferol) was added to a column, and the eluate was collected in 10 ml portions, each of which was evaporated and examined using reversed-phase HPLC as described below. Typically, 99% of the cholecalciferol was recovered in the three fractions eluted with 1% 2-propanol in hexane, 70–80% being in the second fraction. Similarly, the 25-hydroxy derivative was eluted quantitatively with 5% 2-propanol. If the vitamins were discharged later or sooner than prescribed, the amount of water added to the alumina (ranging from 2 to 5%) was adjusted, or the adsorbent was reactivated in an oven following the procedure recommended by the manufacturer.

Purification of cholecalciferol by HPLC on silica

The solvent system (solvent A) for the silica column was a mixture of equal volumes of hexane and cyclohexane containing 0·25% 2-propanol and the flow rate was 2 ml min^{-1}. Standards of cholecalciferol were eluted after 8–10 min; the times of the beginning and end of elution were determined carefully before samples were examined.

Eluate from the alumina column containing vitamin D was evaporated in a centrifuge tube under nitrogen, the residue was dissolved in 200 μl solvent A (or, if greater, 1 ml per 40 mg cholesterol) and 100 μl was injected. The eluate was collected for about one minute in a centrifuge tube, starting 15 s before and ending 15 s after the recorded period of elution of standards. After evaporation of the eluate under nitrogen, the residue was redissolved in 100 μl 2-propanol/water (90 : 10, v/v), rotating the tube for at least a minute to ensure removal of all lipid from the wall. The silica column was flushed with solvent for *c.* 5 min to remove remaining lipids before injection of the next sample. The termination of the run was usually indicated by the elution of a large peak of all-*trans* retinol, which chromatographically was one of the most polar lipids to pass the clean-up on alumina.

Final reverse-phase HPLC of cholecalciferol and ergocalciferol

The column was eluted with dry methanol (1·5 ml min^{-1}); in this system, standards of cholecalciferol and ergocalciferol were separated almost to baseline (Thompson *et al.*, 1982). The solution of the sample (60 μl) was injected and the cholecalciferol content was estimated from the area under the peak (eluted after 10 min) by comparison with the response obtained with standards.

Purification of 25-hydroxycholecalciferol by reversed-phase HPLC

The second fraction from the alumina column was evaporated under nitrogen and the residue dissolved in 100 μl 2-propanol/water (90:10 v/v). A portion (100 μl) was injected to the reversed-phase column eluted with methanol. The eluate was collected during the period of elution of the 25-hydroxy derivative established previously with standards (3·5 min).

Final HPLC of 25-hydroxycholecalciferol on silica

The same silica column was used as in the analysis for cholecalciferol but the solvent system was changed to 4% isopropanol in heptane (solvent B). The eluate from the reversed-phase column was evaporated and redissolved in 100 μl solvent B. The 25-hydroxycholecalciferol was eluted after 7 min and was quantified by comparison of the response with that of standards.

RESULTS AND DISCUSSION

As discussed previously (Thompson *et al.*, 1982), glycerides can be conveniently removed during analysis of foods for vitamin D by saponification, but sterols sometimes remain in quantities that are difficult to apply to analytical HPLC columns. Although removal of polar interfering lipids can be achieved by straightforward clean-up or washing of extracts, preliminary removal of sterols requires more refined time-consuming chromatography, such as with HAPS (Thompson *et al.*, 1977) or reversed-phase columns, and this step was not included in the present method. A short column of alumina was used to remove polar lipids and thus protect the silica column and eliminate the need for protracted washing between runs. The alumina column did not separate cholesterol from vitamin D, but it could be used to separate 25-hydroxycholecalciferol from monohydroxy-sterols.

The selection of extraction solvent in the procedure was based on measurements of the relative solubilities of vitamins A, cholecalciferol and soaps in hexane/dichloromethane mixtures and alkaline digests. Radioactively labelled fats were used in these tests and vitamins were measured by HPLC. Addition of dichloromethane to hexane increased the efficiency of extraction of monohydroxy-lipids, such as cholecalciferol and retinol, from digests, but mixtures containing more than 15% dichloromethane extracted soaps and produced troublesome emulsions when subsequently washed with water (Thompson, 1986).

Although rapid HPLC methods for measuring vitamin D have been proposed for fortified foods and feeds, in the authors' experience, chromatography in two systems is necessary in the examination of foods containing small amounts of vitamin D and significant amounts of fat. A true 'two-dimensional' combination, involving straight-phase and reversed-phase columns (Thompson *et al.*, 1982; AOAC, 1984; Johnsson *et al.*, 1989), can be expected to provide better resolution of the vitamin from impurities than the use of two similar columns (Sertl & Molitor, 1985).

