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SERUM CONCENTRATIONS OF DIHYDROTACHYSTEROL-2 IN THE TREATMENT OF OSTEOPOROSIS AND HYPOPARATHYROIDISM

R. BOSCH,* J. H. H. THIJSSEN AND S. A. DUURSMA

Clinical Research Group for Bone Metabolism, University Hospital, Utrecht, Netherlands

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SUMMARY

A method for the quantitative estimation of dihydrotachysterol-2 is described using high performance liquid chromatography coupled to an UV monitor. Since radioactive labelled DHT_2 is not available, recovery is based on the assumption that dihydrotachysterol-2 and cholecalciferol behave identically during chromatic procedures. Evidence is presented to support this assumption.

In patients treated with DHT_2 the serum concentrations of DHT_2 rose in proportion to the administered dose.

For several decades dihydrotachysterol-2 has been available under the trade names Dihydral (Philips-Duphar) and AT 10 (Merck). It may be regarded as a partial reduction product of vitamin D_2 (Westerhof and Keverling Buisman, 1956).

Although in the last few years several synthetic and natural cholecalciferol analogues have been introduced into clinical practice, DHT_2 is still used in many countries. Its principal advantage, compared with other calciferol analogues, is its shorter duration of therapeutic action and hence its reduced toxicity (Stanbury & Mawer, 1978).

DHT_2 increases serum calcium by increasing absorption of calcium from the intestine and mobilizing calcium from the skeleton, and clinical indications for its use include hypoparathyroidism and renal osteodystrophy. In the last few years some have treated osteoporosis with sodium fluoride and an additional, high dose of vitamin D to achieve adequate mineralization of the newly formed osteoid (Jowsey *et al.*, 1972). Generally the dose of DHT_2 is adjusted to maintain calcium levels within normal limits, but nevertheless, bone histology may be abnormal in such patients. Thus, correlation between serum concentrations of DHT_2 and histopathological studies might serve as a guide to the correct dose of DHT_2.

In contrast to DHT_3 no data are available concerning the metabolism of DHT_2, although comparison of their physiological actions suggests similar metabolism. It is reasonable to assume that the biologically active metabolite of DHT_2 in human serum is

* Correspondence: R. Bosch, Clinical Research Group for Bone Metabolism, University Hospital Catharijnesingel 101, 3500 CG Utrecht, Netherlands.

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25-hydroxydihydrotachysterol-2 (Hallick & DeLuca, 1972). Since the 25-hydroxylation system for DHT_3 and DHT_2 is not subject to feedback inhibition (Bhattacharyya & DeLuca, 1973) one can postulate that the serum concentration of 25-OH–DHT_2 is directly proportional to the administered dose of DHT_2. To help achieve adequate treatment with DHT_2 in patients with metabolic bone diseases measurement of serum DHT_2 may be of value in achieving clinical efficacy.

The radiochemical techniques developed for the quantification of the metabolites of vitamin D_3, which are based on their reaction energies for the D-binding protein, cannot be used for the determination of DHT_2 and D_3 (DeLuca, 1974; Eisman *et al.*, 1976; Clemens *et al.*, 1979; Bouillon *et al.*, 1980). Up to now D_3 has been measured by means of an isotope dilution mass fragmentographic assay (Björkhem & Larson, 1978) and by means of an UV monitor coupled to HPLC equipment (Jones, 1977; Dokoh *et al.*, 1979; Horst *et al.*, 1979). In this study the last method has been adapted for the quantitative measurement of DHT_2.

MATERIALS

Cholecalciferol was purchased from Merck (Darmstadt, Germany), ergocalciferol, dihydrotachysterol-2 and dihydrotachysterol-3 were a gift from Philips-Duphar (Weesp, Netherlands). (1α, 2α(*n*)-^{3}H) cholecalciferol (specific activity 12·3 Ci/mM) was obtained from the Radiochemical Centre (Amersham, United Kingdom). All sterols were purified before use by high performance liquid chromatography (HPLC). All reagents and solvents were obtained from Merck (Darmstadt, Germany) and were of analytical grade. Solvents for HPLC were redistilled before use. Silica gel (0·4–0·1 mm) came from Baker Chemicals (Deventer, Netherlands).

