Gel-permeation high-performance liquid chromatography as a powerful technique for rapid analysis of calcitriol (1,25-dihydroxycholecalciferol) receptors

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(Received 2 August 1982/Accepted 21 September 1982)

We used gel-permeation high-performance liquid chromatography to detect cytosolic and nuclear calcitriol (1,25-dihydroxycholecalciferol) receptors. This new convenient technique allowed accurate separation of the calcitriol receptor from other vitamin D-binding proteins. Compared with sucrose-density-gradient centrifugation, it has several advantages including, in particular, its rapidity.

Calcitriol (1,25-dihydroxycholecalciferol) is the most active metabolite of calcidiol (cholecalciferol; vitamin D₃), at least in relation to intestinal Ca²⁺ absorption (Norman, 1979; Fraser, 1981). It is now considered as the hormonal form of calcidiol and has a mechanism of action similar to that of other steroid hormones (DeLuca, 1980). In its target cells, calcitriol binds to cytosolic and nuclear receptors. One of the results is synthesis *de novo* of specific mRNA coding for vitamin D-dependent Ca²⁺-binding protein (Wasserman *et al.*, 1978). The main target organs for calcitriol are intestine, kidney and bone, but recently several other organs have been reported to contain both calcitriol receptors and Ca²⁺-binding protein (Franceschi *et al.*, 1981; Narbaitz *et al.*, 1981; Wasserman & Fullmer, 1982; Delorme *et al.*, 1982). Calcitriol therefore increasingly emerges as a ubiquitous hormone.

To date, the biochemical methods used to demonstrate the presence of specific calcitriol receptors are mainly sucrose-density-gradient ultracentrifugation and DNA–cellulose chromatography (Kream & DeLuca, 1977; Pike & Haussler, 1979; Christakos & Norman, 1981; Coty *et al.*, 1981; Danan *et al.*, 1981). However, these techniques are time-consuming and can only process a limited number of samples. It was thus of interest to look for a faster, more practical method for identifying specific calcitriol receptors.

Recent progress in the field of g.p.h.p.l.c. of proteins prompted us to test the potency of this technique as a tool for detecting calcitriol receptors.

Abbreviations used: g.p.h.p.l.c., gel-permeation high-performance liquid chromatography.

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Materials and methods

Normal 5 week-old Warren chickens were obtained from Ruvel (France). [26,27-³H]Calcidiol (25-hydroxy[26,27-³H]cholecalciferol; sp. radioactivity, 24 Ci/mmol) and [23,24-³H]calcitriol (sp. radioactivity 90 Ci/mmol) were purchased from Amersham (France). Unlabelled calcidiol was a gift from Roussel Uclaf (France) and 24-hydroxy-calcidiol and calcitriol were kindly supplied by Hoffmann La Roche (Switzerland).

Tissue preparation

Animals were decapitated and bled. For cytosolic preparations, the duodenal loop was extracted, washed three times with 10 ml of ice-cold Tris/KCl buffer (0.01 M-Tris/HCl, pH 7.4, 0.3 M-KCl, 0.0015 M-EDTA, 0.005 M-dithiothreitol and 0.01 M-sodium molybdate). The mucosa was scraped and suspended in Tris/KCl buffer (1:5, w/v). After centrifugation at 800 g for 10 min at 4°C, the pellet was collected and washed another three times under the same conditions. The mucosa was then homogenized in 3 vol. of cold Tris/KCl buffer containing 0.23 unit of proteinase inhibitor (Aprotin from Sigma)/ml by three passes in a Potter–Elvehjem homogenizer. The homogenate was recentrifuged at 9000 g for 15 min at 4°C and the resulting supernatant was re-centrifuged at 120 000 g for 60 min. The final high-speed supernatant without the fluffy layer was taken as cytosol.

To prepare the nuclei, Tris/KCl buffer was replaced for washes and homogenization by 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.0015 M-EDTA and 0.005 M-dithiothreitol. Homogenates

were filtered through a gauze and centrifuged at 1000g for 10 min at 4°C. The pellet was collected and further washed three times in the same manner. The 0.3 M nuclear extract was obtained by trituration of the nuclear pellet in Tris/KCl buffer and subsequent centrifugation at 40 000 g for 45 min at 4°C.

