Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 9, pp. 631–637, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00
© 1992 Pergamon Press Ltd

The use of octadecyl-bonded microparticulate silica in the separation of free and bound fractions during saturation analysis of vitamin D metabolites

R.D. COLDWELL,† J. NOLAN,† D.J.H. TRAFFORD,† E.B. MAWER‡ and H.L.J. MAKIN*†

† *Department of Chemical Pathology, The London Hospital Medical College, Turner Street, London E1 2AD, UK*
‡ *Department of Medicine, University of Manchester, Manchester M13 9PT, UK*

Abstract: The use of octadecyl-bonded microparticulate silica to separate free and bound fractions during the saturation analysis of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D has been investigated. A slurry of octadecyl-bonded silica in an appropriate incubation buffer was prepared and used in parallel with a conventional dextran-coated charcoal suspension in several assay procedures. Standard curves, non-specific binding and plasma values were compared. A competitive protein binding assay for 25-hydroxyvitamin D and two radioreceptor assays and one radioimmunoassay for 1,25-dihydroxyvitamin D were investigated. In most cases the octadecyl-bonded silica preparation gave the more favourable results; its action was rapid, time- and temperature-independent, and it produced low non-specific binding and higher B_0 values in all the assays examined. It was in our hands easier to use than dextran-coated charcoal. The use of octadecyl-bonded silica is recommended as an efficient agent for the separation of free and bound fractions in the saturation analysis of vitamin D metabolites.

Keywords: *Octadecyl-bonded microparticulate silica; dextran-coated charcoal; saturation analysis; vitamin D metabolites.*

Introduction

Dextran-coated charcoal, first introduced by Herbert *et al.* [1], is widely used in saturation analyses described for many vitamin D metabolites. Although it is economical and relatively easy to use, in methods developed for steroid hormones it has been reported to be temperature-dependent and to require between 15 and 30 min to exert its effect [2]. In 1985, Steiner *et al.* described the use of an alternative method for the separation of protein-bound and free steroid [3]. This so-called 'hydrophobic interaction chromatography' used microcolumns of octadecyl-bonded silica (ODS-SIL) in the development of sensitive binding assays and in steroid receptor studies. Columns were kept at 0°C, required preconditioning with methanol, water and buffer before use, and one column was required for each separation. Although this method produced sensitive and reproducible results, it was labour-intensive and impractical to employ for large numbers of assay tubes. In this paper the results obtained from a comparative study of the use of ODS-SIL and dextran-coated charcoal (DCC) as separating agents are described, using both as simple suspensions in buffer. This method of using ODS-SIL can be applied to large numbers of samples quickly and easily.

Materials

Standard 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 were gifts from Roche Products Ltd (Welwyn Garden City, Herts, UK). Standards were purified by normal-phase HPLC and their concentration calculated after measuring UV absorbance at 264 nm, using a molar extinction coefficient of 18,200. Tritiated 25-hydroxyvitamin D_3 (25-hydroxy-[26,27-methyl-^{3}H]-cholecalciferol, 130–180 Ci mmol^{-1}) and tritiated 1,25-dihydroxyvitamin D_3 (1α,25-dihydroxy-[26,27-methyl-^{3}H]-cholecalciferol, 130–180 Ci mmol^{-1}) were purchased from Amersham International plc (Amersham, Bucks, UK). Tritiated standards were purified by solid-phase chromatography on Sep-Pak SIL at approximately 4–6 week intervals. Bond-Elut extraction columns (Analytichem International Inc.) were obtained from Anachem (Luton, Beds, UK) and

* Author to whom correspondence should be addressed.

