COMPETITIVE PROTEIN-BINDING ASSAY
FOR 25-HYDROXYCHOLECALCIFEROL

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SUMMARY

1. A competitive protein-binding assay for plasma 25-hydroxycholecalciferol was
developed, using Sephadex LH-20 columns for the isolation of the sterol and a
partially purified protein from rat serum as the binding protein.
2. The mean plasma 25-hydroxycholecalciferol in eighteen male and female normal
volunteers was 38 ± SD 14 pmol/ml (15.2 ± 5.6 ng/ml).
3. Levels of 25-hydroxycholecalciferol in plasma of four male volunteers main-
tained for 2 months on a diet supplemented with 26.1 nmol (10 µg) of cholecalciferol
a day were found to be significantly higher.
4. High correlation was established between plasma 25-hydroxycholecalciferol
values obtained by the competitive protein-binding assay and values obtained by
bioassay.

Key words: 25-hydroxycholecalciferol, competitive protein-binding assay.

The assay of cholecalciferol levels in animal tissues by physicochemical methods has not
hitherto been possible, due in part to the problems of purification and in part to the need for a
method with high sensitivity (for review see Kodicek & Lawson, 1967; Sheppard, Proser &
Hubbard, 1972). The antimony trichloride colorimetric reaction, which is widely used for the
estimation of cholecalciferol, particularly in high-potency preparations such as fish oils,
lacks sensitivity and specificity.

For a time, gas-liquid chromatography was thought to be sufficiently sensitive and specific.
The difficulties presented by the isomerization of cholecalciferol at the high temperatures
of the gas-chromatograph to give pyro- and isopyro-calciferol were overcome by converting
cholecalciferol into isotachysterol, which is unchanged in the gas-chromatograph (Murray,
Day & Kodicek, 1966). The recovery of the vitamin during the purification is estimated by

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adding ergocalciferol or cholecalciferol to the sample as an internal standard. The sensitivity of this method was improved by the preparation of the halo-esters of isotachysterol and the incorporation of a $^{63}$Ni electron-capture detector into the gas-chromatograph. By this means 10 ng of cholecalciferol could be estimated. However, difficulties were encountered in eliminating substances which co-chromatographed with the isotachysterol heptafluorobutyrates (Wilson, Lawson & Kodicek, 1969).

It is now known that cholecalciferol, to be effective in the target tissues, is hydroxylated first at C-25 to give 25-hydroxycholecalciferol (25-HCC) and subsequently at C-1 to give 1,25-dihydroxycholecalciferol (1,25-DHCC). In most species, including man (Mawer, Lumb, Schaefer & Stanbury, 1971), the principal circulating metabolite in plasma soon after a dose of cholecalciferol is 25-HCC, with cholecalciferol accounting for only a small proportion and 1,25-DHCC contributing less than 5% to the total anti-rachitic activity (Lawson, Wilson & Kodicek, 1969; Lawson, Pelc, Bell, Wilson & Kodicek, 1971).

In the light of these findings there have been renewed attempts to characterize the binding proteins for cholecalciferol and 25-HCC in blood and target organs (Edelstein, Lawson & Kodicek, 1973; Edelstein, 1973). A consequence of these studies has been the development of a competitive protein-binding assay for cholecalciferol and its 25-hydroxy metabolite with sufficient sensitivity to enable the 25-HCC levels of human plasma to be determined.

The first published competitive protein-binding assay for cholecalciferol and its metabolites in plasma utilized a specific binding protein from rat serum (Belsey, DeLuca & Potts, 1971). The problem of the limited solubility of these steroids in water was overcome by inclusion in the assay system of human plasma $\beta$-lipoproteins as a carrier; however, several days were needed for equilibrium to be reached in this system. Soon after, Haddad & Chyu (1971) introduced another competitive protein binding assay, for 25-HCC only, utilizing a specific binding protein from kidney. The steroid was solubilized by including 7% ethanol in the assay system, so that the time needed to reach equilibrium was shortened to 60 min. The free steroid was separated from the bound protein by use of charcoal coated with dextran. In principle this last method provides a simple and sensitive method for the routine estimation of 25-HCC levels in peripheral blood, and has been applied to the estimation of 25-HCC in plasma at levels as low as 4 ng/ml.

Chromatography, on silicic acid columns, was used in both these competitive protein-binding assays to effect a partial purification of the 25-HCC from the other plasma lipids. This technique, we find, interferes with the competitive protein-binding assay and causes erratic blank values because of substances produced during chromatography due to impurities in the silicic acid material and the interaction of the solvents with the silicic acid (Murphy, 1971).