The separation obtainable during HPLC was investigated by collecting all of the eluate from the silica column in 11 fractions collected at one-minute intervals and rechromatographing each fraction on the reversed-phase column (Fig. 1). The isolation of cholecalciferol in the ninth fraction (Fig. 1, fraction j) from tocopherols (Fig. 1, fractions c, d), vitamin A (fractions k, l) and numerous other unidentified substances

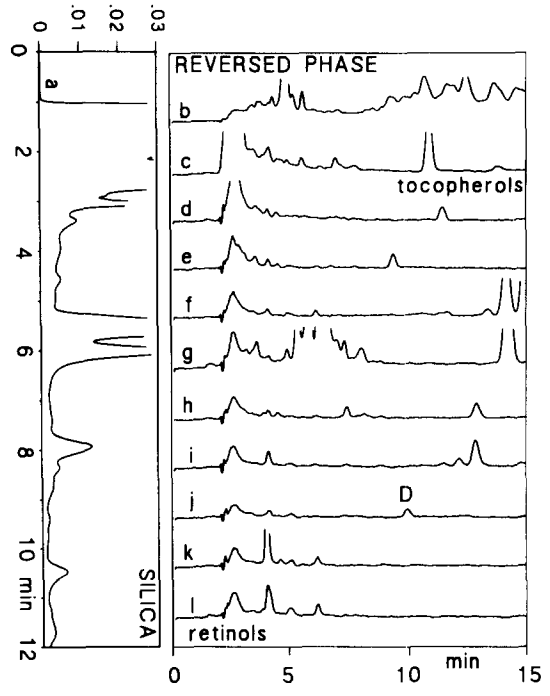

Fig. 1. Isolation of cholecalciferol (2·2 μg per 100 g) from normal pig fat by 2D HPLC. The HPLC records are positioned vertically and horizontally to display the 2D separation. The eluate from first HPLC on silica (a) was collected in 11 fractions between 1 and 12 min. Each fraction was evaporated and run on a reversed-phase column (b–l). Cholecalciferol eluted from silica after 9·5 min and was detected after 10 min on the reversed-phase column (j, marked D). Most of the absorbing material eluted in fraction b, tocopherols eluted in fractions c and d and 13-*cis*-retinol eluted in fractions k and l. Full scale for b was 0·008 absorbance units; for c–l, 0·002 absorbance units.

was confirmed. Although the detector recorded only substances absorbing light at 254 nm, high levels of the vitamin encountered in contaminated fats were isolated in a relatively pure state. In these instances, the identity of the isolated cholecalciferol could be confirmed from absorption spectra, using a photodiode array detector during HPLC (Fig. 2), and from the mass spectra of pooled preparations, which included the expected signals at m/z 384, 364–6, 351 and 271.

HPLC measurements obtained during routine applications are shown in Fig. 3. When levels of vitamin D were excessive (50–200 μg per 100 g) the identification and quantification of vitamin D were unambiguous (Figs 2 and 3(B)). In normal meats, which contained less than 2 μg per 100 g, some doubts usually remained (Figs 3(C) and 3(D)); however, on the basis of published HPLC profiles, other methods applicable to trace amounts of vitamins are suspected to involve at least as great an uncertainty (Jackson *et al.*, 1982; Takeuchi *et al.*, 1984; Kobayashi *et al.*, 1986).

The selection of the silica HPLC column is not considered to be critical, but the Apex column, first recommended by Sertl and Molitor (1985), has proved to be particularly robust and effective. The Waters Radial-Pak reversed-phase columns will separate cholecalciferol and ergocalciferol with methanol as eluate. In the authors' experience, both recommended

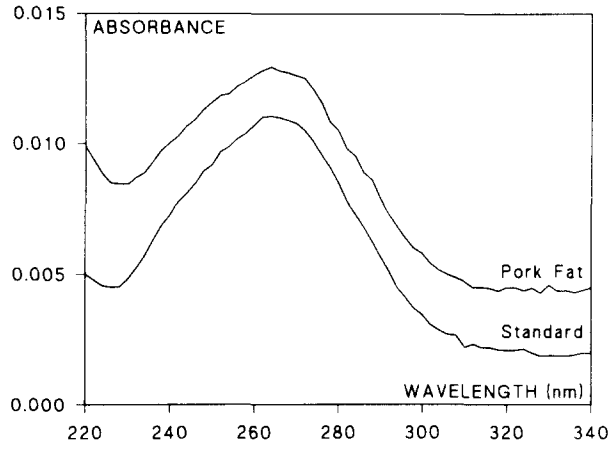

Fig. 2. Absorption spectra of eluate during reversed-phase chromatography of extract of pork fat (34 μg cholecalciferol per 100 g) and of a cholecalciferol standard. Measured with a photodiode array detector.

