Journal of Chromatography, 621 (1993) 33–39
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7085

Advantages of paper chromatography as a preparative step in the assay of 1,25-dihydroxyvitamin D

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(First received June 17th, 1993; revised manuscript received July 29th, 1993)

ABSTRACT

Ether extraction and paper chromatography were used to separate the main metabolites of vitamin D in plasma [25-(OH), 24,25-$(OH)_2$ and 1,25-$(OH)_2$ vitamin D] prior to radio receptorassay. The overall procedural loss of the 1,25-$(OH)_2$ vitamin D was 58 ± 5% (n = 40), corrected for by tracer addition. The sensitivity of the assay was 0.5 fmol/tube, corresponding to 4 pmol/l, and the intra- and inter-assay coefficients of variation were 10.5% and 11.5%, respectively. The range of values measured in healthy controls was 80–200 pmol/l (n = 60), which is in agreement with findings reported in the literature. A comparison of the results of the present procedure with those obtained with a procedure employing C_{18} purification, disclosed a correlation coefficient of 0.92 ($p \leq 0.0001$), a slope of 0.89 ($p \leq 0.0001$) and a small non-significant intercept of 5.0 pmol/l (n = 53).

INTRODUCTION

Vitamin D is hydroxylated in the liver to 25-hydroxyvitamin D (25-(OH)D), the main circulating metabolite of the hormone in human blood [1]. Hydroxylation to 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) and 24,25-dihydroxyvitamin D (24,25-$(OH)_2$D) mainly takes place in the kidney [2,3]. 1,25-$(OH)_2$D is the biologically active form and is involved in many regulating processes, whereas 24,25-$(OH)_2$D has little, if any, biological activity [4], and 25-(OH)D, as far as known, has none. The main role of 1,25-$(OH)_2$D is the regulation of calcium levels in blood [5]. There is growing evidence that it is also involved in insulin synthesis, sex hormone synthesis, cell differentiation and cell growth [6–9].

The most recent and most accurate method for assessing 1,25-$(OH)_2$D is by making use of high-performance liquid chromatography–mass spectrometry (HPLC–MS) [10]. The currently most widely used method is assessment by a radio receptorassay (RRA), using calf thymus tissue for preparation of the receptor. This receptor,

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0378-4347/93/$06.00

though, binds with not only 1,25-$(OH)_2D$ but also the other metabolites. The binding with 25-(OH)D is only weak, but this compound circulates at a concentration *ca.* 1000 times higher than that of 1,25-$(OH)_2D$. Consequently, the assay of 1,25-$(OH)_2D$ is feasible only after separation from the other metabolites. Such purification can be effectively achieved by HPLC [10–13] as well as by a dual- or single-column solid phase extraction [14,15]. The latter methods, however, do not separate 24,25-$(OH)_2D$ from 1,25-$(OH)_2D$.

Because in our laboratory liquid–liquid extraction followed by paper chromatography has proved to be an effective and a convenient purification technique for various other steroid hormones, requiring little time and instrumentation of limited cost, we have investigated its efficacy in the purification of 1,25-$(OH)_2D$ preceding RRA.

EXPERIMENTAL

Samples

Human plasma was obtained (1) from the local blood bank and pooled for control experiments, (2) from 60 healthy individuals (34 men, 26 women) in order to establish a normal range of 1,25-$(OH)_2D$ concentrations, and (3) from 53 patients, selected for the purpose of obtaining a wide range of 1,25-$(OH)_2D$ concentrations (determined at TNO Food and Nutrition Research Laboratories, Zeist, Netherlands, by making use of solid phase chromatography conform a previously described method [16]).

Reagents and chemicals

Radioactive materials were purchased from Dupont (NEN Products, Dreieich, Germany). Non-labelled 1,25-$(OH)_2D$ was kindly donated by Dr. U. Fisher (F. Hoffmann-La Roche, Basel, Switzerland). All other chemicals used were of analytical-reagent grade. C_{18} and silica collumns were purchased from Waters (Millipore, Milford, MA, USA).