The present paper describes a competitive protein-binding assay for 25-HCC, using Sephadex LH-20 columns for the isolation of the 25-hydroxy metabolite and a partially purified protein from rat serum as the binding protein. The method is more sensitive, rapid and reliable than those presently available.

**MATERIALS AND METHODS**

*Sample extraction*

Heparinized blood was collected, and plasma prepared and kept frozen until use. The lipids from 1·0 ml were extracted by shaking the sample with 10·0 ml of chloroform–methanol (2:1,
Assay for 25-hydroxycholecalciferol

v/v). Two millilitres of water were added and the tubes cooled down in an ice bath. Phase separation was achieved by rewarvming the tubes to room temperature or by centrifugation. The bottom chloroform layer was collected with a transfer pipette and the residue was re-extracted with 10.0 ml of chloroform. The bottom layer was collected as before and combined with the first chloroform extract. The combined chloroform extract was dried under a stream of nitrogen and the residue was redissolved in 0.2 ml of 50% (v/v) chloroform in light petroleum (b.p. 40–60°C). Using an internal standard of 25-[26,27-3H]hydroxycholecalciferol (11 Ci/mmole, The Radiochemical Centre, Amersham, Bucks., U.K.), this extraction procedure was found to recover invariably 90% of the 25-HCC present in the blood sample.

Chromatography

The dissolved lipid residue was applied to a column (24.0 cm x 0.5 cm) previously packed with Sephadex LH-20 (Pharmacia Fine Chemicals Ltd, London W5 1BR, U.K.) in 50% (v/v) chloroform in light petroleum (b.p. 40–60°C) and fitted with a 6.0 ml reservoir. The tube containing the lipid residue was then washed twice with 0.2 ml of the same solvent and the washings were transferred to the column, which was developed with the same solvent. The first 6.0 ml eluted was discarded and the following 8.0 ml, containing the 25-HCC, was collected and dried under a stream of nitrogen. A further 8.0 ml was collected and treated in a similar manner. This fraction was used as a column blank in the competitive protein-binding assay. The recovery from the column of the 25-HCC present in the lipid extract in four estimations was 85%, with a range of 84–86%.

The dried fractions were dissolved in 1.0 ml of ethanol and aliquots taken for the competitive protein-binding assay. At least five or six columns could be handled conveniently at the same time. The columns were fitted with solvent-resistant stoppers and were re-used at least three times without any decrease in recovery. The columns were washed thoroughly with eluting solvent before re-use.

Preparation of binding protein

Piebald weanling rats were raised on a vitamin D-deficient diet as described by Numerof, Sassaman, Rodgers & Schaefer (1955). After 2–3 weeks, blood was collected by intracardial puncture, and serum prepared. Serum (4 ml) was subjected to gel filtration on Sephadex G-200 followed by ion-exchange chromatography on DEAE-Sephadex as described before (Edelstein et al., 1973). The protein peak eluted from the DEAE-Sephadex column, which contained the binding protein for 25-HCC, was dialysed extensively at 4°C against 0.02 M-sodium phosphate buffer, pH 7.6, and freeze-dried. The binding protein preparation was obtained by dissolving the freeze-dried material in the phosphate buffer and diluting to a concentration at which approximately 50% binding was obtained with 25-[26,27-3H]HCC.

Biological assay of serum vitamin D activity

A one-point assay was carried out according to the radiographic method of Bourdillon, Bruce, Fischmann & Webster (1931). The serum lipid extracts were dissolved in a small volume of arachis oil and administered to the rachitic rats by stomach tube.

Assay procedure

The ethanol fractions (0.2 ml) obtained from the Sephadex LH-20 chromatography were
Fig. 1. A typical calibration curve for 25-hydroxycholecalciferol assay.

Plasma sample
↓
Extraction with chloroform and methanol
↓
Chromatography on Sephadex LH-20
↓
25-HCC fraction
↓
Equilibration of $^3$H-labelled 25-HCC and the 25-HCC fraction with the assay protein
↓
Adsorption of ‘free’ sterol by charcoal coated with dextran
↓
Counting of ‘bound’ sterol