columns have been obtainable for several years and performance has been consistent. The method finally adopted for the examination of meat and fat is thus similar in many respects to that proposed earlier for margarines (Thompson *et al.*, 1982). Although most methods in the literature are claimed to involve improvements, review of the proposed designs provided little justification for a major change in approach. Jackson *et al.* (1982) precipitated sterols in methanol and used thin-layer chromatography for clean-up before HPLC. In the authors' opinion, these steps compromise quantification because precipitation can involve erratic losses and it is difficult to eluate unstable lipids quantitatively from plates. Kobayashi *et al.* (1986) compared several published methods for foods and described a procedure (Takeuchi *et al.*, 1984) in which, in contrast to the present method, the reversed-phase HPLC is employed first followed by HPLC on silica. In general, the separation achieved in two-dimensional chromatography should not be affected by the order in which the columns are employed. In practice, however, chromatography must be extended for over an hour to elute unidentified substances when a reversed-phase column is used first in the analysis of some foods for vitamin D; these substances presumably elute rapidly from a silica column (Fig. 1(B)). In fact, a silica Sep-Pak clean-up column was used by Takeuchi *et al.* (1984) and Kobayashi *et al.* (1986) to avoid this problem and thus their method actually involved an initial silica chromatography.

When samples contain little vitamin D, the solubility of other sterols (usually predominantly cholesterol) in the solvent system imposes an upper limit on the amount of extract that can be applied to an HPLC column. A five-fold increase in solubility of cholesterol is achieved in the recommended procedure by the addition of cyclohexane to hexane/2-propanol. The elution of sterols was monitored with a refractive index detector; on silica, cholesterol eluted (7·5 min) conveniently before vitamin D (9 min) whereas it was retained longer (12 min) than the vitamin (9 min) on a reversed-phase column.

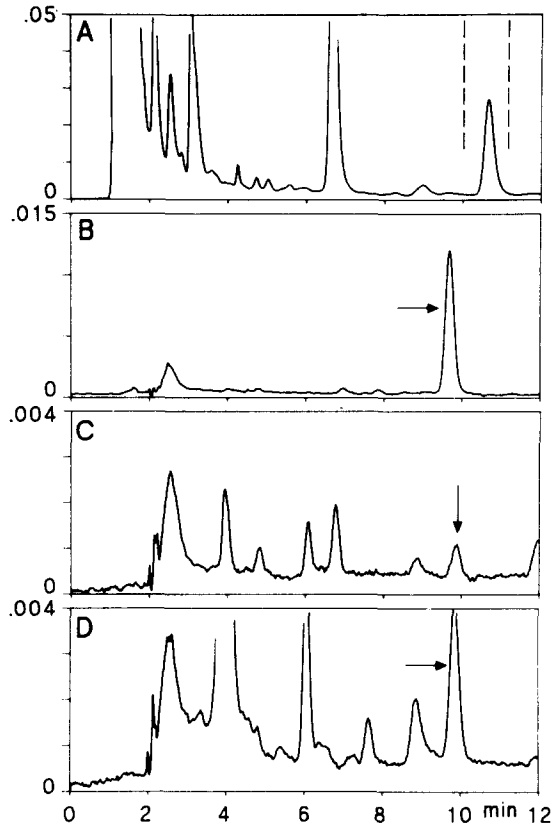

Fig. 3. Typical HPLC profiles obtained in routine analysis. Arrows point at cholecalciferol peaks. A, Extract of fat from overdosed pig, silica column, collected fraction indicated by – – –; B, cholecalciferol (34 μg per 100 g fat) in fat from overdosed pig, reversed-phase column; C, cholecalciferol (1·8 μg per 100 g fat) in normal pork fat, reversed-phase column; D, cholecalciferol (4·9 μg per 100 g tissue) in liver from overdosed pig, reversed-phase column.

Precision in the complete method and the component parts was assessed during development by tests with standards and samples (Table 1). A major source of variation was the dissolution of sample for the final reversed-phase step. Originally, methanol was used as solvent and measurements of recoveries sometimes yielded erratic and high results. The problem was attributed to evaporation of methanol. A less volatile mixture of 2-propanol and water, which has similar chromatographic properties, was used in its place; pure isopropanol was unsuitable because it caused spreading of the vitamin D peak. Overall, precision can be conservatively estimated to be better than 10% (Table 1).