Sep-Pak cartridges (Waters Chromatography Division) were purchased from Millipore (UK) Ltd (Watford, Herts, UK). HPLC was carried out using a WISP 712 sample injector, 590 pump and 990 photodiode array detector (Waters Associates, Northwich, Cheshire, UK) and Zorbax SIL columns from HPLC Technology Ltd (Macclesfield, Cheshire, UK). The calf thymus radioreceptor assay for 1,25-dihydroxyvitamin D was performed using the kit supplied by Incstar Ltd (Wokingham, Berks, UK). Tritium was counted (10 min or 10,000 counts) on a Kontron Betamatic I scintillation counter (Kontron Instruments Ltd, Watford, Herts, UK). Dextran-coated charcoal, unless otherwise indicated, was prepared as 0.05% dextran T20 (Sigma Chemical Co. Ltd, Poole, Dorset, UK)/0.5% activated charcoal in an appropriate buffer. All extraction solvents were AR grade, from BDH Ltd (Poole, Dorset, UK) or May & Baker (Dagenham, Essex, UK). HPLC grade solvents were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). Absolute alcohol (99.86%, v/v) was obtained from Hayman Ltd (Witham, Essex, UK) and was redistilled before use. Glassware to be used during the concentration or drying down of sample extracts was silanized by soaking overnight in 1% dichlorodimethylsilane (BDH Ltd, Poole, Dorset, UK) in toluene (v/v) and rinsed with methanol before use.

Preparation of ODS-SIL for separation of free and bound steroid

ODS-SIL was prepared from prepacked Sep-Pak C18 cartridges or Bond-Elut C18 columns which had been used five times for extraction purposes [e.g. 4]. The ODS-SIL was removed and thoroughly washed with several volumes of methanol, and then the appropriate buffer was gradually incorporated into the suspension until the solvent was 100% buffer. The ODS-SIL slurry thus produced was stored at 4°C, suspending approximately 25 g ODS-SIL in 100 ml buffer.

Methods

Four different assay procedures were compared, one competitive protein binding assay for 25-hydroxyvitamin D (using human plasma vitamin D binding protein) and three assays for 1,25-dihydroxyvitamin D (two radioreceptor assays (one using calf thymus receptor and one chick gut receptor protein) and a radioimmunoassay). Duplicate assays were performed, as per the standard procedures, and either ODS-SIL or dextran-coated charcoal was used to separate bound and free fractions at the end of the incubation period. Standard curves, non-specific binding and plasma values were compared for each method.

In addition, the competitive protein binding assay for 25-hydroxyvitamin D was used to examine the use of dextran-coated charcoal, and to compare its performance with that of ODS-SIL. The effect of incubation temperature and length of incubation time were compared, since dextran-coated charcoal has been reported to 'strip' bound ligand after relatively short periods of incubation.

Method 1: 25-Hydroxyvitamin D by competitive protein binding assay

This is an in-house method developed at LHMC.

Sample preparation. Approximately 2000 cpm [^{3}H] 25-hydroxyvitamin D_3 were added to 0.5 ml plasma or serum and the sample volume was made up to 1 ml with distilled water. After equilibration at room temperature for 30 min, the sample was extracted with an equal volume of acetonitrile, vortex mixed and centrifuged at 1000 ***g*** for 10 min. The supernatant was made 65% aqueous with 0.4 M phosphate buffer, pH 10.5, and applied to a Bond-Elut C18 cartridge which had been preconditioned with methanol and water. After washing the cartridge with water and methanol–water (60:40, v/v), vitamin D and its metabolites were eluted with methanol. The extract was dried down in a vacuum oven at 37°C and reconstituted in isopropanol–hexane (1:99, v/v) before being applied to a silica Sep-Pak which had been washed with both methanol and isopropanol–hexane (1:99, v/v). The silica Sep-Pak was washed with a further 10 ml isopropanol–hexane (1:99, v/v) to remove vitamin D, cholesterol and other contaminants. 25-Hydroxyvitamin D was then eluted with 10 ml isopropanol–hexane (3:97, v/v) and the solvent removed prior to competitive protein binding assay.

Competitive protein binding assay. The dried sample extract was redissolved in 500 µl redistilled ethanol. Percentage recovery of the analyte through the extraction procedure was assessed at this stage by measuring the radio-

label recovered in 250 μl of the ethanol extract. Duplicate 50 μl aliquots were also taken for competitive protein binding assay. The binding protein used in this assay procedure is vitamin D binding globulin (VDBG), the plasma transport protein for vitamin D metabolites. Thus normal human plasma was used as the source of binding protein, after dilution (1/15,000) with 0.1 M Tris buffer, pH 9.0, containing 0.01% gelatin. Assay tubes therefore contained: 50 μl standard, sample or redistilled ethanol, 5000 cpm [^{3}H] 25-hydroxyvitamin D_3 (in 50 μl redistilled ethanol), and 1 ml diluted human plasma (or buffer for non-specific blanks). After 90 minutes at 4°C, the free fraction was separated by the addition of 500 μl of either OD-SIL or DCC suspension. After mixing and standing at 4°C for 15 min, the tubes were centrifuged for 10 min at 1000 ***g***. Aliquots (1 ml) of supernatant were taken for counting.

