

Determination of 1,25-dihydroxyvitamin D Concentrations in Human Colon Tissues and Matched Serum Samples

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Abstract. *Background:* A novel method to measure 1,25-dihydroxyvitamin D (1,25(OH)₂D) in human tissue was developed and validated. The objectives of this study were to determine whether 1,25(OH)₂D is present in human colon tissue and to characterize the relationship between human colon tissue and serum 1,25(OH)₂D concentrations. *Materials and Methods:* Normal colon tissue specimens and matched serum samples were obtained from 30 patients who had undergone colectomy. Colon 1,25(OH)₂D was measured by lipid extraction followed by enzyme immunoassay (EIA). Serum 1,25(OH)₂D and 25-hydroxyvitamin D (25(OH)D) were measured by EIA. Vitamin D binding protein (DBP) was measured in a subset of serum and tissue samples. *Results:* Regression analysis indicated a significant positive correlation between serum and colon 1,25(OH)₂D concentrations ($r=0.58$, $p=0.0008$). The corresponding intercept at zero serum 1,25(OH)₂D was 21.5 pmol/kg (95% CI=16.95-25.98; $p<0.001$). Colon 1,25(OH)₂D did not correlate significantly with serum 25(OH)D. DBP levels in tissue samples were negligible. *Conclusion:* The hormone 1,25(OH)₂D can be successfully detected in human colon at physiologically relevant concentrations, partly determined by serum 1,25(OH)₂D. The results support the notion of *in vivo* synthesis of 1,25(OH)₂D within colon tissues.

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Key Words: Vitamin D, 1,25-dihydroxyvitamin D, colon, colorectal, tissue level, colon cancer, methodology.

Epidemiological data have demonstrated that the risk of colon cancer and other malignancies decreases with increasing exposure to UV-B light and vitamin D (1). A recent meta-analysis indicated that higher circulating 25-hydroxyvitamin D (25(OH)D), the clinical indicator of vitamin D status, was associated with a 33% reduced risk of colorectal cancer (2). Gorham *et al.* have shown that higher serum 25(OH)D (≥ 82 nmol/L) or vitamin D intake (≥ 1000 IU/d) reduced colorectal cancer incidence by 50% (3, 4). In a prospective study of colorectal patients, higher plasma 25(OH)D levels were associated with a 48% reduction in overall mortality (5). A 4-year randomized controlled trial of vitamin D₃ (1100 IU/d) and calcium (1400-1500 mg/d) revealed that vitamin D alone decreased all-cancer risk by up to 77% in post-menopausal women (6).

Several tissues, including the colon, express the vitamin D receptor (VDR) and the vitamin D-activating enzyme CYP27B1 (7-11), which mediate the anti-proliferative effects of 1,25-dihydroxyvitamin D (1,25(OH)₂D), the active vitamin D metabolite. Accordingly, it has been postulated that 1,25(OH)₂D accumulates in various tissues to locally induce growth inhibitory, pro-differentiating and pro-apoptotic actions on cells (12-16). Although tissue 1,25(OH)₂D measurement has been reported in mice (17), no clinical observations in human tissues are available because of the technical difficulty in measuring 1,25(OH)₂D in human tissue. Furthermore, the relationship between tissue and serum levels of vitamin D metabolites has not been evaluated.

We have developed a novel, robust method to measure 1,25(OH)₂D concentration in human tissue. The objectives of this study were to validate the tissue 1,25(OH)₂D extraction method, determine whether 1,25(OH)₂D is present in human colon tissue and characterize the relationship between human colon and serum 1,25(OH)₂D concentrations. Our findings

support the analytical validity of the tissue extraction method and we demonstrate that 1,25(OH)₂D is present in human colon tissue at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D, with some evidence of local colonic synthesis.