Samples were kept frozen at -80°C in 1 ml fractions.

To limit lipid adsorption to the columns, (NH₄)₂SO₄ fractionation was performed by adding 100% saturated ice-cold (NH₄)₂SO₄ to the 1 ml cytosolic preparation to a final concentration of 38% saturation. After 20 min equilibration, the suspension was centrifuged at 9000 g for 10 min at 4°C. The precipitate was redissolved in 0.5 ml of Tris/KCl buffer yielding a fraction termed 'resolubilized cytosol'. Preparations were incubated in 0.5 ml of Tris/KCl buffer containing 1–4 nM of the appropriate ³H-labelled sterol for 20 min at 4°C, and for a further 20 min at 25°C. To study sterol binding, 30–60 µl portions of the incubation mixture were submitted to g.p.h.p.l.c., and 200 µl portions concomitantly underwent sucrose-density-gradient analysis.

G.p.h.p.l.c.

G.p.h.p.l.c. was performed through a Waters I125 gel-exclusion h.p.l.c. column equipped with a Waters 6000A solvent pump and a Waters U6K injector. The temperature of the column was lowered to 4°C by an outside refrigerated water circuit. The 0.1 M-Tris/acetate buffer, pH 7, containing 0.15 M-NaCl was kept in ice and run at a flow rate of 0.4 ml/min; 45 fractions (0.4 ml each) were routinely collected and, after addition of 10 ml of Picofluor 15 (Packard), were counted for radioactivity. Bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* purchased from Boehringer were used as molecular-weight markers for calibration of the column. Sample elution was monitored by u.v. absorbance at 280 nm. After each set of experiments, care was taken to wash the column, first with water to eliminate halide ions, and then with methanol, to desorb the lipids from the column.

Other methods

Sucrose-density-gradient analysis (with a 10–30% sucrose gradient) was performed as described in detail previously (Danan *et al.*, 1981).

Protein contents of the samples were determined by a modified version of Lowry's method (Bensadoun & Weinstein, 1976).

Results

G.p.h.p.l.c. analysis of calcitriol-binding proteins from chick duodenal mucosa

Resolubilized cytosol obtained from duodenal mucosa of normal chicken was incubated with 1 nM-[³H]calcitriol as described in the Materials and

methods section. The incubation mixture was then fractionated by g.p.h.p.l.c. Fig. 1(a) shows that the radioactivity eluted as a single radioactive peak at fractions 20–21. When incubations with [³H]calcitriol were performed in the presence of unlabelled calcitriol (500 nM), the radioactive peak greatly decreased. Such a decline was not observed when incubations were performed in the presence of unlabelled calcidiol or 24-hydroxycalcidiol (both at 500 nM). This indicated that a macromolecule present in the resolubilized cytosol specifically bound calcitriol.

Parallel sucrose-density-gradient analysis of the resolubilized cytosol incubated with 1 nM-[³H]calcitriol confirmed the existence of a specific calcitriol-binding protein sedimenting at 3.3 S (Fig. 1b), generally considered to be the calcitriol receptor (Franceschi *et al.*, 1981).

When the resolubilized cytosol was incubated with 4 nM-[³H]calcidiol, a small radioactive peak was detected by g.p.h.p.l.c. in fraction 15 (Fig. 1c). This peak greatly declined when incubations with [³H]calcidiol were performed in the presence of 1 µM unlabelled calcidiol. Sucrose-density-gradient analysis of the resolubilized cytosol incubated with [³H]calcidiol revealed the presence of a radioactive peak sedimenting at 5.5 S (result not shown), corresponding to the 5.5 S vitamin D-binding protein-actin complex (Van Baelen *et al.*, 1980).

Fig. 1(d) shows the radioactive profile of native cytosol incubated with either [³H]calcitriol or [³H]calcidiol. The protein binding calcitriol and the protein binding calcidiol eluted at fractions 21 and 15 respectively. These results are in complete agreement with those obtained for the cytosol precipitated with (NH₄)₂SO₄ and resolubilized (Figs. 1a and 1c). (NH₄)₂SO₄ precipitation is thus a useful step that enabled lipid contamination to be avoided and reduced protein loading of the g.p.h.p.l.c. column without affecting the analysis of the specific calcitriol-binding protein.