Method 2: 1,25-Dihydroxyvitamin D_3 by chick gut cytosol receptor assay

Based on France and Lalor [5], with a modified extraction procedure.

Sample preparation. Approximately 1000 cpm [^{3}H] 1,25-dihydroxyvitamin D_3 were added to 2 ml plasma or serum and equilibrated at room temperature for 30 min. The sample was extracted with 2 ml acetonitrile, vortex mixed and centrifuged at 100 ***g*** for 10 min. The supernatant produced was made 60% aqueous with 0.4 M phosphate buffer, pH 10.5, before being applied to a prewashed Bond-Elut C18 cartridge. After washing with water and methanol–water (60:40, v/v) vitamin D and its metabolites were eluted with methanol. The extract produced was dried down and applied to a silica Sep-Pak as described in Method 1. After washing with 10 ml isopropanol–hexane (1:99, v/v) and 10 ml isopropanol–hexane (3:97, v/v), polyhydroxylated vitamin D metabolites, including 1,25-dihydroxyvitamin D, were eluted with 10 ml isopropanol–hexane (30:70, v/v). This fraction was purified further by normal-phase HPLC (5μ Zorbax SIL, 25 × 0.46 cm, mobile phase isopropanol–methanol–hexane (7:3:90, v/v/v) at 1.5 ml min^{-1}, monitoring 264 nm) in order to isolate 1,25-dihydroxyvitamin D_3 from other polar vitamin D metabolites prior to radioreceptor assay.

Chick gut cytosol receptor assay. The solvent was removed from the purified 1,25-dihydroxyvitamin D_3 extract and the sample redissolved in 300 μl redistilled ethanol. An aliquot of 100 μl was counted to calculate the percentage recovery through the extraction and purification procedures. Two 50 μl sample aliquots were used in the radioreceptor assay. A specific 1,25-dihydroxyvitamin D receptor was partially purified from chick duodena and was stored as the lyophylysate at −20°C. Due to avian discrimination against vitamin D_2 metabolites, the chick gut receptor assay measures 1,25-dihydroxyvitamin D_3 only. Assay tubes contained: 50 μl standard, sample or redistilled ethanol, 5000 cpm [^{3}H] 1,25-dihydroxyvitamin D_3 (in 50 μl redistilled ethanol), and 1 ml chick gut cytosol receptor preparation (2.5 mg lyophylysate per ml 0.05 M phosphate buffer, pH 7.4, containing 0.05 M KCl and 1 g l^{-1} dithiothreitol) or 1 ml buffer for non-specific blank tubes. Incubation was at room temperature for 90 min. Assay tubes were cooled to 4°C prior to the addition of 300 μl of either ODS-SIL or DCC. After vortex mixing and standing at 4°C for 15 min, the tubes were centrifuged at 1000 ***g*** for 10 min. Aliquots (1 ml) of supernatant were removed for counting.

Method 3: calf thymus receptor assay kit for 1,25-dihydroxyvitamin D

This method, although supplied in kit form, is based upon that described by Reinhardt *et al.* [6].

Sample preparation. Approximately 1000 cpm [^{3}H] 1,25-dihydroxyvitamin D_3 were added to 1 ml plasma and allowed to equilibrate at room temperature for 30 min. The sample was extracted with 1 ml acetonitrile, vortex mixed and then centrifuged at 1000 ***g*** for 10 min. The supernatant was mixed with 1 ml 0.4 M phosphate buffer, pH 10.5 and applied to a C18OH 'phase switching' cartridge, which had been washed with isopropanol, methanol and water. The cartridge was then washed with a series of solvents: distilled water, methanol–water (60:40, v/v) dichloromethane–hexane (10:90, v/v) and isopropanol–hexane (1:99, v/v). 1,25-dihydroxyvitamin D was finally eluted with 6 ml isopropanol–hexane (3.5:96.5, v/v).