HPLC was performed on a Perkin-Elmer Series 2 liquid chromatography system (Norwalk, Connecticut) and a UV-absorbance detector Model 440 (Waters Associates, Milford, Massachusetts). Samples were introduced via a Model 7280 valve (Rheodyne, Berkeley, California). The column used for HPLC was a Zorbax Sil 850 column (0·62 × 25 cm; Dupont de Nemours, Den Bosch, Netherlands).

METHODS

Extraction of serum

20 000 d.p.m. (1α, 2α(*n*)-^{3}H) cholecalciferol in 20 μl of ethanol is added to 2 ml of serum and preincubated for at least 2 h at room temperature. 4 ml of methanol is added, followed by 16 ml of diethylether and 12 ml of distilled water. After each addition the emulsion was shaken vigorously. The final emulsion is centrifuged for 3 min at 3000 r.p.m. and cooled down to −70°C for 30 min. The ether layer is removed with a capillary Pasteur pipette and evaporated at 40°C under nitrogen.

Silica gel chromatography

The total residual of the serum lipid extract is redissolved in 1 ml of *n*-hexane/diethyl-ether (2 parts to 1 part by volume), applied to a dry silica gel column (0·5 × 3 cm; 0·5 g), packed in a capillary Pasteur pipette and eluted with 9 ml of the same solvent mixture. The eluent is dried at 40°C under nitrogen and taken up in 25 μl *n*-hexane/propanol-2 (98 parts to 2 parts by volume). This solution is stored at −70°C in the dark until use.

Liquid chromatography and UV monitoring

The UV detector is adjusted to a wavelength of 251 nm, the absorption maximum of DHT_2. 5 μl of the silica gel extract is subjected to HPLC on the Zorbax column equilibrated in *n*-hexane/propanol-2 (98 parts to 2 parts by volume) with a constant flow rate of 1 ml/min at a pressure of 400 to 500 pounds per square inch. After four samples the column is calibrated with 2 μl of a reference preparation containing 20 ng of dihydrotachysterol-2; 20 ng of cholecalciferol and 2000 d.p.m. of (1α, 2α(*n*)-^{3}H) cholecalciferol. The peak heights of standards and samples are measured on a Pye Unicam recorder (Philips, Eindhoven, Netherlands).

The eluate of each sample and reference is collected for 25 min after injection. Each single eluate is evaporated at 40°C under nitrogen, redissolved in 1 ml of ethanol and transferred to polyethylene counting vials; 10 ml of liquid scintillator (0·25% PPO and 0·025% POPOP in toluene) is added. The samples and references are counted for 10 min in a Packard 2750 Tricarb liquid scintillation counter (Packard Instruments Co., Downers Grove, Illinois) at 70% efficiency to assess the recovery of DHT_2.

RESULTS

Extraction and purification of serum

The non-specific total lipid extraction with diethylether, methanol and water has been chosen because of a satisfactory recovery of all compounds under study. The recovery of (1α, 2α(*n*)-^{3}H) cholecalciferol after extraction was 65% (s.d. = 4%; $n = 5$). It was necessary to purify the extract by means of chromatography on silica gel before HPLC was performed. The recovery of cholecalciferol after elution over a silica gel column was 96% (s.d. = 2%; $n = 5$). Now cholecalciferol and dihydrotachysterol-2 could be completely resolved by HPLC using *n*-hexane/propanol-2 (98 parts to 2 parts by volume) as the eluent (Fig. 1). The overall recovery of (1α, 2α(*n*)^{3}H cholecalciferol after extraction, purification and chromatography was 46% (s.d. = 9·6%; n = 15).

