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Protein Binding Assays for 25-Hydroxy, 24,25-Dihydroxy and 1,25-Dihydroxy Metabolites of Vitamin D¹⁾ in Human Plasma

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Summary: In this study we describe protein binding assays for 25-hydroxy, 24,25-dihydroxy and 1,25-dihydroxy metabolites of vitamin D in 6 ml of plasma. The procedure involves methanol/methylene chloride extraction of plasma followed by separation and purification of mono- and dihydroxylated vitamin D metabolites on two LH 20 columns with different elution solvents. The separation of dihydroxylated vitamin D metabolites is carried out by high performance liquid chromatography (HPLC). 25-Hydroxy and 24,25-dihydroxy vitamin D are measured by competitive protein binding assays with diluted homogenate from vitamin D-deficient rat kidneys. 1,25-Dihydroxy vitamin D is measured by competitive protein binding assay with diluted cytosol from vitamin D-deficient chick intestine.

The concentration of 25-hydroxy vitamin D in human plasma, as determined by this assay, was $12.2 \pm 5.9 \mu\text{g/l}$; of 24,25-dihydroxy vitamin D $1.5 \pm 1.0 \mu\text{g/l}$, and of 1,25-dihydroxy vitamin D $25.8 \pm 11.7 \text{ ng/l}$ (means $\pm$ S.D., $n = 17$, October, Düsseldorf). In April, the concentration of the vitamin D metabolites of the same subjects were: 25-hydroxy vitamin D $3.3 \pm 0.9 \mu\text{g/l}$; 24,25-dihydroxy vitamin D $0.17 \pm 0.04 \mu\text{g/l}$ and 1,25-dihydroxy vitamin D $38.3 \pm 10.2 \text{ ng/l}$.

Mean values in nonselected human plasma samples of hospitalized patients ($n = 84$) taken during one year were: 25-hydroxy vitamin D $5.7 \pm 2.9 \mu\text{g/l}$; 24,25-dihydroxy vitamin D $0.64 \pm 0.44 \mu\text{g/l}$; 1,25-dihydroxy vitamin D $53.3 \pm 27.6 \text{ ng/l}$.

Mean values in pancreatectomized subjects were: 25-hydroxy vitamin D $4.2 \pm 2.6 \mu\text{g/l}$; 24,25-dihydroxy vitamin D $0.27 \pm 0.15 \mu\text{g/l}$; 1,25-dihydroxy vitamin D $19.6 \pm 11.9 \text{ ng/l}$ ($n = 10$).

Proteinbindungssassays für die 25-Hydroxy-, 24,25-Dihydroxy- und 1,25-Dihydroxy-Metabolite von Vitamin D¹⁾ im Plasma des Menschen

Zusammenfassung: Wir beschreiben in dieser Arbeit Proteinbindungssassays aus einer 6 ml Plasmaprobe für die 25-Hydroxy-, 24,25-Dihydroxy- und 1,25-Dihydroxy-Metabolite von Vitamin D. Die Metabolite werden mit einer Methanol/Methylenchlorid-Mischung aus dem Plasma extrahiert. Anschließend werden die Mono- von den Dihydroxyderivaten auf zwei LH 20-Säulen getrennt und mit einem zweiten Elutionsmittel gereinigt.

Die Trennung der Dihydroxyderivate erfolgt durch Hochdruckflüssigchromatographie (HPLC). 25-Hydroxy- und 24,25-Dihydroxy-Vitamin D werden in einem kompetitiven Proteinbindungssassay mit einem verdünnten Nierenhomogenat von Vitamin D-arm ernährten Ratten gemessen. Die Bestimmung von 1,25-Dihydroxy-Vitamin D erfolgt ebenfalls in einem kompetitiven Proteinbindungssassay mit dem verdünnten Cytosol aus den Mucosazellen von Vitamin D-arm ernährten Küken.