Calf thymus radioreceptor assay. The 1,25-dihydroxyvitamin D extract was dried down in a vacuum oven at 37°C and redissolved in 200 μl ethanol buffer. Aliquots of 50 μl were taken for assessment of recovery and, in duplicate, for assay. The receptor protein preparation supplied with this kit is derived from calf thymus and specifically binds 1,25-dihydroxyvitamin D_2 and 1,25-dihydroxyvitamin D_3 with equal affinity. Assay tubes initially contained 50 μl standard, sample or ethanol buffer, and 400 μl calf thymus receptor in phosphate buffer containing KCl (or 400 μl buffer for non-specific blank tubes). After mixing and incubating for 1 h at room temperature, 5000 cpm [^{3}H] 1,25-dihydroxyvitamin D_3 were added and incubation continued for a further hour. At the end of this time the tubes were cooled to 4°C and 100 μl of either ODS-SIL or dextran-coated charcoal suspension were added. After mixing and incubating for 20–30 min the tubes were centrifuged at 1000 *g* for 15 min. The supernatant was decanted and counted for 10 min.

Method 4: radioimmunoassay for 1,25-dihydroxyvitamin D using a monoclonal antibody

This method has been described by Mawer *et al.* [7].

Sample preparation. Recovery tracer was added to 3 ml plasma or serum and after a short incubation period the sample was extracted with 3 ml acetonitrile and centrifuged. The supernatant produced was applied to a preconditioned Sep-Pak C18 cartridge, which was washed with methanol–water (60:40, v/v) and then the vitamin D metabolites were eluted with acetonitrile. The vitamin D extract was purified further by normal phase HPLC (Zorbax GOLD, mobile phase isopropanol–methanol–hexane (1:1:28, v/v/v) monitoring at 254 nm). This system separates 1,25-dihydroxyvitamin D_2 from 1,25-dihydroxyvitamin D_3. Both fractions were collected and the 1,25-dihydroxyvitamin D_3 fraction was used for assay.

Radioimmunoassay. The 1,25-dihydroxyvitamin D_3 extract was dried down under nitrogen and redissolved in 100 μl ethanol. Samples of 20 μl were taken for assay and recovery through the extraction procedure was also assessed at this stage. The monoclonal antibody used in this assay (5F2), has equal affinity for both 1,25-dihydroxyvitamin D_2 and 1,25-dihydroxyvitamin D_3 and was produced as described by Mawer *et al.* [8]. Assay tubes contained: 20 μl standard, sample or ethanol, 100 μl 0.07 M phosphate buffer, pH 6.0, containing 0.1% gelatin, 5000 cpm [^{3}H] 1,25-dihydroxyvitamin D_3 (in 10 μl ethanol), and 500 μl monoclonal antibody 5F2 in 0.07 M phosphate buffer, pH 6.0, containing 0.1% gelatin (or heat-treated plasma in buffer for blank tubes). Tubes were incubated for 17–24 h at 4°C, after which 100 μl of either ODS-SIL (50 g per 100 ml buffer) or dextran coated charcoal (0.15% dextran, 1.5% charcoal) were added, mixed and incubated for 15 min. Aliquots of 500 μl supernatant were taken for counting.

Results

The shape of standard curves obtained using ODS-SIL and dextran-coated charcoal as separating agents were essentially similar for each of the four assays compared although there

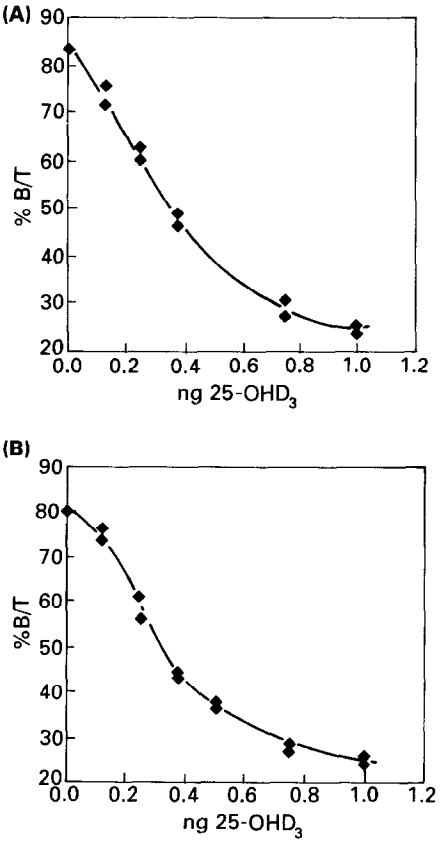

Figure 1
Standard curves for the competitive protein binding assay for 25-hydroxyvitamin D using (A) ODS-SIL and (B) dextran-coated charcoal as separating agents.