Measurement in serum

Known aliquots of DHT_2 added to normal serum could be recovered on the basis of the recovery of (1α, 2α(*n*)-^{3}H) cholecalciferol (Fig. 2). The within-assay coefficients of variation, estimated by parallel analysis at two levels of DHT_2, were 6·2% (50 ng DHT_2/ml serum; $n = 6$) and 8·6% (75 ng DHT_2/ml serum; $n = 6$). The between-assay coefficient of variation, measured on five separate occasions, was 8·6% (50 ng DHT_2/ml serum). DHT_2 concentrations in patients' blood samples were measured in duplicate. The coefficient of variation in serum DHT_2 concentrations between 4·5 and 7·5 ng/ml was 9·4% ($n = 13$), between 7·5 and 12·5 ng/ml it was 8·8% (n = 13) and above 12·5 ng/ml 6·9% ($n = 12$). The sensitivity of the measurement can be calculated from the final detection at twice the signal/noise ratio. The noise measured on the base line was 4×10^{-5} extinction units. This resulted in a limit of detection of 0·7 ng/ml. Sera containing no DHT_2 did not show any absorption at the DHT_2 retention position in the chromatograms.

The specificity of the system is illustrated in Fig. 1. The width of the base of the DHT_2 peak was about 0·8 ml. In most sera the distance in retention volumes of neighbouring peaks was at least 1·5 ml. Sometimes a small, less polar peak was observed as a shoulder of the DHT_2 peak or as a single peak at a distance 0·6 ml from the DHT_2 peak. Yet this little

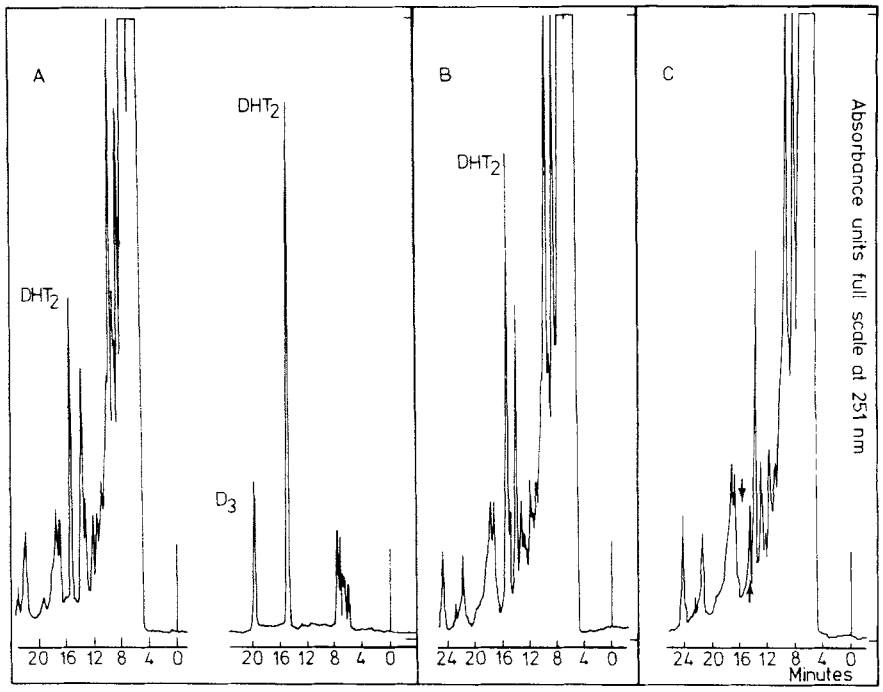

Fig. 1a. HPLC profiles on the right, pure DHT_2 and D_3, 20 ng each, at sensitivity 0·01 Absorbance Units Full Scale (AUFS); on the left, D_3 fraction of DHT_2-containing serum at 0·005 AUFS.

1b. HPLC profile of D_3 fraction from a patient suffering from hypoparathyroidism and treated with DHT_2. Note the slight peak to the right of the DHT_2 peak. Sensitivity: 0·005 AUFS.

1c. HPLC profile of the D_3 fraction of a normal serum at sensitivity of 0·005 AUFS. Rarely a small peak (indicated by bottom arrow) is observed some 0·6 earlier than the DHT_2 peak. The top arrow indicates the position of DHT_2.

Conditions: see text; ordinate—UV absorption at 251 nm; abcissa: minutes. Flow rate 1 ml/minute.

peak could easily be distinguished from the DHT_2 peak. The peak of dihydrotachysterol-2 always had a sharp Gaussian distribution.