Die Konzentration von 25-Hydroxy-Vitamin D im Plasma des Menschen beträgt nach diesem Assay $12,2 \pm 5,9 \mu\text{g/l}$, von 24,25-Dihydroxy-Vitamin D $1,5 \pm 1,0 \mu\text{g/l}$ und von 1,25-Dihydroxy-Vitamin D $25,8 \pm 11,7 \text{ ng/l}$ (Mittelwerte

¹⁾ The term cholecalciferol is used for vitamin D₃ and its metabolites; vitamin D is used in so far as it cannot be excluded that metabolites of ergocalciferol and/or cholecalciferol are present.

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± S.D., n = 17, Oktober, Düsseldorf). Im April betrug die Konzentration der Vitamin D-Metabolite bei denselben Personen: 25-Hydroxy-Vitamin D $3,3 \pm 0,9 \mu\text{g/l}$, 24,25-Dihydroxy-Vitamin D $0,17 \pm 0,04 \mu\text{g/l}$ und 1,25-Dihydroxy-Vitamin D $38,3 \pm 10,2 \text{ ng/l}$.

Die Werte von nicht ausgewählten, stationären Patienten (n = 84) betrugen während eines Jahres durchschnittlich: 25-Hydroxy-Vitamin D $5,7 \pm 2,9 \mu\text{g/l}$, 24,25-Dihydroxy-Vitamin D $0,64 \pm 0,44 \mu\text{g/l}$ und 1,25-Dihydroxy-Vitamin D $53,3 \pm 27,6 \text{ ng/l}$.

Die Werte bei 10 pankreatektomierten Patienten sind: 25-Hydroxy-Vitamin D $4,2 \pm 2,6 \mu\text{g/l}$, 24,25-Dihydroxy-Vitamin D $0,27 \pm 0,15 \mu\text{g/l}$ und 1,25-Dihydroxy-Vitamin D $19,6 \pm 11,9 \text{ ng/l}$.

Introduction

It is now well documented that the physiological effects of vitamin D are exerted by hydroxylated metabolites rather than the parent vitamin itself. Vitamin D₃ is produced photochemically in the skin from 7-dehydrocholesterol or absorbed from the diet and initially hydroxylated in the liver to 25-hydroxy-cholecalciferol, the major circulating form of vitamin D₃ (1). This metabolite can serve in the kidney as the substrate for further hydroxylation to 24,25-dihydroxy- (2), to 1,25-dihydroxy- (3) and to 1,24,25-trihydroxy-cholecalciferol (4).

Of these metabolites, 1,25-dihydroxycholecalciferol on the basis of its mechanism of action, must be regarded as a hormone which plays a central role in the maintenance of calcium and phosphorus homeostasis, whereas the physiological importance of 24,25-dihydroxy- and 1,24,25-trihydroxycholecalciferol still has to be clarified. Quantitative methods for the determination of 25-hydroxy, 24,25-dihydroxy and 1,25-dihydroxy metabolites of vitamin D have been described (tab. 1).

We have developed a practicable method for simultaneous measurement of these vitamin D metabolites in a single serum sample.

Materials and Methods

Materials

[1,2(n)-³H]Cholecalciferol (lot No. 998-180) and [1,2(n)-³H]cholesterol (lot No. 1009-094) were obtained from New England Nuclear. [23,24(n)-³H]25-Hydroxycholecalciferol (code TRK. 558, batch 8), [23,24(n)-³H]24,25-dihydroxycholecalciferol (code TRK. 616, batch A 2), [23,24(n)-³H]1,25-dihydroxycholecalciferol (code TRK. 558, batch A 8), were from Amersham Buchler, Braunschweig.

The commercial vitamin D₃ derivatives were purified by water cooled Sephadex LH 20 columns (1.5 × 50.0 cm, Pharmacia Fine Chemicals, Uppsala, Sweden), elution solvent: *n*-hexane/chloroform/methanol (90 ml + 10 ml + 10 ml) followed by HPLC (Laboratory Data Control, Florida, USA), utilising LiChrosorb SI 100 (0.4 × 30.0 cm, 10 μm), elution solvent: dichloromethane/methanol (98 ml + 2 ml). The organic solvents and LiChrosorb SI 100 were obtained from Merck-Darmstadt.