Fig. 2. Outline of the competitive protein-binding assay for the determination of 25-hydroxycholecalciferol in human plasma.
pipetted into tubes in duplicate and dried under a stream of nitrogen. Ethanol (80 μl) containing 4000 d.p.m. of 25-[26,27-3H]HCC was added followed by sodium phosphate buffer, pH 7·6 (0·02 mol/l; 1·0 ml) and the binding-protein preparation (50 μl). A standard curve was prepared in the same way with increasing amounts of unlabelled 25-HCC replacing the plasma lipid fraction. The tubes were stoppered, mixed well and allowed to stand at room temperature for 30 min. The tubes were then placed in an ice bath for 10 min and 1·0 ml of a suspension of charcoal (Norit GSX, Hopkin & Williams Ltd, Chadwell Heath, Essex, U.K.), coated with dextran (mol. wt. 60 000–90 000, Koch–Light Laboratories Ltd, Colnbrook, Bucks., U.K.) (Murphy, 1967), was added to each tube. The contents of the tubes were mixed and then replaced in the ice bath for 5 min and centrifuged for 5 min at 1500 rev./min at 4°C. Aliquots (1·0 ml) of the supernatant were transferred to counting vials containing 10·0 ml of a solution of Triton X-100 in toluene (1:2, v/v) containing 0·03% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0·5% 2,5-diphenyloxazole, and counted in a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3375.

The calibration curve was constructed by plotting the percentage of radioactivity bound against the amount of non-radioactive steroid. Unknown amounts of 25-HCC were determined by reference to the calibration curve obtained simultaneously with the serum extracts and appropriate corrections were made for the aliquots of serum and serum extracts used. Fig. 1 represents a typical calibration curve for 25-HCC assay. An outline of this assay is illustrated in Fig. 2.

**Precision**

In order to study the precision of the assay, five separate estimations were carried out on the same sample of plasma. The results of these experiments are shown in Table 1. The mean value was found to be 23±SD 3·6 pmol/ml (9·2±1·44 ng/ml).

A single estimation in duplicate, of the same sample 1 week later, gave a mean value of 22 pmol/ml (8·8 ng/ml).

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>25-HCC pmol/ml</th>
<th>25-HCC ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22·7</td>
<td>9·1</td>
</tr>
<tr>
<td>2</td>
<td>23·5</td>
<td>9·4</td>
</tr>
<tr>
<td>3</td>
<td>24·2</td>
<td>9·7</td>
</tr>
<tr>
<td>4</td>
<td>22·0</td>
<td>8·8</td>
</tr>
<tr>
<td>5</td>
<td>22·0</td>
<td>8·8</td>
</tr>
<tr>
<td>Mean</td>
<td>23·0</td>
<td>9·2</td>
</tr>
<tr>
<td>SD</td>
<td>3·6</td>
<td>0·39</td>
</tr>
</tbody>
</table>

TABLE 1. Precision of the competitive protein-binding assay for plasma 25-HCC. Five separate estimations of the 25-HCC on a single sample of human plasma were made in duplicate.
Sensitivity

The smallest amount of unlabelled steroid that can be detected by the present method is less than 1.25 pmol (0.5 ng) per assay tube. However, for routine estimations we aim for levels of 2.5-6.25 pmol per assay tube. In cases where smaller values are obtained, the measurement is repeated and the amount of lipid taken for chromatography is doubled or tripled. It was established that large amounts of plasma lipids obtained from as much as 3 ml of plasma do not interfere with the assay. 25-HCC was undetectable in plasma lipids obtained from rachitic animals.

Specificity

The specificity of the method is aided by the efficient separation of 25-HCC from competing vitamin D metabolites. By using $^3$H- and $^{14}$C-labelled metabolites, it was established that the present chromatographic system separated the 25-HCC fraction from any other vitamin D metabolite (Fig. 3).

![Fig. 3. Chromatographic separation of cholecalciferol (D$_3$), 25-hydroxycholecalciferol (25-HCC) and 1,25-dihydroxycholecalciferol (1,25-DHCC) on a Sephadex LH-20 column. For experimental details see the text.](image_url)

Recovery of added material

This was assessed by experiments in which 6.25 pmol of 25-HCC was added to 1 ml plasma samples containing 23 pmol (9.2 ng) of 25-HCC. The results of five such experiments are shown in Table 2.

RESULTS AND DISCUSSION

The method was used to measure plasma 25-HCC in eighteen normal male and female adult volunteers, and four normal male volunteers taking a daily cholecalciferol supplement of 26.1 nmol (10 µg) for 2 months (Table 3). These investigations were carried out with the
informed consent of the volunteers concerned and with the approval of the Medical Research Council.