Extraction and paper chromatography

To 1.0 ml of plasma, 25 μl (10 000 dpm) of ^3H-labelled 1,25-$(OH)_2D_3$ (166.4 Ci/mmol, 26,27-^3H) in ethanol were added. The samples were incubated for 15 min at room temperature and extracted with 15 ml of diethyl ether. The solvent was removed by drying under a stream of dry nitrogen. The residue was dissolved in an aliquot of diethyl ether and applied to a sheet of paper (Whatman N°. 1) divided into 8 lanes (2.5 × 42 cm), prewashed with ethanol in order to reduce the reagent blank. The paper was chromatographed for 3 h in a descending system [17] of petroleum ether (b.p. 80–110°C)–toluene–methanol–water (333:167:400:100, v/v/v/v). The tracer label was located by radioscanning with a Model 7201 radiochromatogram scanner (Packard Instruments). The appropriate area of paper was cut out, and the 1,25-$(OH)_2D$ was eluted with 2 ml of ethanol for 1 h at room temperature. The ethanol was then evaporated under a stream of dry nitrogen, and the residue was dissolved in 200 μl of 25% ethanol in phosphate buffer (0.04 *M* Na_2HPO_4, 0.01 *M* NaH_2PO_4, 0.10 m*M* monothioglycerol) (pH 7.4) in order to concentrate the sample.

Purification

To purify samples by means of solid-phase columns, and to obtain processed water blanks and recovery data, two different procedures were employed. The first was a two-column purification, employing C_{18} and silica columns as described by Reinhardt *et al.* [14], and the second was a single-column purification employing only a C_{18} column, as described by Hollis [15].

Preparation of calf thymus receptor

Calf thymus was obtained from the local slaughterhouse and stored until use at −80°C. To make a receptor preparation, 20 g of calf thymus was cryopulverized in a motor-driven mortar. The thymus, vat and piston were all cooled with liquid nitrogen. A 45-ml volume of 0.05 *M* phosphate buffer was added, containing 0.4 *M* KCl, 1.5 m*M* EDTA, 10 m*M* Na_2MoO_4, 0.1 m*M* monothioglycerol and 0.1 m*M* bacitracin, and allowed to stand for 30 min at 4°C. The thymus tissue was then further suspended with an Ultra

Turrax for *ca.* 5 min. The suspension was centrifuged at 100 000 *g* for 60 min at 4°C, and the supernatant was stored in 1-ml aliquots at −80°C until use. Prior to assay, the solution was diluted 1:30 with 0.05 *M* phosphate buffer containing 0.1 *M* KCl, 1.5 m*M* EDTA, 10 m*M* Na_2MoO_4, 0.1 m*M* monothioglycerol and 0.1 m*M* bacitracin, to achieve optimal initial binding. No significant loss of binding capacity of the receptor preparation was detected even after 1 year of storage.

Radio receptorassay

The RRA was performed as described by Hollis [15] with a few modifications. A standard curve (0–1200 pmol/l) was set up in duplicate. To 50 μl of standard, control or unknown, 50 μl of ^{3}H-labelled 1,25-$(OH)_2D_3$ (10 000 dpm) in ethanol–phosphate buffer (1:3, v/v) and 500 μl of receptor solution were added in a glass tube. After 3-h incubation at room temperature, bound and unbound 1,25-$(OH)_2D$ were separated using dextran-coated charcoal to adsorb the unbound fraction. This separation went as follows. An aliquot of 150 μl of 1% charcoal suspension in 0.05 *M* phosphate buffer containing 0.1 *M* KCl and 0.1% Dextran T70 was pipetted into the cavity of a plastic test-tube cap, where, owing to adhesion, the slurry stayed in position until shaken out. The caps thus filled were put on the tubes and then the tubes were shaken simultaneously to mix the suspension with the contents of the tubes. After 3 min, the samples were centrifuged at 2000 *g* for 10 min at room temperature. The supernatants were decanted simultaneously into counting vials using the device described by Vecsei and Gless [18]. After the addition of 4 ml of scintillation liquid, the radioactivity was determined.