The buffer used in our assay system consisted of 0.05 mol/l Tris-HCl, 0.15 mol/l KCl (Merck, Darmstadt) and 12 mmol/l thioglycerol (Fluka AG, Buchs, Switzerland), pH 7.4 at 23 °C.

Preparation of binding protein

The binding protein for determination of 25-hydroxy and 24,25-dihydroxy vitamin D was prepared from rachitic rat kidneys by the method of Haddad & Chyu (5) and immediately frozen and stored at -80 °C. Before use in the assay it was diluted 1:40 with Tris-HCl. The binding protein for 1,25-dihydroxy vitamin D was prepared by the method of Lambert et al. (10) from the mucosa of white leghorn chickens housed in cages maintained at 28 °C without UV

Tab. 1. Quantitative methods for determination of vitamin D metabolites.

Method	25-Hydroxy vitamin D	24,25-Dihydroxy vitamin D	1,25-Dihydroxy vitamin D
Protein binding assays	<i>Haddad & Chyu</i> (5)	<i>Taylor</i> et al. (6) <i>Shepard</i> et al. (7)	<i>Shepard</i> et al. (7) <i>Brumbaugh</i> et al. (8) <i>Eisman</i> et al. (9) <i>Lambert</i> et al. (10) <i>Haussler</i> et al. (11)
A ₂₅₄ quantitation	<i>Lambert</i> et al. (10) <i>Haussler</i> et al. (11)	<i>Lambert</i> et al. (10) <i>Haussler</i> et al. (11)	
Radioimmunoassays			<i>Fairney</i> et al. (12) <i>O'Riordan</i> et al. (13) <i>Peacock</i> et al. (14) <i>Schäfer & Goldsmith</i> (15)
Isotope dilution mass fragmentography	<i>Björkhem</i> et al. (16)		<i>Björkhem</i> et al. (16)
Bioassays			<i>Hill</i> et al. (17) <i>Stern</i> et al. (18)

light and provided ad libitum with a vitamin D-deficient purified soy protein diet and deionized water, until sacrifice by decapitation at 4–5 weeks. The cytosol was frozen in an acetone dry ice bath and stored under N₂ gas at –80 °C until use. The protein concentration of the cytosol used in the assay was 0.9 g/l.

Extraction of plasma (Scheme 1)

To 6 ml of plasma [³H]25-hydroxycholecalciferol (1500 counts/min, specific activity 4.07 PBq/mol), [³H]24,25-dihydroxycholecalciferol (1000 counts/min, specific activity 3.05 PBq/mol) and [³H]1,25-dihydroxycholecalciferol (1500 counts/min, specific activity 4.07 PBq/mol) in ethanol were added. The sample was divided into two 3 ml portions. Each portion was placed in a 20 ml glass centrifuge tube in an ice bath and the sample was extracted with 3 vol. of cold methanol/methylene chloride (200 ml + 100 ml) per vol. of plasma and vortexed for 3 min. One vol. of cold methylene chloride was added, the sample was vortexed for 3 min and then centrifuged at 1400 g for 15 min at 4 °C (MSE, Fisons, Colara, Düsseldorf). The extraction was repeated with 2 vol. of cold methylene chloride. All organic phases of the two 3 ml portions were combined, placed in a 100 ml separatory funnel, washed twice with an equal volume of phosphate buffer (0.05 mol/l, pH 8.5) and evaporated to dryness on a rotary evaporator under reduced pressure.