Materials and Methods

Extraction and measurement of 1,25(OH)₂D in human colon tissue. Serum and normal colon specimens were obtained from 30 patients who had undergone colectomy surgery. For each patient, resected colonic mucosa and muscularis regions were sampled and pooled for 1,25(OH)₂D analysis. The pooled colon tissue was washed in PBS to remove blood and feces, and 200 mg samples were minced with a scalpel to disrupt connective tissue prior to transfer into borosilicate glass tubes. The vitamin D-containing fraction was extracted using a modification of a previously described method (18). Briefly, tissue samples were reconstituted in distilled water (1 mL) and homogenized in 2:1 methanol:methylene chloride (3.75 mL) at high speed for 5 min using a hand-held homogenizer (PRO 200, PRO Scientific Inc., CT, USA). Next, methylene chloride was added to the mixture (1.25 mL), followed by distilled water (1.25 mL). All samples were vortexed thoroughly after the addition of each reagent. After centrifugation (3500 rpm, 20 min), the lower lipid-containing layer was collected and the remaining protein residue was re-extracted with methylene chloride (1.25 mL). The collected methylene chloride extracts were dried under nitrogen gas and reconstituted in 1,25(OH)₂D-stripped human sera. This sera was prepared by treating pooled human serum with a charcoal-dextran suspension (Sigma Chemicals, Saint Louis, MO, USA), followed by mixing (2 h) and centrifuging (3500 rpm, 20 min) to collect the 1,25(OH)₂D-free supernatant. Absence of artifactual 1,25(OH)₂D in the stripped serum was confirmed by enzyme immunoassay (EIA). Reconstituted tissue extracts were filtered using micro-filtration tubes (Nanosep MF, Pall Corporation, Port Washington, NY, USA) prior to 1,25(OH)₂D assay.

The 1,25(OH)₂D levels in tissue extracts and serum samples were measured by a commonly-used EIA (Immunodiagnostic Systems, Scottsdale, AZ, USA). Serum 25(OH)D was measured by a chemiluminescent immunoassay (DiaSorin LIAISON, Stillwater, MN, USA). Vitamin D binding protein (DBP) was measured in a subset of patient serum (n=13) and colon homogenates (mucosa, n=5; muscularis, n=8) by ELISA (Immunodiagnostik, Bensheim, Germany).

Validation of human colon 1,25(OH)₂D assay. As there is no reference method or material for 1,25(OH)₂D measurement in human tissue, validation experiments were conducted to evaluate the ability of the extraction method to accurately and reproducibly extract 1,25(OH)₂D from a tissue matrix. Bovine muscle tissue (200 mg) was homogenized (PRO 200), spiked and left to incubate (1 h) with either radiolabelled [³H]1 α ,25(OH)₂D₃ (17,940 cpm/mL) dissolved in ethanol, n=10; crystalline 1 α ,25(OH)₂D₃ dissolved in ethanol (5 μ g/mL), n=10; or pooled control human serum containing known, physiological levels of 1,25(OH)₂D (\approx 163 nmol/L), n=10. All samples were extracted as described above and 1,25(OH)₂D was quantified, respectively, by liquid scintillation radioactivity analysis (TRI-CARB 2900TR Liquid Scintillation Analyzer; PerkinElmer, Shelton, CT, USA); HPLC (column: Zorbax SIL [5 μ m particles, 4.6 mm i.d., 25 cm length; Agilent, Mississauga, ON, Canada], mobile phase: hexane:isopropanol (9:1)) and EIA (Immunodiagnostic Systems). The extraction

methodology was also tested on unspiked neat human serum (200 μ L) in place of tissue with EIA quantification.

EIA measurement confirmed the absence of artifactual 1,25(OH)₂D in the bovine muscle tissue and stripped serum used for method development. Results of the various quantification platforms were used to calculate the % recovery of added 1,25(OH)₂D achieved by our extraction method. A subset of colon and serum samples, as well as the stripped serum, was assayed multiple times to determine the method's precision.

Statistical analyses. Colon 1,25(OH)₂D and DBP values are expressed as pmol/kg and nmol/kg (wet tissue weight), respectively. Serum 1,25(OH)₂D and DBP values are expressed as pmol/L and nmol/L, respectively. The concentrations of free 1,25(OH)₂D were calculated as the ratio between the molar concentrations of 1,25(OH)₂D and DBP ("free 1,25(OH)₂D index") as described previously (19). All data were analyzed by SPSS software (version 13.0) and graphs were created using GraphPad Prism.