Calculations

Measurement results were corrected for procedural losses (recovery), reagent blank, and the effects caused by the use of recovery tracer itself. The standard curve was fitted to a four-parameter model as described by Healey [19], using a non-linear fit algorithm according to Marquardt [20]. The concentration of hormone present in the sample eluate was read from the standard curve after correction of the total counts for the contribution of the radioactivity by the recovery tracer. The concentration was then corrected for the mass contribution of the recovery tracer and the reagent blank. Finally, a correction for procedural losses was performed.

Student's t-test and Spearmann correlations were performed with the SAS program on a VAX/VMS computer.

RESULTS

The 1,25-$(OH)_2D$ tracer was extracted from plasma by diethyl ether with an efficiency of 89 ± 4%. After paper chromatography and elution of the central area of the peak, the overall recovery of 1,25-$(OH)_2D$ was 42 ± 5% (n = 40).

The three main metabolites of vitamin D in plasma, 25-(OH)D, 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$, were efficiently separated by paper chromatography, as shown in Fig. 1.

Radiolabelled 1,25-$(OH)_2D$ comigrated with non-labelled 1,25-$(OH)_2D$, as was demonstrated

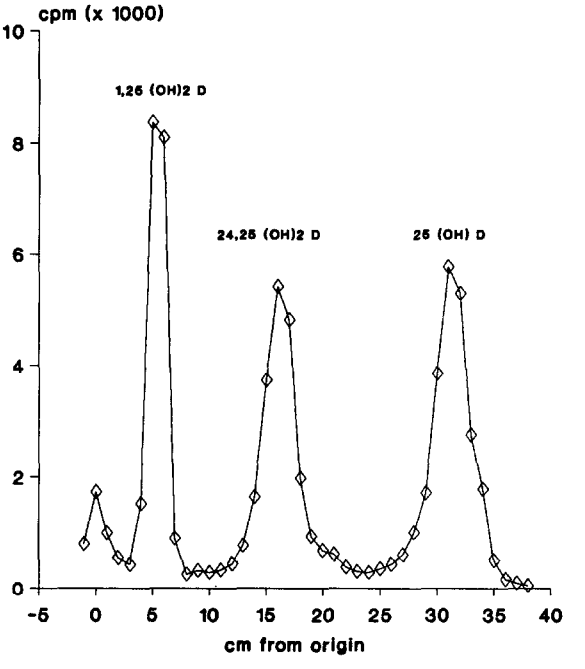

Fig. 1. Separation of 1,25-$(OH)_2D$, 1,24-$(OH)_2D$, and 25-(OH)D by paper chromatography using a descending system [17].

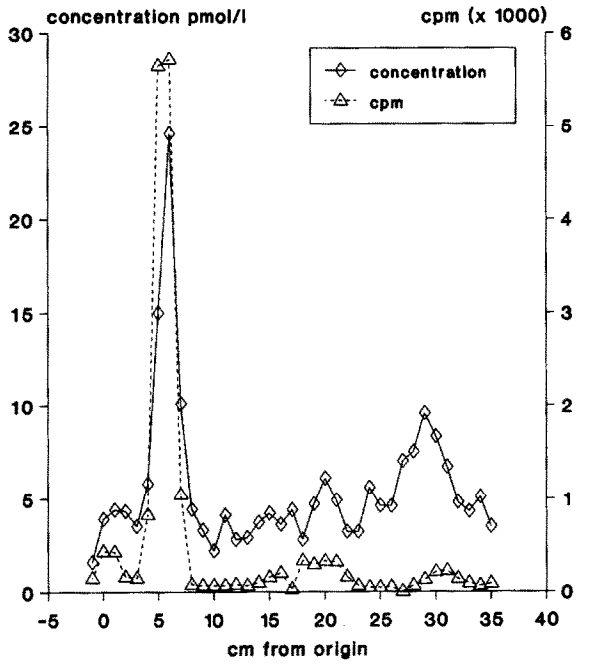

Fig. 2. Radioactivity (cpm) of ^{3}H-labelled 1,25-$(OH)_2$D along the paper chromatogram and concentration of 1,25-$(OH)_2$D measured in plasma after chromatography. Radiolabelled 1,25-$(OH)_2$D comigrates with non-labelled 1,25-$(OH)_2$D.