Gel chromatography

The methanol/methylene chloride plasma extracts were solubilized in 500 µl of *n*-hexane/chloroform/methanol (90 ml + 10 ml + 10 ml) and chromatographed on a 0.9 × 10.0 cm Sephadex LH 20 column equilibrated with the same solvent system. The tube was rewashed twice with 100 µl of the solvent. The first 5 ml of elution was discarded, the next 10 ml was collected as the 25-hydroxy vitamin D region and the next 25–35 ml, depending on the individual LH 20 column calibration profile, as the 24,25- and 1,25-dihydroxy vitamin D cut. Further purification of the 25-hydroxy vitamin D and the dihydroxylated fractions from the initial Sephadex LH 20 column was accomplished with a second LH 20 column (0.9 × 10.0 cm; *n*-hexane/chloroform 100 ml + 100 ml). The 4–30 ml fraction contained the 25-hydroxy vitamin D, and the 30–110 ml fraction of the corresponding LH 20 column the dihydroxylated metabolites. Each peak from the second LH 20 gel chromatographies was evaporated to dryness on a rotary evaporator under reduced pressure.

High performance liquid chromatography of 25-hydroxy vitamin D and of the mixture of dihydroxylated metabolites

The residues from the 25-hydroxy vitamin D and the 24,25-/1,25-dihydroxy vitamin D cuts were dissolved in 250 µl dichloromethane/methanol (98 ml + 2 ml) and separately subjected to HPLC with a flow rate of 1.0 ml/min of dichloromethane/methanol (98 ml + 2 ml) at a pressure of 86 pounds per square inch (psi).

The [³H]25-hydroxycholecalciferol eluted at 4.5–5.5 ml, the [³H]24,25-dihydroxycholecalciferol at 9.0–10.0 ml and the [³H]1,25-dihydroxycholecalciferol at 14.5–15.5 ml. These positions were checked with a [³H]25-hydroxycholecalciferol, a [³H]24,25- and a [³H]1,25-dihydroxycholecalciferol standard for each run of samples. The 25-hydroxy vitamin D region of the first run and the 24,25- and 1,25-dihydroxy vitamin D regions of the second run were collected separately, evaporated under a stream of dry nitrogen and resuspended in different volumes (1600 µl for 25-hydroxy, 600 µl for 24,25- and 1,25-dihydroxy vitamin D) of dichloromethane/methanol (98 ml + 2 ml) for assay and for counting, in order to estimate recovery.

Competitive binding assays

25-Hydroxy vitamin D assay

500 µl of the 1600 µl of resuspended 25-hydroxy vitamin D was taken for counting to estimate recovery (Tricarb, Packard, Warrenville, USA). Two 250 µl samples were evaporated under a stream of dry nitrogen, redissolved in 60 µl of absolute ethanol, and 50 µl out of 60 µl were taken for assay. The sample incubations were performed in duplicate.

24,25-Dihydroxy vitamin D assay

50 µl out of the 600 µl of resuspended 24,25-dihydroxy vitamin D were taken for the estimation of recovery. Two 250 µl samples were processed as in the 25-hydroxy vitamin D assay.

Each assay mixture consisted of 1 ml diluted binding protein, as described in 'preparation of binding protein', 50 µl of [³H]25-hydroxy- or [³H]24,25-dihydroxycholecalciferol (10000 counts/min in absolute ethanol) and varying amounts of non-radioactive 25-hydroxy or 24,25-dihydroxy vitamin D in an additional 50 µl of absolute ethanol. To keep the quantity of ethanol small

Scheme 1. Outline of the multiple assay procedure for the analysis of 25-hydroxy, 24,25- and 1,25-dihydroxy metabolites of vitamin D in human plasma.

in each tube (below 5% of final assay volume) the 50 μ l of ethanol containing [3 H]25-hydroxy- or [3 H]24,25-dihydroxycholecalciferol (10000 counts/min) were evaporated under a stream of dry nitrogen in all assay tubes, except in those tubes for specific and unspecific binding. Standard curves were constructed over a range of 0.15–20.0 ng using crystalline 25-hydroxy- or 24,25-dihydroxycholecalciferol. Samples for assay were introduced in 50 μ l of absolute ethanol as was the crystalline 25-hydroxy- and 24,25-dihydroxycholecalciferol used to produce the standard curves. No compensation was made for the [3 H]25-hydroxy- or [3 H]24,25-dihydroxycholecalciferol, added to measure recovery, because there were only 100 counts/min in the 25-hydroxy vitamin D and 200 counts/min in the 24,25-dihydroxy vitamin D final assay sample after the separation procedure, with an average recovery of 50 per cent.