Results

Validation of human colon 1,25(OH)₂D assay. Table I shows the 1,25(OH)₂D extraction recoveries across the various quantification methods used to confirm recovery and analytical validity of the tissue extraction procedure. The results indicated that the extraction method consistently yielded excellent recovery of 1,25(OH)₂D (mean: 100.1 \pm 6.5 %). The tissue 1,25(OH)₂D assay was reproducible as the within-assay variation (mean: 6.7 \pm 2.4%) was comparable to those reported by the kit manufacturer (3-5%) and other immunoassays (Table II). Taken together, these results demonstrated that the developed tissue extraction method was suitable for assay of 1,25(OH)₂D in human colon.

Human colon and serum vitamin D metabolite levels. Figure 1 shows the relationship between human colon and serum levels of 1,25(OH)₂D. Colon tissue 1,25(OH)₂D concentrations correlated significantly with serum 1,25(OH)₂D levels (r=0.58, p<0.001). Regression analysis indicated a significantly positive slope (0.12, 95% CI=0.05-0.18; p<0.001) of colon tissue 1,25(OH)₂D (pmol/kg) with serum 1,25(OH)₂D (pmol/L). The corresponding intercept at zero serum 1,25(OH)₂D was also significantly positive, 21.5 pmol/kg (95% CI=16.95-25.98; p<0.001), suggesting that the colon tissue 1,25(OH)₂D response could not be attributable to serum 1,25(OH)₂D alone.

Serum 1,25(OH)₂D levels did not exceed the upper limit of the normal reference range (200 pmol/L) in any patient. On average, colon 1,25(OH)₂D levels were 50% lower than serum 1,25(OH)₂D concentrations (p<0.001) (Table III). DBP levels in colon were deemed negligible as measured values were below or near the concentration of the lowest calibrator (1717 nmol/L), substantially below the normal reference range of serum DBP (3903-10733 nmol/L), and significantly lower than serum DBP concentrations

Table I. Recovery of 1,25(OH)₂D achieved with tissue extraction procedure followed by confirmatory quantification methods.

Matrix	n	Added 1,25(OH) ₂ D	1,25(OH) ₂ D quantification method	1,25(OH) ₂ D recovery (%±CV)
Bovine muscle tissue	10	None (ethanol vehicle)	EIA	Not detected
Bovine muscle tissue	10	[³ H]1 α ,25(OH) ₂ D ₃ (17,940 cpm/mL)	Liquid scintillation radioactivity analysis	90.5±3.8
Bovine muscle tissue	10	1 α ,25(OH) ₂ D ₃ (5 μ g/mL)	HPLC	104.9±4.1
Bovine muscle tissue	10	Pooled human serum [1,25(OH) ₂ D: ~163 pmol/L]	EIA	101.9±3.4
Serum ^a	10	None	EIA	103.1±10.3

^aSerum samples were extracted with described tissue extraction method, reconstituted in stripped serum, and quantified by enzyme immunoassay (EIA). Results were compared to those obtained using the same serum sample assayed neat (*i.e.* no tissue extraction step) by EIA. CV: coefficient of variation.

Table II. Precision of 1,25(OH)₂D assay.