in an experiment in which a 10 000 dpm tracer was added to 1 ml of plasma. After extraction and chromatography, the chromatogram was scanned for radioactivity and then cut into pieces 1 cm long. Each piece of paper was eluted and the eluate was assayed for 1,25-$(OH)_2$D. Both the radioactivity and the concentration along the entire chromatogram are shown in Fig. 2. The peak in concentration observed at *ca.* 30 cm from the origin corresponds to the location of 25-(OH)D.

In order to establish the appropriate dilution of the receptor preparation for the 1,25-$(OH)_2$D assay, serial dilutions of the calf thymus receptor preparation were made. Fig. 3 shows the binding of ^{3}H-labelled 1,25-$(OH)_2$D at different dilutions of receptor preparation. To obtain an initial binding of *ca.* 40%, a 1:30 (v/v) dilution was used for the assay.

Fig. 4 shows a typical standard curve for the RRA using the appropriate 1:30 dilution of the receptor preparation. The sensitivity was calculated as three times the standard deviation from the data on the zero sample. The detection limit was found to be 0.5 fmol/tube, corresponding to 4 pmol/l when 1 ml of plasma was assayed, recovery being taken into account.

The intra- and inter-assay coefficients of variation (C.V.) were 10.5% (n = 15 at 93.4 pmol/l)

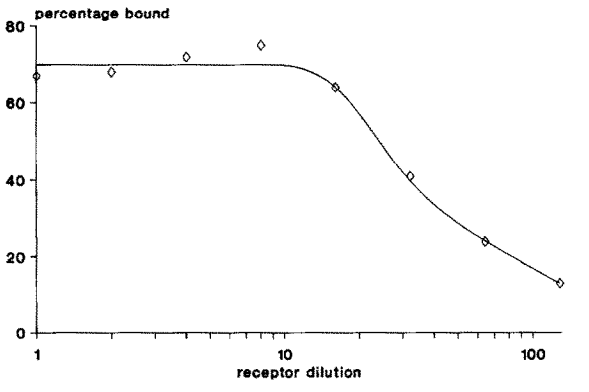

Fig. 3. Percentage binding of ^{3}H-labelled 1,25-$(OH)_2$D after incubation for 3 h at room temperature with different dilutions of calf thymus receptor preparation. The preparation was used in a 1:30 dilution.

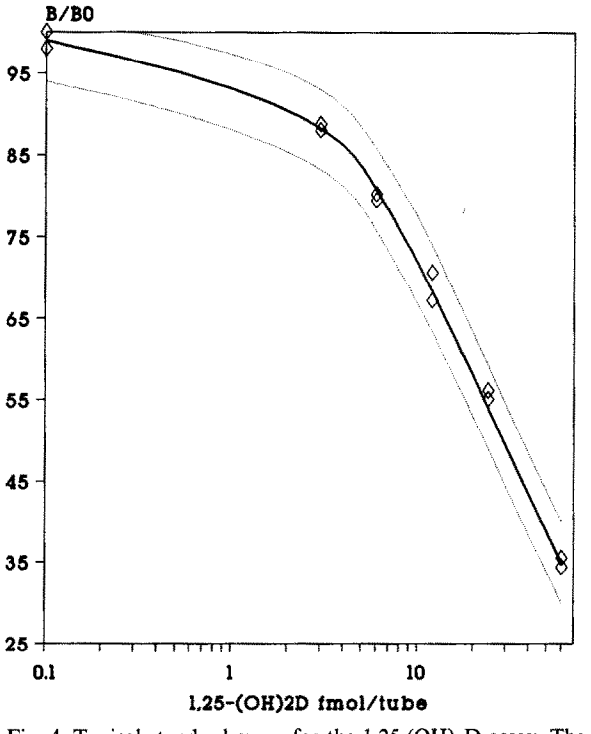

Fig. 4. Typical standard curve for the 1,25-$(OH)_2$D assay. The sensitivity was 0.5 fmol/tube.