The 0.3 M-KCl nuclear extracts from chick duodenal mucosa incubated with 1 nM-[³H]calcitriol also displayed the radioactive peak at fraction 21 after g.p.h.p.l.c. analysis (results not shown). These results, together with those already reported for sucrose-density-gradient analysis, confirm the biochemical similarity between the specific calcitriol-binding protein present in cytosol and nucleus of vitamin D target organs.

Note that in all present experiments, removal by charcoal treatment of the excess of free hormone before g.p.h.p.l.c. analysis was not necessary, since [³H]calcitriol and [³H]calcidiol were found to be strongly retained by the column. Only a small fraction of the injected ³H-labelled sterol eluted at fractions 31–32 without interfering with detection of the binding proteins.

Fig. 1. *G.p.h.p.l.c. and sucrose-gradient analysis of calcitriol and calcidiol binding to chicken duodenum cytosol*
 Resolubilized chick duodenum cytosol (250 µl; 1.5 mg of protein) was incubated with 1–4 nM of ^3H -labelled sterol for 30 min at 4°C, and for 20 min at 25°C. A 30 µl portion was injected into the h.p.l.c. column eluted at a flow rate of 0.4 ml/min with 0.1 M-Tris/acetate, pH 7, containing 0.15 M-NaCl and 0.4 ml fractions were collected. Temperature of the column was kept constant at 4°C. Of the remaining 220 µl, 200 µl was submitted to 10–30%-sucrose-density-gradient centrifugation, and 29 fractions were collected from the top of the gradient. (a) G.p.h.p.l.c. analysis of resolubilized chicken duodenum cytosol incubated with 1 nM- ^3H calcitriol either alone (x) or in the presence of 500 nM unlabelled calcitriol (Δ). (b) Sucrose-density-gradient analysis of chicken duodenal resolubilized cytosol, incubated with 1 nM- ^3H calcitriol, either alone (x) or in the presence of 500 nM unlabelled calcitriol (Δ). (c) G.p.h.p.l.c. analysis of resolubilized chicken duodenum cytosol, incubated with 4 nM- ^3H calcidiol, either alone (▲) or in the presence of 500 nM unlabelled calcidiol (○). (d) G.p.h.p.l.c. analysis of 50 µl (0.4 mg of protein) of native cytosol, incubated with 1 nM- ^3H calcitriol (x) or with 4 nM- ^3H calcidiol (▲). Arrows indicate positions in which standard proteins, detected by their u.v. absorbance at 280 nm, eluted from the h.p.l.c. column. [^{14}C]Ovalbumin (OV) and [^{14}C]bovine serum albumin (BSA) were used to calibrate the sucrose gradient. Other abbreviations: chymo., chymotrypsinogen; cyto., cytochrome c.

G.p.h.p.l.c. of plasma vitamin D-binding protein

It was important to test whether g.p.h.p.l.c. was able to discriminate between the specific calcitriol-

binding protein and the native serum vitamin D-binding protein. For this purpose, 1:20-diluted chick plasma was incubated with 4 nM- ^3H calcidiol and

30 μ l portions were submitted to g.p.h.p.l.c. As shown in Fig. 2, the radioactive profile of the g.p.h.p.l.c. indicated the presence of a radioactive peak at fraction 17. This corresponded to the 4S native vitamin D-binding protein, which therefore possessed an elution volume different from that of the specific calcitriol-binding protein.