were slight differences in initial binding, ODS-SIL producing higher B_0 values. An example of the two curves produced using the competitive protein binding assay for 25-hydroxyvitamin D is shown in Fig. 1. Non-specific binding data obtained using both separating agents for the four different assays are summarized in Table 1, and Fig. 2 shows comparisons of the plasma–serum values produced using both methods of separation for all four assays.

Table 1
Non-specific binding data obtained using ODS-SIL and DCC as separating agents, a comparison of four assays for vitamin D metabolites

NSB %B/T	ODS-SIL	DCC
25-OH CPBA	6.5	10.0
$1,25(OH)_2D_3$	9.5	10.3
Chick gut receptor $1,25(OH)_2D$	10.2	6.7
Calf thymus receptor $1,25(OH)_2D$ RIA	3.0	1.7

The effect of varying the temperature of the separation stage was also investigated. Standard curves were prepared for the competitive protein binding assay for 25-hydroxyvitamin D and separation of free and bound ligand was carried out at 4, 15°C and room temperature (22°C). Figure 3 shows the B_0 (%B/T) values obtained using both ODS-SIL and dextran-coated charcoal at these different temperatures. The use of ODS-SIL produces a higher B_0 value than dextran coated charcoal which may be due to a closer approach to equilibrium or to 'stripping' by DCC. The ODS-SIL B_0 also deteriorates more slowly than the DCC value as the incubation temperature increases. The differences between the maximum standard (1 ng) and B_0 (i.e. the drop of %B/T over the range of the curve) produced at the three incubation temperatures by both separating agents are also shown. The effect of contact time on B_0 and the drop of the curve using both ODS-SIL and dextran-coated charcoal was also compared at 4°C. These results are pre-