The assays were carried out in a shaking water bath at 25 °C for 2 h and then the tubes were immersed in an ice water bath. 0.5 ml of 10 g/l charcoal (Norit A, Serva, Heidelberg) in Tris-buffer was added to each assay tube, which was then vortexed vigorously for one minute. The tubes were centrifuged at 1400 g for 20 min at 4 °C, and 1 ml of the supernatant was taken for counting.

1,25-Dihydroxy vitamin D assay

The fractions of interest from the HPLC were resuspended in 600 μ l of dichloromethane/methanol (98 ml + 2 ml), and 50 μ l was taken for counting to estimate recovery. The remaining 550 μ l was evaporated under a dry stream of nitrogen, dissolved in 30 μ l of absolute ethanol, and 25 μ l of this solution was taken for assay. Each assay mixture consisted of 500 μ l of cytosol (0.9 g/l protein), [3 H]1,25-dihydroxycholecalciferol (5000 counts/min in 25 μ l of absolute ethanol), and varying amounts of 1,25-dihydroxy vitamin D in an additional 25 μ l of absolute ethanol. The ethanol in all tubes except those for specific and unspecific binding was evaporated under a stream of dry nitrogen as described for the 25-hydroxy and the 24,25-dihydroxy vitamin D assays. Standard curves were constructed over a range of 9.0–1200 pg using crystalline 1,25-dihydroxycholecalciferol. After calculation of the recovery, the amount of radioactivity was corrected in the tubes containing the samples so that all tubes contained the same amount of radioactivity at the time of final assay. The assays were carried out in a shaking water bath at 25 °C for 2 h and then the tubes were immersed in an ice water bath. 250 μ l of ice cold, specially prepared charcoal was added to each tube. The tubes were allowed to stand for 30 min and then centrifuged at 1400 g for 15 min at 4 °C. A fraction of the supernatant (500 μ l) was collected and counted in a scintillation solution (Insta-Gel, Packard Instrument Company, Warrenville, USA) to a total of 10000 counts/min at 33–36% efficiency.

To the charcoal used for the 25-hydroxy and the 24,25-dihydroxy vitamin D assay, human plasma was added to give a final concentration of 50 g/l (19). This mixture was shaken gently for 8–12 hours at 4 °C and then centrifuged at 110 g for 15 min at the same temperature. The residue was resuspended in buffer, in an ice water bath, to a final concentration of 50 g/l charcoal.

Results

Extraction of plasma

The extraction ratios of [3 H]25-hydroxy-, [3 H]24,25- and [3 H]1,25-dihydroxycholecalciferol with methanol were 88.0%, 95.0% and 87.3% respectively, which was significantly higher than the extraction ratios with chloroform (23.1%, 50.4% and 73.0% respectively (20)). The extraction ratio with methanol is high, but the subsequent evaporation procedure, even under reduced pressure, very slow. When dichloromethane is used (9) to extract plasma, the amount of extracted lipophilic material is greatly reduced but there is a significant decrease in the recovery of [3 H]25-hydroxycholecalciferol (10). The use of methanol/methylene chloride offers a compromise between reduction in these interfering lipophilic substances and satisfactory recovery of the vitamin D metabolites. The extraction ratios of [3 H]25-hydroxy-, [3 H]24,25- and [3 H]1,25-dihydroxycholecalciferol were 92%, 94% and 83% respectively (tab. 2).

Separation procedure

An elution profile of labelled 25-hydroxy-, 24,25- and 1,25-dihydroxycholecalciferol from the initial Sephadex LH 20 column by *n*-hexane/chloroform/methanol is shown in figure 1. 25-Hydroxycholecalciferol is clearly separated from the dihydroxylated metabolites. Both collected fractions were further purified on a second Sephadex LH 20 column with *n*-hexane/chloroform as the eluent. Again, mono- and dihydroxylated metabolites are clearly separated (fig. 2). An elution profile of labelled and unlabelled vitamin D₃ metabolites from HPLC is shown in figure 3. The three metabolites were collected separately and assayed by competitive protein binding assays.