The mean value for the normal volunteers was $38 \pm 14$ pmol/ml ($15.2 \pm 5.6$ ng/ml); the values were, however, skewly distributed, 60% of subjects having values lower than the mean. This mean is in agreement with that found by Stamp, Round, Rowe & Haddad (1972), also

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>pmol/ml</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.0</td>
<td>10.4</td>
</tr>
<tr>
<td>2</td>
<td>30.7</td>
<td>12.3</td>
</tr>
<tr>
<td>3</td>
<td>27.7</td>
<td>11.1</td>
</tr>
<tr>
<td>4</td>
<td>27.2</td>
<td>10.9</td>
</tr>
<tr>
<td>5</td>
<td>28.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Mean</td>
<td>28.0</td>
<td>11.2</td>
</tr>
<tr>
<td>SD</td>
<td>1.75</td>
<td>0.7</td>
</tr>
</tbody>
</table>

TABLE 2. Recovery of added 25-HCC from plasma. 6.25 pmol of 25-HCC was added to 1 ml of plasma which was used for the studies reported in Table 1. The level of this steroid now in the sample was determined in duplicate as outlined in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Daily cholecalciferol supplement (nmol)</th>
<th>25-HCC (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>38 ± 14</td>
<td>15.2 ± 5.6</td>
</tr>
<tr>
<td>Vitamin D-supplemented</td>
<td>4</td>
<td>89.7 ± 37.5</td>
<td>35.9 ± 15.0</td>
</tr>
</tbody>
</table>

$P$ value $< 0.001$

(1) Values represent mean ± SD.

in subjects in the United Kingdom, but lower than the mean level of $68.3 \pm 29.5$ pmol/ml ($27.3 \pm 11.8$ ng/ml) found in normal subjects in U.S.A. (Haddad & Chyu, 1971). This is due most probably to differences in dietary vitamin D intake and exposure to sunshine.

A significantly higher value ($89.7 \pm 37.5$ pmol/ml) was obtained in those subjects who had received a daily cholecalciferol supplement (Table 3). This observation suggests that plasma 25-HCC can be used for the assessment of vitamin D nutritional status.
TABLE 4. Comparison of plasma 25-HCC values obtained by competitive protein-binding assay with values obtained by bioassay. Plasma from nine individuals was analysed for 25-HCC by competitive protein-binding assay, and for total vitamin D activity by bioassay (reference: cholecalciferol). (1 ng/ml = 2.5 pmol/ml.)

<table>
<thead>
<tr>
<th>Competitive protein-binding assay (pmol/ml)</th>
<th>Bioassay (pmol/ml)</th>
<th>Cholecalciferol equivalents(^{(1)}) (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.5</td>
<td>28.6</td>
<td>43.0</td>
</tr>
<tr>
<td>22.5</td>
<td>31.3</td>
<td>32.8</td>
</tr>
<tr>
<td>35.0</td>
<td>23.4</td>
<td>51.0</td>
</tr>
<tr>
<td>28.2</td>
<td>23.4</td>
<td>41.1</td>
</tr>
<tr>
<td>36.8</td>
<td>20.8</td>
<td>53.6</td>
</tr>
<tr>
<td>30.0</td>
<td>32.0</td>
<td>43.8</td>
</tr>
<tr>
<td>80.0</td>
<td>109.4</td>
<td>116.7</td>
</tr>
<tr>
<td>71.7</td>
<td>48.2</td>
<td>104.7</td>
</tr>
<tr>
<td>11.5</td>
<td>8.6</td>
<td>16.7</td>
</tr>
</tbody>
</table>

\(^{(1)}\) See the text.

FIG. 4. Histogram of plasma 25-hydroxycholecalciferol values in normal adult volunteers.
Assay for 25-hydroxycholecalciferol

When values obtained by both competitive protein-binding assay and biological assay in the same sample were compared, a correlation coefficient of 0·85 was obtained (Table 4). When the values obtained by the binding assay were expressed as 'cholecalciferol-equivalents', i.e. ng of 25-HCC \times 1·4 (Blunt, Tanaka & DeLuca, 1968; The Royal Society, 1972), the levels were equal or higher than the bioassay values. This may indicate that the principal circulating metabolite of cholecalciferol in blood is in fact the 25-hydroxylated metabolite.

In summary, the method provides a simple, reliable and sensitive means for the routine estimation of 25-HCC in peripheral blood samples. Diluted rat serum can be employed instead of the partially purified protein from rat serum, but the sensitivity of the assay is then reduced. By using columns of Sephadex LH-20 instead of silicic acid, the blank values were low, erratic displacements were not observed and larger quantities of lipids could be chromatographed. The same solvent was used throughout the chromatography and the columns were used several times.

Attempts to develop a similar competitive protein-binding assay for cholecalciferol have so far been unsuccessful. Although specific binding proteins for cholecalciferol exist (Edelstein et al., 1972, 1973), the limited solubility of the steroid in water prevents competitive displacement from taking place. The estimation of 1,25-dihydroxycholecalciferol awaits further information on the properties of the specific binding proteins for this hormone. However, when this is achieved, the present chromatographic system can be used for the separation of these steroids as well.

ACKNOWLEDGMENTS

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REFERENCES