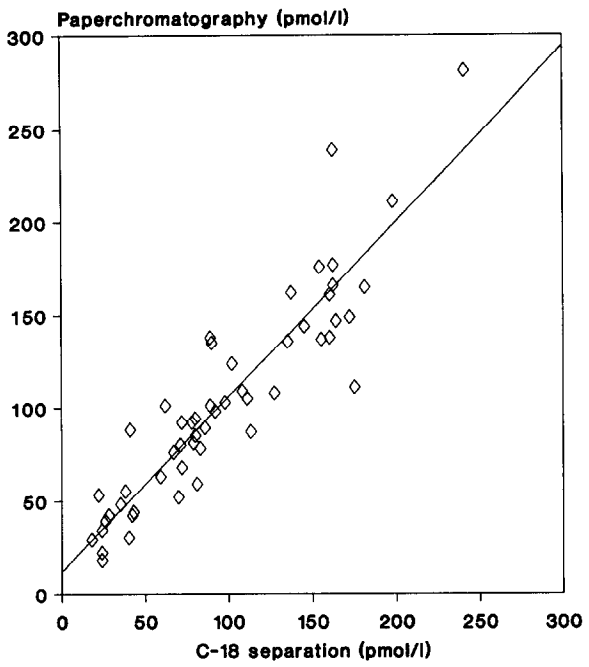

Fig. 5. Correlation of measured values after solid-phase C_{18} chromatography and paper chromatography (n = 53, r = 0.92, $p \leq 0.0001$).

and 11.5% (n = 8 at 103.3 pmol/l), respectively. Of one and the same plasma sample, 4, 2, 1 and 0.5 ml were assayed to determine parallelism (n = 5). The values (± S.D.) measured were 99 (± 2), 95 (± 12), 95 (± 11) and 96 (± 18) pmol/l, respectively. To investigate the efficacy of the recovery corrections, different aliquots of standard 1,25$(OH)_2$D were added to a plasma sample. The recovery of this standard was 107% ± 5% (100 pmol/l added) (n = 9) and 115% ± 8% (200 pmol/l added) (n = 8). Normal levels of 1,25-$(OH)_2$D were obtained from plasma of 34 healthy men and 26 healthy women with ages ranging from 22 to 79 yr (men 50 ± 16; women 42 ± 13). The normal range for men was 81–195 pmol/l, the normal range for women was 83–200 pmol/l. There was no significant difference in these ranges. Nor was there any significant difference in concentration related to age, either in men or in women.

The present assay was compared with a 1,25-$(OH)_2$D assay using C_{18} purification; 53 samples were assayed with both methods. The results are shown in Fig 5. The coefficient of correlation was 0.92 ($p \leq 0.0001$), the slope was 0.89 ($p \leq 0.0001$) and the intercept was 5.0 pmol/l (N.S.).

The processed water blanks were obtained by assaying 1 ml of water instead of plasma. The water blank obtained after C_{18} purification (0.60 ± 0.28 fmol/tube) (n = 20) was slightly lower than that obtained after paper chromatography (0.95 ± 0.33 fmol/tube) (n = 20) ($p \leq 0.05$). The processed water blank after combined C_{18} and silica purification was significantly higher than those of the other two (1.95 ± 1.25 fmol/tube) (n = 16) ($p \leq 0.01$).

The water blank after extraction and chromatography was found to be mainly due to the chromatographic step. The water blank resulting from ether extraction was *ca.* 2.5 times lower than that obtained from paper chromatography and did not significantly contribute to the latter.