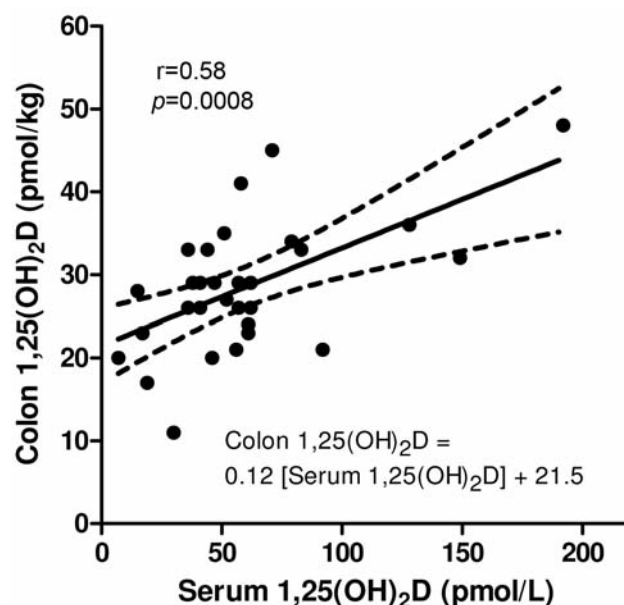
Matrix	n	Mean 1,25(OH) ₂ D (pmol/L)	Within-assay	
			SD	CV%
Bovine muscle tissue	10	Not detected	N/A	N/A
Stripped serum	10	Not detected	N/A	N/A
Colon 1	2	24.5	2.1	8.7
Colon 2	2	28.5	2.1	7.4
Colon 3	2	43.8	1.8	4.0
Serum 1 (neat)	3	44.0	3.6	8.2
Serum 2 (neat)	3	104.3	2.1	2.0
Serum 3 (neat)	3	162.3	9.3	5.7

SD: Standard deviation; CV: coefficient of variation; N/A: not applicable.

($p < 0.001$) (Table III). Furthermore, colon 1,25(OH)₂D levels did not correlate significantly with DBP concentrations separately in colon mucosa ($r = 0.63$, $p = 0.37$), colon muscularis ($r = -0.50$, $p = 0.39$) or both ($r = 0.51$, $p = 0.16$). Colon 1,25(OH)₂D did not correlate significantly with serum 25(OH)D ($r = 0.09$, $p = 0.66$) or serum DBP ($r = -0.14$, $p = 0.71$). The free 1,25(OH)₂D index was significantly higher in colon tissue (2.4 ± 1.0) compared to serum (1.2 ± 0.4) ($p = 0.006$).

Discussion

Evaluations of vitamin D with various health outcomes including cancer have been investigated predominantly in the context of serum levels of vitamin D metabolites. However, these outcomes have not been associated with the tissue levels of vitamin D metabolites. Intracellular concentrations of vitamin D may indeed be more important than serum levels, particularly given the multitude of cells that differentially express the genes that activate (*i.e.* *CYP27B1*) and respond to (*i.e.* *VDR*) vitamin D (7-11). Thus, it has been hypothesized that it is the local accumulation of 1,25(OH)₂D in tissues, not serum *per se*, that drives the biological effects of vitamin D in

Figure 1. Correlation of human colon tissue and serum concentrations of 1,25(OH)₂D.

cell growth regulation and immunomodulation (12-16). However, the direct measurement of 1,25(OH)₂D in human tissue has not been reported. We have developed a novel and robust method to measure 1,25(OH)₂D concentration in human colon tissue.

Our results describe for the first time that the 1,25(OH)₂D hormone can be detected in human colon tissue at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D. Colon tissue 1,25(OH)₂D levels were ~50% of those in serum, and correlated significantly with serum 1,25(OH)₂D as would be expected for a hormone and its target tissue. Although the precise origin of the colonic 1,25(OH)₂D (*i.e.* local synthesis vs. tissue uptake) is difficult to ascertain without the administration of radio-labeled agent, the data also provided evidence of some degree of

Table III. Vitamin D metabolite and DBP measurements in human colon tissue and serum.