Recovery

Table 2 shows the recoveries from human plasma of radioactive labelled vitamin D₃ derivatives during the separation procedure. Radioactive internal standard for each metabolite was added to 6 ml of plasma followed by methanol/methylene chloride extraction (step 1), first Sephadex LH 20 gel chromatography (elution sol-

Tab. 2. Recoveries of labelled cholecalciferol metabolites during the separation procedure according to scheme 1.

	Recovery (%)		
	[23,24(n)- 3 H]25-hydroxycholecalciferol	[23,24(n)- 3 H]24,25-dihydroxycholecalciferol	[23,24(n)- 3 H]1,25-dihydroxycholecalciferol
1 st Step (extraction)	92	94	83
2 nd Step (1 st LH 20 column)	73	69	66
3 rd Step (2 nd LH 20 column)	54	61	58
4 th Step (HPLC)	48	55	52

vent: *n*-hexane/chloroform/methanol) (step 2), second Sephadex LH 20 gel chromatography (elution solvent: *n*-hexane/chloroform) (step 3), HPLC (step 4) and scintillation counting. The final recovery following step 4 was nearly 50% of the initially added radioactivity for all derivatives.

Fig. 1. Elution profile of the labelled vitamin D₃ metabolites 25-hydroxycholecalciferol (—), 24,25-dihydroxycholecalciferol (---) and 1,25-dihydroxycholecalciferol (·····) on two LH 20 columns (0.9 × 10.0 cm, elution solvent: *n*-hexane/chloroform/methanol, 90 ml + 10 ml + 10 ml). Fractions of 0.5 ml each were collected and counted for radioactivity.

Fig. 2. Elution profile of labelled vitamin D₃ metabolites 25-hydroxycholecalciferol (25-OH D₃), 24,25-dihydroxycholecalciferol (24,25-(OH)₂ D₃) and 1,25-dihydroxycholecalciferol (1,25-(OH)₂ D₃) on the second LH 20 column (0.9 × 10.0 cm elution solvent: *n*-hexane/chloroform, 100 ml + 100 ml). Fractions of 1.0 ml each were collected and counted for radioactivity.

Fig. 3. Elution profile of the labelled and unlabelled vitamin D₃ metabolites 25-hydroxycholecalciferol (25-OH D₃), 24,25-dihydroxycholecalciferol (24,25-(OH)₂ D₃) and 1,25-dihydroxycholecalciferol (1,25-(OH)₂ D₃) on high performance liquid chromatography (HPLC, LiChrosorb SI 100, 10 μm, elution solvent: dichloromethane/methanol, 98 ml + 2 ml, flow rate 1 ml/min, 86 pounds per square inch). Fractions of 0.5 ml each were collected and counted for radioactivity. The absorbance at 254 nm was measured in parallel.

Standard curves

Standard curves for each of the cholecalciferol metabolites are shown in figures 4–6.

Fig. 4. Standard curve for 25-hydroxycholecalciferol.

Fig. 5. Standard curve for 24,25-dihydroxycholecalciferol.

Fig. 6. Standard curve for 1,25-dihydroxycholecalciferol.

Sensitivity

The absolute sensitivity per assay tube was 150 pg/tube for 25-hydroxy- and 24,25-dihydroxy-, 9 pg/tube for 1,25-dihydroxycholecalciferol. Figures 7 and 8 show typical standard curves for the 25-hydroxy, 24,25- and 1,25-dihydroxy vitamin D assay. After subtraction of the non-specific binding the counts bound in the supernatant were plotted on the vertical axis, whereas the amount of unlabelled vitamin D derivative present in each incubation tube was plotted on the horizontal axis. The first point of each curve was significantly different from the zero point for 25-hydroxycholecalciferol ($t = 3.59$, $p < 0.01$), for 24,25-dihydroxycholecalciferol ($t = 3.27$, $p < 0.025$) and for 1,25-dihydroxycholecalciferol ($t = 7.62$, $p < 0.005$). Taking these values as limits of detection of each derivative gives a sensitivity of 0.05 $\mu\text{g/l}$ plasma for 25-hydroxy- and 24,25-dihydroxycholecalciferol and of 3 ng/l plasma for 1,25-dihydroxycholecalciferol.