CLINICAL RESULTS

Values were obtained in adult patients with a normal height and body weight. They were treated with DHT_2 for at least 3 months; 15 patients were suffering from hypoparathyroidism (all women), 13 patients from osteoporosis (9 women and 4 men), 2 women from hyperparathyroidism, 1 woman was suffering from pseudo-hypoparathyroidism, and 5 patients were suffering from uraemia (3 women and 2 men).

A linear correlation was obtained between the administered dose and the serum level of DHT_2 in all patients (Fig. 3; $P<0{\cdot}001$; $n=36$). One hypoparathyroid patient, on a constant DHT_2 therapy of 1·0 mg daily, was followed for 5 months. The mean serum concentration was fairly constant at 13·3 ng/ml (s.d. = 12·9%; $n=5$). In patients with osteoporosis, treated with 0·1 mg DHT_2 daily, the mean concentration of DHT_2 was 9·0 ng/ml (s.d. = 28·8%; $n=13$).

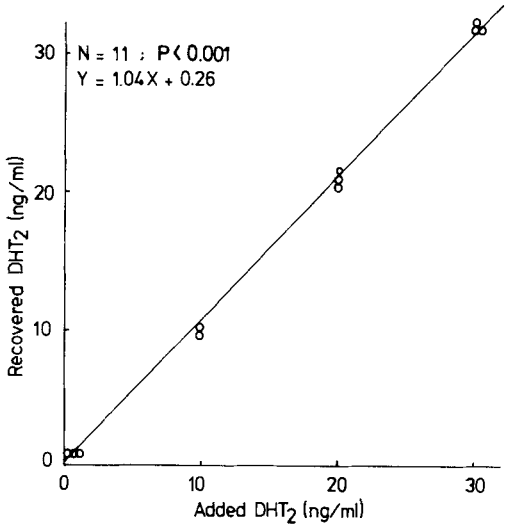

Fig. 2. Recovery of DHT_2 added to normal serum samples. Correlation coefficient = 0·998; ordinate—observed concentration of DHT_2 in ng/ml; abcissa—concentrations of DHT_2 added in ng/ml serum.

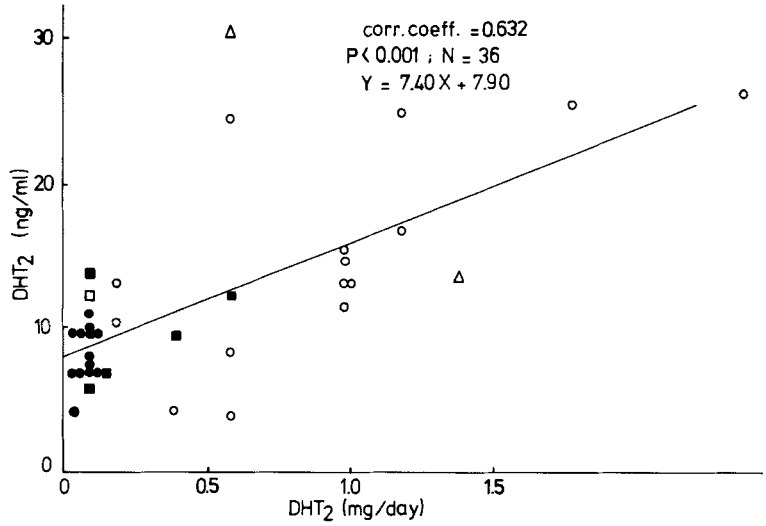

Fig. 3. Comparison of the observed DHT_2 concentrations in sera from patients with various bone diseases after treatment with DHT_2. ○ = hypoparathyroidism; □ = pseudoparathyroidism; △ = hyperparathyroidism; ● = osteoporosis; ■ = chronic renal failure. Ordinate—concentration of DHT_2 in ng/ml serum; abscissa—DHT_2 dose in mg per day.