Cholecalciferol was recovered quantitatively during saponification, extraction and chromatography. Standards were stored at room temperature and thus contained 9% previtamin D (Keverling Buisman *et al.*, 1968). Brief digestion in refluxing aqueous ethanol caused formation of more isomer but the process appeared to reverse during extraction and clean-up which extend in routine work over two days. The measured recoveries, using commercial lard (which contained no detectable vitamin D) spiked with 44–155 μg cholecalciferol per 100 g, thus ranged from 95–114% with a mean value of 100%; in the practical

Table 1. Precision of cholecalciferol measurements in various steps of the procedure

Test sample	Procedure	Number	RSD (%)	Recovery (%)
Standard	rp	12	0·6	nm
Standard	ev/rp	12	3·5	100
Standard	si	11	1·1	nm
Standard	al/si	6	2·1	104
Standard	dg/ex/si	5	6·9	93
Lard + standard	dg/ex/al/si	4	2·1	92
Lard + standard	dg/ex/al/si/ev/rp	4	3·6	101
Pork fat + standard	dg/ex/al/si/ev/rp	5	8·0	nm

Procedure codes: al, alumina; dg, digestion; ev, evaporated/redissolved; ex, extraction; rp, HPLC reversed-phase; si, HPLC silica.
nm, recovery not measured.

Table 2. Levels of cholecalciferol in meat and fat from pigs given feed high in vitamin D

Animal	Fat (%)	Meat cholecalciferol (μg per 100 g)	Meat cholecalciferol (μg per 100 g fat)	Fat cholecalciferol (μg per 100 g)
A1	14·1	10·3	73·0	53·4
A2	15·3	17·2	112·4	148·4
A3	19·1	4·6	24·1	25·0
A4	18·1	8·3	45·8	31·4
B1	4·8	2·7	56·2	40·1
B2	3·6	1·8	50·0	24·1
B3	3·2	1·0	31·2	22·6
B4	3·2	2·2	68·8	63·9
B5	4·7	1·8	38·3	21·4

Pigs A1–A4 slaughtered 3 weeks after a 24-day period on high vitamin D; B1–B5 examined later after 2 weeks of restricted feed intake.
25-Hydroxycholecalciferol levels were below 2 μg per 100 g fat.

examination of foods, further consideration of the formation of previtamin was not considered worthwhile.

From the appearance of chromatograms obtained with lard, pork fat (Fig. 3(C)) and liver, the limit of detection was estimated to be close to levels (0·2–1·0 μg per 100 g) suspected to occur in normal beef, pork and veal (Rastas *et al.*, 1989).

In the analysis of most samples, a peak attributable to vitamin D was visible in the first HPLC on silica. It is emphasized, however, that when numerous peaks are detected at only one wavelength (254 nm) in extracts from foods, one cannot assume that a peak eluting at the expected time in a single HPLC run represents entirely or partially the vitamin D content. More certain identification, which is mandatory when evaluating and perhaps condemning meats intended for human consumption, is obtained by chromatography in a second system (Figs 1 and 3). Other tests can be applied to the purified vitamin, such as the formation of previtamin on heating (Keverling Buisman *et al.*, 1968).

A photodiode array detector proved to be valuable in that it could be used to confirm that the peaks eluted both in the silica and reversed-phase chromatography had maximum absorbance between 240 and 280 nm. The detector in this application, however, must be sensitive, measuring absorbances below 0·005 units, and the HPLC column must be thoroughly washed between runs, to avoid confusing undulations in the baseline. Such a detector, employed in the development of the present method to measure absorbance continuously between 220 and 400 nm, revealed that the vitamin D was eluted in the silica HPLC just before 13-*cis*-retinol. The two substances are easily confused when eluates are monitored at only one wavelength. When a photodiode array detector is used in routine analysis, identification of vitamin D is relatively straightforward, even when levels are low.

Vitamin D was not detected in three samples of pork fat from various retail sources, but peaks representing 2·2, 1·8, 0·17, 0·72 and 0·5 μg cholecalciferol per 100 g were observed respectively in two pork fats, one bacon fat and two samples of chicken fat.

In an example of a practical application in the examination of overdosed animals, liver, meat and fat were examined from a consignment of pigs held at an abattoir because they were part of a large group fed in error 2–3 mg cholecalciferol per day for 2–3 weeks during rearing. Vitamin D levels in three livers were higher than normal (0·3, 3·6 and 4·9 μg per 100 g) but, because of the limited consumption and value of liver, most resources were applied to the examination of meat and fat. The levels of cholecalciferol in fat (ranging up to 180 μg per 100 g) were concluded to be the most significant in the protection of consumers because they were slow to fall and appeared to determine the levels in meat. The amounts of vitamin D found in meat were reduced after food restriction, but much of the change could be attributed to a fall in fat content (Table 2), the levels in fat declining slowly during a period of a month to 8–37 μg per 100 g.

Relatively small amounts of 25-hydroxycholecalciferol (0·7–2·0 μg per 100 g) were detected in the fat from only the most contaminated pigs, equivalent by weight to 1–4% of the cholecalciferol and thus contributing 5–20% to the biological activity (Reeve *et al.*, 1982). Although an extension of the method is described for the measurement of 25-hydroxycholecalciferol, the authors conclude that in the assessment of safety, a simple addition of 20% to the measured levels of vitamin D probably allows for the contribution of metabolites, previtamin D and losses during analysis.

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