The 1,25-dihydroxy vitamin D content of the plasma extracts was determined from that portion of 1,25-dihydroxycholecalciferol standard curve corresponding to the range 7–16 times the sensitivity. The same range of the standard curve was chosen for determination of 25-hydroxy and 24,25-dihydroxy vitamin D.

Specificity

Ergocalciferol, cholesterol, cortisol and testosterone did not react in or interfere with the assay systems. However, there was a complete cross reaction of the renal receptor with 25-hydroxy- and 24,25-dihydroxycholecalciferol; therefore the separation of 25-hydroxy from 24,25-dihydroxy vitamin D had to be done prior to assay.

The values reported are the sum of ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) metabolites. A separation of the analogous metabolites of these two vitamins could not be achieved in this assay system. 25-Hydroxyergocalciferol and 25-hydroxycholecalciferol are equally recognized by the rat binding protein (21,

Fig. 7. Typical standard curves for 25-hydroxycholecalciferol (25-OH D₃) and 24,25-dihydroxycholecalciferol (24,25-(OH)₂ D₃) for estimation of the sensitivity. Each point is the mean of 10 replicates.

Fig. 8. Typical standard curve for 1,25-dihydroxycholecalciferol for estimation of the sensitivity. Each point is the mean of 10 replicates.

22); this seems likely to be true for 24,25-dihydroxyergocalciferol and 24,25-dihydroxycholecalciferol as well. 1,25-Dihydroxyergocalciferol and 1,25-dihydroxycholecalciferol also are equally recognized by chicken cytosol binding protein and can be assayed together (23). A separation of 25-hydroxyergocalciferol from 25-hydroxycholecalciferol is described by *Shepard* (7).

The displacement potency of ergocalciferol analogs in competitive protein binding assays for 25-hydroxy-, 24,25- and 1,25-dihydroxycholecalciferol was measured by *Jones* (24). He found that 24,25-dihydroxyergocalciferol was 1.7 times less potent than 24,25-dihydroxycholecalciferol, 25-hydroxyergocalciferol or 25-hydroxycholecalciferol in the displacement of [³H]25-hy-

droxycholecalciferol from rat serum binding proteins, and that 1,25-dihydroxyergocalciferol was 1.3 times less potent than 1,25-dihydroxycholecalciferol in the displacement of [^3H]1,25-dihydroxycholecalciferol from a chick intestinal binding receptor.

Accuracy

Plasma containing high levels of vitamin D metabolites exhibited a proportional reduction of the measured values upon serial dilution. 20 ng of 25-hydroxycholecalciferol was added to 6 ml of plasma and assayed. At this level, following correction for procedural losses, 86 to 112% of the added 25-hydroxycholecalciferol was recovered.

Precision and reproducibility

A pooled plasma sample was divided into five aliquots which were processed for the assays. At mean 25-hydroxy vitamin D concentrations of 10 $\mu\text{g/l}$, the coefficient of variation was 15% and at mean 24,25-dihydroxy vitamin D concentrations of 1.0 $\mu\text{g/l}$ the coefficient of variation was 13%. When duplicate plasma samples were analyzed on different days, the mean difference of paired duplicates was 16.6 ± 11.2 (S.D.) % and 15.8 ± 9.7 (S.D.) % for 25-hydroxy and 24,25-dihydroxy vitamin D respectively. In five aliquots of a pooled plasma sample the mean concentration of 1,25-dihydroxy vitamin D was 19.5 ng/l and the coefficient of variation 14%. On different days, when 10 duplicate plasma samples were analyzed, the mean difference of paired duplicates was 17.3 ± 13.4 (S.D.) %.