Relationship between the g.p.h.p.l.c. and sucrose-density analysis

It was of interest to further characterize the calcitriol-binding activity revealed by g.p.h.p.l.c. in fraction 21. The resolubilized cytosol was therefore incubated with 1 nM-[3 H]calcitriol and submitted to g.p.h.p.l.c. A portion (200 μ l) of fraction 21 (corresponding to the top of the radioactive peak) was immediately layered at the top of a 10–30% sucrose density gradient. Gradient analysis revealed the

Fig. 2. *G.p.h.p.l.c. of calcitriol binding to chicken plasma* Diluted (1:20) chicken plasma (250 μ l) was incubated with 4 nM-[3 H]calcitriol for 30 min at 4°C and for 20 min at 25°C, either alone ($\blacktriangle$) or in the presence of 1 μ M unlabelled calcitriol (O). Portions (30 μ l) were analysed by g.p.h.p.l.c. using 0.1 M-Tris/acetate buffer, pH 7, containing 0.15 M-NaCl, and run at a rate of 0.4 ml/min. The temperature of the column was kept constant at 4°C. Fractions were collected and counted for radioactivity. Arrows indicate positions in which standard proteins, detected by their u.v. absorbance, eluted from the column. The abbreviations are defined in the legend to Fig. 1.

Fig. 3. *Sucrose-density-gradient analysis of the calcitriol-binding protein and of the calcitriol-binding protein eluted from the g.p.h.p.l.c. column*

Resolubilized cytosol (150 μ l; 0.9 mg of protein) was incubated with 1 nM-[3 H]calcitriol and submitted to g.p.h.p.l.c. A portion (200 μ l) of fraction 21 eluted from the g.p.h.p.l.c. column was immediately analysed by 10–30% sucrose-density-gradient ultracentrifugation (x). Native cytosol (150 μ l; 1.2 mg of protein) was incubated with 4 nM-[3 H]calcitriol and subjected to g.p.h.p.l.c. A portion (200 μ l) of fraction 15 eluted from the g.p.h.p.l.c. column was immediately analysed by 10–30% sucrose-density-gradient ultracentrifugation ($\blacktriangle$).

presence of a radioactive peak sedimenting at 3.3S (Fig. 3).

In the same type of experiment, in which cytosol was incubated with 4 nM-[3 H]calcitriol instead of [3 H]calcitriol and then fractionated by g.p.h.p.l.c., fraction 15 gave a single radioactive peak sedimenting at 5.5S on sucrose-density-gradient analysis. These two sets of experiments clearly demonstrated that the binding activities present in fractions 15 and 21 respectively corresponded to the 5.5S vitamin D-binding protein-actin complex and to the 3.3S specific calcitriol receptor.

Discussion

Our results clearly show that g.p.h.p.l.c. is a suitable method for characterizing calcitriol recep-

tors in vitamin D target organs. The results for g.p.h.p.l.c. are in complete agreement with those for sucrose-gradient analysis, since, like sucrose-density-gradient ultracentrifugation, g.p.h.p.l.c. accurately separated calcitriol receptors from vitamin D-binding protein and 5.5S vitamin D-binding protein-actin complex. However, g.p.h.p.l.c. has the marked advantage of being faster and extremely reproducible. It allows rapid analysis of calcitriol receptors, as it reduces to 30 min the separation step that formerly required 18 h of centrifugation. This rapidity might minimize proteinase action and dissociation of the [^3H]calcitriol-receptor complex. In addition, an entire experiment, including the killing of the animals, preparation of cytosol or nuclear extracts, incubation with [^3H]calcitriol and g.p.h.p.l.c. analysis can be complemented and the results obtained in a single day. Alternatively, 10 samples can easily be analysed routinely in 1 day.

The powerful potential of h.p.l.c. can now be used to investigate all the steps of vitamin D metabolism and mode of action. First, substantial progress in analysing and isolating many new vitamin D metabolites has been made through the h.p.l.c. technique. In addition we recently used g.p.h.p.l.c. for characterizing intestinal and renal vitamin D-dependent Ca^{2+} -binding proteins. Lastly the present study demonstrates the importance of g.p.h.p.l.c. for analysing the calcitriol receptor in vitamin D target organs.

While the present work was in progress Pavlik *et al.* (1982) also used g.p.h.p.l.c. to detect oestrogen and progesterone receptors. In the near future, g.p.h.p.l.c. will undoubtedly be the ideal technique for steroid receptor analysis in both basic and clinical research.

The skilful assistance of Mrs. C. M. Fattaccini was much appreciated. We are also indebted to Mrs. M. Courat, M. Dreyfus and Mr. P. Schuman for their help in the preparation of this manuscript.

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