DISCUSSION

Liquid–liquid extraction and paper chromatography were successfully used to separate 1,25-dihydroxyvitamin D from 25-(OH)D and 1,24-$(OH)_2$D. This separation is generally achieved by HPLC [10,13] or C_{18} and silica columns. However, HPLC and the C_{18}–silica method of Reinhardt *et al.* [14] are rather expensive and time-consuming. The single-column method of Hollis [15] takes less time but does not separate 1,25-$(OH)_2$D from 24,25-$(OH)_2$D. This limitation is of no consequence when in the assay no cross-reaction occurs between these two metabolites, as is the case in the present RRA. Otherwise, as for example in the radioimmunoassay of Clemens *et al.* [21], Hollis' method fails.

The recovery of 1,25-$(OH)_2$D after paper chromatography (42 ± 5%) (n = 40) was not significantly different from the recovery after C_{18} chromatography (38 ± 10%) (n = 12). The processed water blank after paper chromatography (0.95 ± 0.33 fmol/tube) was slightly higher than that after C_{18} chromatography (0.60 ± 0.28 fmol/tube). The intra- and inter-assay C.V. (10.5% and 11.5%) of the present assay are similar to those reported by Hollis [15].

The sensitivity of our assay was 0.5 fmol/tube, calculated as three times the standard deviation of the counts of the zero sample. Reinhardt *et al.* [14] using twice the standard deviation of the zero sample, reported a sensitivity of 3.6 fmol/tube, and Hollis [15], also using twice the standard deviation, reported a sensitivity of 1.7 fmol/tube. A possible explanation for these differences may be found in different qualities of the receptor preparations used, since the decline of the standard curve, and thus the sensitivity, is dependent on the quality of the receptor. The intra- and inter-assay C.V. at normal range values were *ca.* 10% in all three assays, which means that, even though we found a lower detection limit, the values within the normal range have a similar degree of accuracy.

Although the reported ranges of 1,25-$(OH)_2$D concentrations in healthy people differ between laboratories, the range of our assay (80–200 pmol/l) is comparable with those in the literature (*e.g.* Reinhardt *et al.* [14] 24–192 pmol/l; Bouillon *et al.* [24] 92–168 pmol/l).

In accordance with Sherman *et al.* [22] and Orwoll and Meier [23], we found no correlation between age and the concentration of 1,25-$(OH)_2$D in plasma. This is in contrast with the results of Bouillon *et al.* [24] and Tsai *et al.* [25], who found that concentration and age are inversely related. These two studies, however, were not confined to healthy volunteers, but also comprised diseased subjects, so there is no point in comparing our results with theirs.

Because the production of 1,25-$(OH)_2$D is an enzymic process, low production in healthy persons must result either from low availability of 25-(OH)D or from an age-related decrease in enzymic activity. Because we exclusively assessed plasma of healthy subjects with no history of any disease related to bone metabolism or vitamin D uptake, abnormally low 25-(OH)D levels were not to be expected. Neither was there, in view of results published by other authors, any reason to expect a correlation between enzyme activity and age [22–24]. So the fact that we did not find such a correlation did not come as a surprise.

Interestingly, Bouillon *et al.* [24], in common with Sherman *et al.* [22], found seasonal differences in 1,25-$(OH)_2$D concentrations. Such fluctuations might influence the range of normal values.

We measured 53 samples using both paper chromatography and C_{18} chromatography to purify the samples. The results were very similar, as is apparent from the slope of 0.89 and a correlation of 0.92 (Fig. 5).

To conclude, the application of the old technique of paper chromatography has proven to be equally effective and reliable for the purification of 1,25-$(OH)_2$D as state of the art techniques, except HPLC–MS. The latter procedure, however, requires costly instrumentation and, like solid-phase and HPLC purification, produces considerably more chemical waste per sample than paper chromatography. Solid-phase extraction and HPLC also require far more time per sample than paper chromatography: in our laboratory, 64 samples can be purified simultaneously with paper chromatography, compared with just one sample with HPLC and ten with solid-phase extraction.