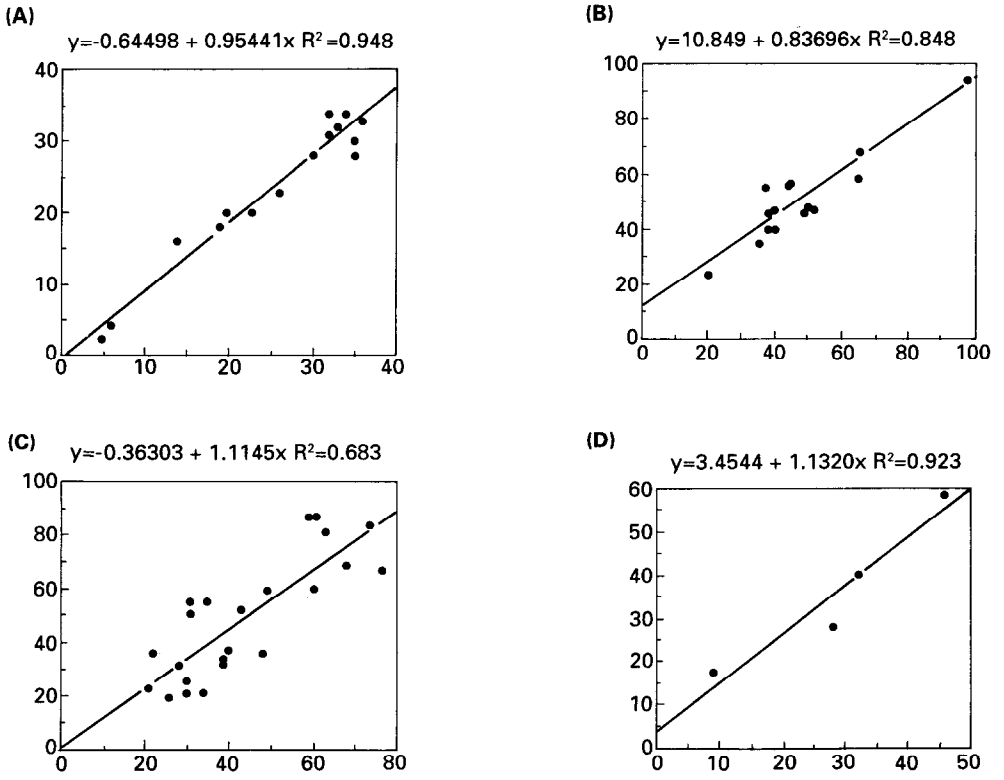

Figure 2
Comparison of plasma values obtained from duplicate samples measured using (A) competitive protein binding assay for 25-hydroxyvitamin D, (B) chick gut cytosol receptor assay for 1α,25-dihydroxyvitamin D, (C) calf thymus receptor assay for 1α,25-dihydroxyvitamin D or (D) radioimmunoassay for 1α,25-dihydroxyvitamin D and either ODS-SIL (y-axis) or dextran-coated charcoal (x-axis). Units are ng ml^{-1} (A) or pg ml^{-1} (B) (C) and (D). The equation of the regression line is shown above each graph. (R^2, the square of the correlation coefficient.)

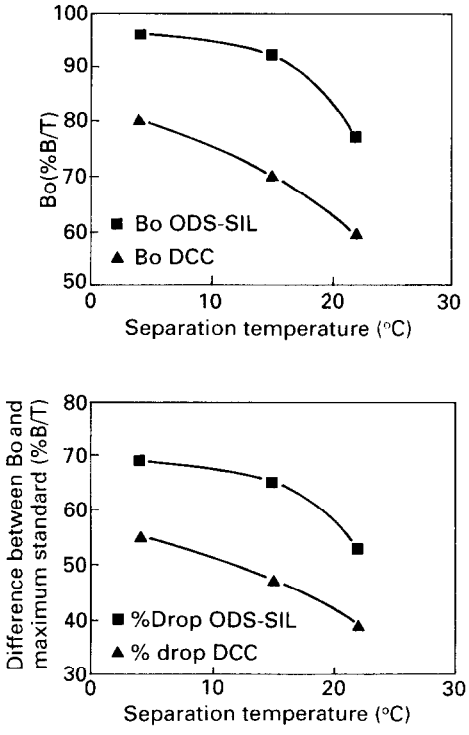

Figure 3
Effect of temperature on the separation of free and bound ligand by ODS-SIL and dextran-coated charcoal during the assay of 25-hydroxyvitamin D (Method 1).

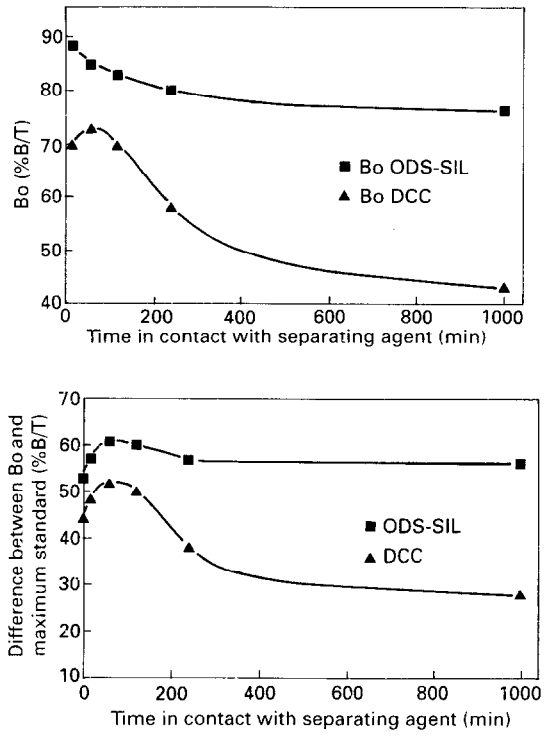

Figure 4
Effect of contact time on the separation of free and bound ligand by ODS-SIL and dextran-coated charcoal during the assay of 25-hydroxyvitamin D (Method 1).

sented out of interest only in Fig. 4, as it is unrealistic that anyone would incubate with separating agent for 200 min. An increased 'stripping' effect is seen using dextran-coated charcoal, but is much less than that reported for steroid hormone assays. This surprising stability of vitamin D assays to dextran-coated charcoal is worthy of comment.

