Short Communications

An Improved System for the Separation of Metabolites of Isotopically Labelled Vitamin D₃ on Silicic Acid Columns

By E. BARBARA MAWER and JOAN BACKHOUSE

*Division of Metabolism, University Department of Medicine, The Royal Infirmary,
Manchester, M13 0JJ*

(Received 23 December 1968)

The recent availability of isotopically labelled vitamin D of reasonably high specific radioactivity has resulted in several studies of vitamin D metabolism both in mammals and the chick (reviewed by DeLuca, 1967).

A more polar metabolite that possessed variable biological activity in the rat line test assay was first described by Norman, Lund & DeLuca (1964). This material, which was designated 'Peak IV', was separated from the parent vitamin by means of gradient elution from silicic acid columns. The biological activity of the peak was variable and it was suspected that the material was not homogeneous. Two reports have recently appeared, describing improved chromatographic systems that separate the major biologically active compound from other components of the originally described 'Peak IV' (Blunt, DeLuca & Schnoes, 1968; Haussler, Myrtle & Norman, 1968).

Work in our laboratory has produced a system that affords further resolution of the more-polar metabolites; up to six peaks of radioactivity in material more polar than vitamin D₃ have been separated in this system.

Exact reproduction of the gradients of diethyl ether in hexane described by Norman *et al.* (1964) has proved difficult, and our modification employs stepwise elution.

After the administration of [1,2-³H₂]- or [4-¹⁴C]-cholecalciferol (vitamin D₃) to whole animals or to tissue preparations, samples of biological fluids or tissue homogenates are extracted by the method of Bligh & Dyer as modified by Avioli, Lee, McDonald, Lund & DeLuca (1967). The chloroform extract thus obtained is partitioned between iso-octane and aqueous methanol (Chen, Terepka, Lane & Marsh, 1965), the phases are separated and backwashed once, and the washings are added to the appropriate phases, which are then concentrated for application to silicic acid columns.

The chloroform extract from the original extraction may be applied directly to a column, but if large amounts of vitamin D are present it is preferable to partition it between iso-octane and aqueous methanol and run each extract separately.

More-polar artifacts can be formed from vitamin D on storage, and if the aqueous methanol phase is removed from the iso-octane phase, which contains the vitamin, interference by artifacts can be minimized.

Most of the extraction operations are done at temperatures below 10° and extracts are stored under nitrogen at -25° and in the dark. Columns are run under nitrogen in a cold-room at 5-8°.

A 24 g. portion of silicic acid (-325 mesh; Sigma Chemical Co., St Louis, Mo., U.S.A.) that has not been heat-activated is slurried with hexane into a glass column (50 cm. x 1.4 cm.). The extract is applied to the column in hexane, and is eluted sequentially with: 250 ml. of diethyl ether-hexane (1:3, v/v); 50 ml. of diethyl ether-hexane (1:1, v/v); 200 ml. of diethyl ether; 100 ml. of methanol-diethyl ether (1:19, v/v); 100 ml. of methanol-diethyl ether (1:1, v/v); 100 ml. of methanol; 100 ml. of water; 10 ml. fractions are collected and monitored for radioactivity by means of liquid-scintillation counting.

The iso-octane extract yields three peaks, all eluted by diethyl ether-hexane (1:3, v/v), of which peak III is cholecalciferol, peak II is eluted in the position of pre-cholecalciferol and peak I in that of cholecalciferol esters. No material more polar than cholecalciferol is present, so in practice these columns can be stopped after the diethyl ether-hexane (1:3, v/v) step.

The aqueous-methanol extract (which in this communication refers to the aqueous-methanol phase obtained by partitioning the original chloroform extract between iso-octane and aqueous methanol, and not to the aqueous-methanol phase left behind after removal of the original chloroform extract according to the procedure of Bligh & Dyer) contains traces only (<1%) of cholecalciferol, but all of the more-polar metabolites. For these columns the extract is applied in diethyl ether-hexane (1:3, v/v), and the 250 ml. of this eluent is collected batchwise, as the radioactivity eluted is very low. Peaks of radioactivity may be eluted by each of the other solvent mixtures. In each case the peak is well separated and reasonably

Table 1. *Silicic acid chromatography of aqueous-methanol extracts of serum after intravenous injection of [4-¹⁴C]cholecalciferol*

Rat serum was obtained 18 hr. after a 5 μ g. dose; human sera for each subject were the pooled specimens obtained 4–14 days after a 130 μ g. dose. Negligible amounts of radioactivity appeared in peaks I–III, since the less-polar compounds had been previously removed into the iso-octane phase. The results are expressed as percentages of total radioactivity in the aqueous-methanol extract recovered in individual fractions IVa–VIII. Fraction IVb from human serum has been shown to possess antirachitic activity in the rat.

Peak designation	IVa	IVb	IVc	V	VI	VII	VIII
Eluent	Ether-hexane (1:1, v/v)	Ether	Ether	Methanol-ether (1:19, v/v)	Methanol-ether (1:1, v/v)	Methanol	Water
Rat control	0	6.7	54.5	20.5	10.9	2.7	4.7
Human control	3.2	77.2	0	4.1	11.2	3.4	0.9
Human renal failure	0.8	42.0	0	26.2	19.7	5.7	5.6

symmetrical except for peaks IVb and IVc, which overlap slightly.

The peaks obtained vary qualitatively and quantitatively with such factors as species, tissue, time and the size of the administered dose of cholecalciferol. Typical patterns are shown in Table 1.

The total radioactivity of the aqueous-methanol fraction increases with time after administration of labelled cholecalciferol *in vivo* and gives a measure of the rate of metabolism of the vitamin (Mawer & Stanbury, 1968).

The present communication describes a readily reproducible method for the separation of the aqueous-methanol fraction into its components. This is a useful tool for the routine examination of differences in the metabolism of vitamin D₃ in

various tissues and in pathological states (see Table 1).

This work is supported by a Grant from the Medical Research Council.

Avioli, L. V., Lee, S. W., McDonald, J. E., Lund, J. & DeLuca, H. F. (1967). *J. clin. Invest.* **46**, 983.

Blunt, J. W., DeLuca, H. F. & Schnoes, H. K. (1968). *Biochemistry*, **7**, 3317.

Chen, P. S., jun., Terepka, A. R., Lane, K. & Marsh, A. (1965). *Analyt. Biochem.* **10**, 421.

DeLuca, H. F. (1967). *Vitam. & Horm.* **25**, 315.

Haussler, M. R., Myrtle, J. F. & Norman, A. W. (1968). *J. biol. Chem.* **243**, 4055.

Mawer, E. B. & Stanbury, S. W. (1968). *Biochem. J.* **110**, 53P.

Norman, A. W., Lund, J. & DeLuca, H. F. (1964). *Arch. Biochem. Biophys.* **108**, 12.