Matrix	1,25(OH) ₂ D	DBP	Free 1,25(OH) ₂ D index (×10 ⁵)	25(OH)D
Colon	28±8 pmol/kg	1194±507 nmol/kg ^a	2.4±1.0	Not measured
Serum	63±41 pmol/L	4929±821 nmol/L	1.2±0.4	62±26 nmol/L
P-value (colon vs. serum)	<0.001	<0.001	0.006	N/A

^aMean of 5 colon mucosa and 8 colon muscularis samples. DBP: Vitamin D binding protein.

local 1,25(OH)₂D production within colon tissue *in vivo*. Had the 1,25(OH)₂D detected in colon been simply the result of serum deposition, then one would also expect to detect physiological levels of DBP, the carrier protein of circulating vitamin D metabolites, in the colon. However, colon DBP levels (1194±507 nmol/kg) were negligible as these values were below or near the lower limit of assay sensitivity and far below the normal reference range of serum DBP (3903-10733 nmol/L). Specifically, DBP levels were four-fold lower in colon than serum, and could not account for the two-fold difference in 1,25(OH)₂D concentrations observed between colon and serum. Furthermore, the free 1,25(OH)₂D index was twice as high in colon (2.4±1.0) compared to serum (1.2±0.4), suggesting increased accessibility and physiological activity of 1,25(OH)₂D hormone at the tissue level. This was an important finding as it shows a higher accumulation of active agent at the target organ compared to the circulation. Another indication of local, paracrine production of 1,25(OH)₂D in tissue was the significantly positive intercept of colon 1,25(OH)₂D (21.5 pmol/kg) at zero serum 1,25(OH)₂D, implying a basal level of 1,25(OH)₂D production in the colon independent of serum 1,25(OH)₂D. Although serum 25(OH)D was not associated with colon 1,25(OH)₂D, the low serum 25(OH)D levels (62 nmol/L) in this patient population may have prevented the detection of 1,25(OH)₂D in colon tissue. Only three patients had serum 25(OH)D concentrations greater than 100 nmol/L, the level which is believed to be optimal for extra-renal metabolism and cancer prevention (4, 7, 20). Of note, we have recently completed a Phase II clinical trial of high-dose vitamin D₃ administration to prostate cancer patients (NCT00741364), which will allow us to address such issues in the context of intra-prostate vitamin D metabolism.

The data also demonstrated that our method for measuring 1,25(OH)₂D in tissue is accurate and precise. Despite having developed our protocol independently from Nittke *et al.* (17), both methods are similar in principle in that they employ a double extraction procedure coupled to EIA. However, the recovery of 1,25(OH)₂D achieved by the present method (100.1±6.5%) was higher than that of Nittke *et al.* (85 %) and was confirmed by several quantification platforms. The higher recovery could be attributed to differences in the lipid extraction step and the present use

of 1,25(OH)₂D-stripped serum to reconstitute the tissue extract prior to EIA. Future work should optimize the tissue 1,25(OH)₂D method and render it applicable to other types of tissues and vitamin D metabolites.

This study had some limitations. The small quantity of available colon tissue (~300 mg) necessitated the pooling of colonic tissue regions and restricted the analyses to 1,25(OH)₂D and DBP. Therefore, the 1,25(OH)₂D or other vitamin D metabolites (*e.g.* 25(OH)D) could not be measured separately in colon mucosa and muscularis regions. Regional differences in vitamin D metabolism across various tissue types should be explored in future studies, and will be investigated in our clinical trial of vitamin D₃ in prostate cancer (NCT00741364).

Higher risk of colon cancer has been consistently associated with lower levels of serum 25(OH)D (1-5). A plausible explanation for this relationship is the inadequate local colonic conversion of inactive circulating 25(OH)D into active 1,25(OH)₂D as the colon expresses the vitamin D-activating enzyme CYP27B1. However, direct evidence for this hypothesis has been lacking because there has been no reported method to quantify 1,25(OH)₂D concentration in human colon. The present measurement of tissue 1,25(OH)₂D shows that this hormone can be found in human colon at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D, and also provides preliminary evidence of local synthesis of 1,25(OH)₂D within colon tissue *in vivo*. The colon 1,25(OH)₂D method described here will enable researchers to quantify vitamin D metabolites in accessible human tissue (*e.g.* surgical specimens) and facilitate an enhanced understanding of vitamin D metabolism at the tissue level. Such a tool should stimulate research examining the relationship between tissue vitamin D metabolites and the various health outcomes that are believed to be linked with vitamin D intakes.

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Received September 26, 2011

Revised November 12, 2011

Accepted November 15, 2011