Concentrations of vitamin D metabolites in healthy controls and patients

The mean plasma level of 17 normals (males and females, aged 23–36 years, October, Düsseldorf) are shown in table 3. The mean values are within a normal

t-distribution. Table 3 also shows the concentrations of vitamin D metabolites in the same subjects measured in April.

The mean metabolite concentrations for a one year period were determined by assaying the pool serum of all hospitalized patients each month for 12 months. The mean values for the 12 determinations are shown in table 3, together with the corresponding plasma values of 10 pancreatectomized subjects.

Discussion

The assays described were used to measure the metabolites of vitamin D in plasma of normal male and female adult volunteers at different times of year. They offer an accurate and reproducible method for determination of these analytes in biological fluids.

We found a marked decrease of 25-hydroxy and 24,25-dihydroxy vitamin D in human plasma samples in April compared to those measured in October, whereas 1,25-dihydroxy vitamin D was relatively unchanged.

The average concentration of 25-hydroxy and 24,25-dihydroxy vitamin D of non-selected hospitalized patients during the whole year was between the two extreme values measured in normal human plasma, but the concentration of 1,25-dihydroxy vitamin D was higher in the hospitalized patients than in normal adults.

Our results are in good agreement with those reported in the literature. The mean value of 25-hydroxy vitamin D in October is of the same magnitude as those of Taylor (25) and Kremer (26), but lower than that reported by Shepard (7). We find the same for the mean value of 24,25-dihydroxy vitamin D, which is in good agreement with the data of Taylor (25), Lambert

Tab. 3. Concentrations of 25-hydroxy, 24,25- and 1,25-dihydroxy metabolites of vitamin D determined in two collectives and at different seasons.

	25-Hydroxy vitamin D $\bar{x} \pm s$ ($\mu\text{g/l}$)	24,25-Dihydroxy vitamin D $\bar{x} \pm s$ ($\mu\text{g/l}$)	1,25-Dihydroxy vitamin D $\bar{x} \pm s$ (ng/l)
Healthy probands October, Düsseldorf n = 17	12.2 ± 5.9	1.5 ± 1.0	25.8 ± 11.7
Healthy probands April, Düsseldorf n = 17	3.3 ± 0.9	0.17 ± 0.04	38.3 ± 10.2
Hospitalized patients all months of the year, Düsseldorf n = 84	5.7 ± 2.9	0.64 ± 0.44	53.3 ± 27.6
Pancreatectomized patients all months of the year, Düsseldorf n = 10	4.2 ± 2.6	0.27 ± 0.15	19.6 ± 11.9

(10) and Horst (27) but lower than that reported by Shepard (7). This may be a reflection of lower vitamin D intakes in Europe as compared with the U.S.A. The plasma level of 1,25-dihydroxy vitamin D is in the same range as those reported by Stern (18), Eisman (9), Lambert (10, 19), and Shepard (7).

In non-selected hospitalized patients we found a mean concentration of 1,25-dihydroxy vitamin D during the whole year identical to that reported by Björkhem (16). He used isotope dilution mass fragmentography, the only known method which is independent of binding proteins and may therefore measure the "real" concentration of 1,25-dihydroxy vitamin D.

In patients pancreatectomized 2–6 years prior to assay and not substituted with vitamin D, we found low levels of 25-hydroxy, 24,25- and 1,25-dihydroxy vitamin D.

In developing the four step purification procedure it was realized that chromatographic purification of the plasma lipid abstracts was extremely important for the removal of UV absorbing compounds and contaminants which may interfere in the competitive binding assays. The contaminants that were washed out with the first 5 ml of the elution solvent of the second LH 20 column lowered a known 24,25-dihydroxy vitamin D value of 1.2 ng/tube by approximately 20% when incubated in the assay. Perhaps this contaminant is identical with the peak X reported by Shepard (7), and this is one of the reasons why we measure 24,25-dihydroxy vitamin D by competitive binding assay and not by UV absorption.

It is evident that these assays will be of value not only in studying vitamin D metabolism but also as a diagnostic tool in the management of patients with disorders of calcium and phosphate metabolism.

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