DISCUSSION

Dihydrotachysterol-2 is a useful vitamin D analogue for patients with renal osteodystrophy or hypoparathyroidism. It is also used in patients with osteoporosis, treated with fluoride. To ascertain the optimal dose of DHT_2, serum concentrations should be compared with histomorphometric measurements in bone tissue.

The binding of DHT_2 to the vitamin D-binding globulin in blood is very small, so this protein cannot be used for a competitive protein-binding assay for DHT_2 (Morris & Peacock, 1976). Unfortunately, the same holds for the 1,25-DHCC receptor assay (Eisman *et al.*, 1976) and the two radio-immunoassays described for 1,25-DHCC (Clemens *et al.*, 1979; Bouillon *et al.*, 1980). Since we did not have access to a mass spectrophotometer (Björkhem & Larson, 1978) we attempted to measure DHT_2 using HPLC with UV detection, in a comparable manner to the determinations of D_2 and D_3 (Jones, 1977; Dokoh *et al.*, 1979; Horst *et al.*, 1979).

Recovery measurements for DHT_2 were based on those obtained for (1α, 2α(*n*)-^{3}H) cholecalciferol, since tritiated DHT_2 was not available.

The quantification of DHT_2 follows the general scheme previously used by others for vitamin D_3 (Jones, 1977; Dokoh *et al.*, 1979; Horst *et al.*, 1979). With our extraction method we observed a lower extraction rate for (1α, 2α(*n*)-^{3}H) cholecalciferol than that reported by others (Dokoh *et al.*, 1979). Extraction of tachysterols/calciferols with diethylether/methanol was chosen since we aimed to optimally extract all relevant tachysterols/calciferols simultaneously. In spite of a lower extraction rate our final recovery is as high as that of others (Preece *et al.*, 1974; Eisman *et al.*, 1976; Morris & Peacock, 1976; Jones, 1977; Skinner & Wills, 1977; Björkhem & Larson, 1978; Clemens *et al.*, 1979; Dokoh *et al.*, 1979; Horst *et al.*, 1979; Bouillon *et al.*, 1980).

Since DHT_2 and D_3 are highly apolar sterols an extensive prepurification of serum extracts is essential to obtain adequate specificity. In order to separate DHT_2 from other apolar compounds we introduced a small silica gel column purification (Preece *et al.*, 1974; Skinner & Wills, 1977) which proved a highly efficient and simple technique.

Chromatographic purification of serum extracts on micro silica gel columns separates DHT_2 and D_3 almost completely from their metabolites and removes some interfering lipids. Although DHT_2 is less polar than D_3 and could be more contaminated with lipid, under the conditions used DHT_2 can be identified in the HPLC chromatogram because there is no aspecific absorption at the DHT_2 position. Hence, it is not necessary to stretch the chromatogram using a less polar eluent. Consequently, the time taken for elution of DHT_2 and D_3 and subsequent rinsing is no longer than that taken for D_3 quantification alone.

Since the molar extinction coefficient of DHT_2 ($\varepsilon_{251\,nm} = 39\,200$; Lawson & Bell, 1974) is about two times higher than that of D_3 ($\varepsilon_{251\,nm} = 17\,000$; personal observation) it is not surprising that the sensitivity of the DHT_2 quantification procedure exceeds the sensitivity of similar D_3 procedures (Jones, 1977; Horst *et al.*, 1979).

The presumption in the quantification of DHT_2 by means of UV monitoring was that recoveries of DHT_2 and D_3 are virtually identical. This presumption has been affirmed (Fig. 2) by the recovery of DHT_2 added to serum ($n = 11$; $R = 0{\cdot}998$; slope = 1·04). Serum extracts without DHT_2 do not show peaks on the DHT_2 position in HPLC chromatograms. This confirms the reliability of the method for clinical use. With reverse phase columns, which should increase recovery, the peaks of DHT_2 and D_3 coincide. Thus it was

not feasible to correlate our results with those obtained using another separation technique.

In patients receiving more than 0·1 mg DHT_2 daily, the serum concentration of DHT_2 rises in proportion to the administered dose and the same trend has been observed by Hallick (Hallick & DeLuca, 1972) using intravenous administered DHT_3.

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