The data presented here have been confined to assays used for the measurement of vitamin D and its metabolites. However similar comparisons have been carried out on two immunoassays, one for testosterone after extraction from plasma (Dr M.J. Wheeler, St Thomas' Hospital, London SE1, UK) and one for cortisol, a direct assay on serum (Farmos kit, Pharmacia Ltd, Milton Keynes, Beds, UK). Comparison of results obtained using polyethylene glycol and second antibody for testosterone with those obtained using ODS-SIL gave a straight line, y(ODS-SIL) = 0.98 × (PEG) − 0.04 (correlation coefficient 0.98). Comparison of the results using polyethylene glycol for cortisol with those obtained using ODS-SIL also gave a straight line y(ODS-SIL) = 0.85 × (PEG) + 25.7 (correlation coefficient = 0.99). This data is presented to indicate that ODS-SIL can also be of value in the saturation analysis of steroids other than vitamin D and its metabolites.

Discussion

Octadecyl-bonded silica has been shown to be an efficient agent in the separation of bound and and free steroid during saturation analysis when used as a slurry. A comparison of the use of ODS-SIL and dextran-coated charcoal during the separation stages of four assays for the measurement of two vitamin D metabolites showed no significant difference in assay results between the two agents. To achieve these results, however, greater precautions were needed using dextran-coated charcoal. Similar standard curves, non-specific binding and plasma–serum concentrations were obtained from all four methods examined, although B_0 values were generally higher using ODS-SIL. Previous reports have suggested that the use of dextran-coated charcoal is sensitive to changes in temperature. It has also been reported that dextran-coated charcoal will begin to 'strip' bound ligand as incubation time increases, so that assays may have to be staggered to allow precise time-keeping. The

results presented here do not seem to support these views, although a deterioration in the performance of dextran-coated charcoal was noticed after excessive incubation times during the separation stage. The use of ODS-SIL produced greater zero binding in all the assays studied, and caused substantially less deterioration of the curve during the incubation time and temperature experiments. Even so, these improvements might not be sufficient to justify the use of ODS-SIL rather than dextran-coated charcoal in laboratories where solid-phase extraction cartridges were not in regular use. In our experience, however, ODS-SIL was preferred to dextran-coated charcoal for several practical reasons. It was easier to handle than dextran-coated charcoal and the slurry could be stored in the appropriate buffer at 4°C for an unlimited time. Separation of bound and free ligand appeared to be instantaneous, and time- and temperature-independent. ODS-SIL formed a heavy precipitate which produced a clean and discrete pellet upon centrifugation. In our hands dextran-coated charcoal regularly produced a scum on top of the supernatant, and the pellet did not appear to be as stable as that produced using ODS-SIL. Our laboratory now uses ODS-SIL as the separating agent of choice in its routine competitive protein binding assay for 25-hydroxyvitamin D.

Acknowledgements — We should like to thank Mrs P. Still for her assistance in the use of the monoclonal immunoassay for 1,25-dihydroxyvitamin D.

References

[1] V. Herbert, K.-S. Lau, C.W. Gottlieb and S.J. Bleicher, *J. Clin. Endocrinol. Metab.* **25**, 1375–1384 (1965).
[2] E.J. Peck and J.H. Clark, *Endocrinology* **101**, 1034–1043 (1987).
[3] Th. Steiner, G.E. Theintz, P.C. Sizonenko and W.L. Herrmann, *J. Steroid Biochem.* **23**, 955–965 (1985).
[4] R.D. Coldwell, D.J.H. Trafford, M.J. Varley, H.L.J. Makin and D.N. Kirk, *Biomed. Environ. Mass Spectrom.* **16**, 81–85 (1988).
[5] M.W. France and B. Lalor, *Irish J. Med. Sci.* **150**, 310–313 (1981).
[6] T.A. Reinhardt, R.L. Horst, J.W. Orf and B.W. Hollis, *J. Clin. Endocrinol. Metab.* **58**, 91–98 (1984).
[7] E.B. Mawer, J.L. Berry, J.P. Cundall, P.E. Still and A. White, *Clin. Chim. Acta* **190**, 199–209 (1990).
[8] E.B. Mawer, J.L. Berry, J. Bessone, S. Shany, H. Smith and A. White, *Steroids* **46**, 741–748 (1985).

[*Received for review 3 February 1992; revised manuscript received 4 June 1992*]