An additional advantage is that paper chromatography separates all three main metabolites of vitamin D in human plasma, in contradistinction to solid-phase extraction.

ACKNOWLEDGEMENT

We thank H. Schutte for translational assistance.

REFERENCES

1 J. W. Blunt, H. F. DeLuca and H.K. Schnoes, *Biochemistry*, 7 (1968) 3317.
2 D. R. Fraser and E. Kodicek, *Nature*, 228 (1970) 764.
3 M. F. Holick, H. K. Schnoes, H. F. DeLuca, R. W. Gray, I. T. Boyle and T. Suda, *Biochemistry*, 11 (1972) 4251.
4 H. F. DeLuca, *Am. J. Med.*, 58 (1975) 39.
5 H. Reichel, H. P. Koeffler and A. W. Norman, *New Engl. J. Med.*, 320 (1989) 980.
6 C. Cade and A. W. Norman, *Endocrinology*, 120 (1987) 1490.
7 F. Meggouh, P. Lointier and S. Saez, *Cancer Res.*, 51 (1991) 1227.
8 H. A. P. Pols, J. C. Birkenhager, J. C. Foekens and J. P. T. M. Van Leeuwen, *J. Steroid. Biochem. Mol. Biol.*, 37 (1990) 873.

9 R. J. Frampton, S. A. Omond and J.A. Eisman, *Cancer Res.*, 43 (1983) 4443.
10 D. Watson, K. D. R. Setchell and R. Ross, *Biomed. Chromatogr.*, 5 (1991) 153.
11 P. F. Brumbaugh, D. H. Haussler, R. Bressler and M. R. Haussler, *Science*, 183 (1974) 1089.
12 T. A. Reinhardt, R. L. Horst, E. T. Littledike and D. C. Beitz, *Biochem. Biophys. Res. Comm.*, 106 (1982) 1012.
13 J. A. Eisman, A. J. Hamstra, B. E. Kream and H. F. DeLuca, *Arch. Biochem. Biophys.*, 176 (1976) 235.
14 T. A. Reinhardt, R. L. Horst, J. W. Orf and B. W. Hollis, *J. Clin. Endocrinol. Metab.*, 58 (1984) 91.
15 B. W. Hollis, *Clin. Chem.*, 32 (1986) 2060.
16 A. H. Mudde, H. Van den Berg, P. G. Boshuis, F. C. Breedveld, H. M. Markusse, P. M. Kluin, O. L. M. Bijvoet and S. E. Papapoulos, *Cancer*, 59 (1987) 1543.
17 I. E. Bush, *The Chromatography of Steroids*, Pergamon Press, Oxford, 1961, pp. 48–50.
18 P. Vecsei and K. H. Gless, *Aldosteron-Radioimmunoassay*, Ferdinand Enke Verlag, Stuttgart, 1975.
19 M. J. R. Healey, *Biochem. J.*, 130 (1972) 207.
20 D. W. Marquardt, *J. Soc. Ind. Appl. Math.*, 11 (1963) 431.
21 T. L. Clemens, G. N. Hendy, R. F. Graham, E. G. Baggiolini, M. R. Uskokovic and J. L. H. O'Riordan, *Clin. Sci. Mol. Med.*, 54 (1978) 329.
22 C. S. Sherman, B. W. Hollis and J. D. Tobin, *J. Clin. Endocr. Metab.*, 71 (1990) 405.
23 E. S. Orwoll and D. E. Meier, *J. Clin. Endocr. Metab.*, 63 (1986) 1262.
24 R. A. Bouillon, J. H. Auwerx, W. D. Lissens and W. K. Pelemans, *Am. J. Clin. Nutr.*, 45 (1987) 755.
25 K. Tsai, H. Heath III, R. Kumar and B. L. Riggs, *J. Clin. Invest.*, 73 (1984) 1668.