Measurement of 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and estradiol from a single plasma sample

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Introduction

Estrogen and the vitamin D_3 metabolites 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3 or calcitriol) and 25-hydroxyvitamin D_3 (25-OH-D_3), are intimately involved in the regulation of calcium metabolism, particularly in bone and egg calcification (in the laying hen). Current methods for the extraction of estradiol and the vitamin D_3 metabolites require the use of different solvents and separate plasma samples. This is not only inefficient, but may be a problem when only small amounts of blood are obtainable, as in small animals.

For several years we have been interested in possible mechanisms of control of egg calcification by estrogen and the vitamin D metabolites and in due course have developed a relatively simple and inexpensive method for the simultaneous extraction and separation of these compounds from a single plasma sample (1 mL or less) for assay by specific antiserum, protein, or receptor binding methods.

Methods and materials

All solvents were of reagent grade and were used as obtained. 25-hydroxy[26,27-methyl-^{3}H]vitamin D_3 (S.A. 173.5 Ci/mmole) and estradiol-17B(2,4,6,7-^{3}H(N) (S.A. 105 Ci/mmole) were obtained from Amersham (Oakville, Ontario, Canada). These radioactive compounds were used as internal tracers for correction of procedural losses and in radioimmuno/receptor-assays for estrogen and vitamin D metabolites, respectively. The purity of the radioactive steroids was checked by thin layer chromatography before use.

Extraction of estrogen and vitamin D metabolites

Approximately 2,000 dpm (equivalent to 3 to 4 pg) of each of the tritiated compounds, estradiol (in 0.05 mL methanol), 25-OH-D_3 (in 0.05 mL ethanol), and calcitriol (in 0.05 mL ethanol) are added to a 50-mL polypropylene centrifuge tube to serve as internal standards and to correct for procedural losses of each sample. The solvent is evaporated

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under a gentle stream of nitrogen in a water bath at 45° C. One milliliter of plasma (or less depending on the amount of the steroids present) is added, followed by 1 mL (or an equal volume) of 18% NaCl (to promote phase separation), and the mixture is then gently vortexed to ensure uniform mixing of the tracers with the endogenous steroids. Total estrogens (estradiol, estrone, and the corresponding 3-sulfate conjugates) and the vitamin D_3 metabolites are then extracted twice with 4 mL freshly prepared tetrahydrofuran-ethyl acetate (1:1), each time vortexing for 2 min and then centrifuging at 2,000 *g* for 10 min. The upper (organic) phase containing the steroids is transferred to a 5-mL siliconized test tube and evaporated to dryness under a stream of nitrogen. The dry plasma extract can be stored at −20° C for at least 2 weeks before chromatography without evidence of breakdown, based on the recovery of the added tritated standards mentioned above.

Preparation of Sephadex LH-20 column

One gram of Sephadex LH-20 (Pharmacia, Piscataway, NJ, USA) is poured gently through a funnel into a column made from a disposable, 10-mL Pyrex pipette (ID ca 0.9 mm) fitted with a small glass bead. The column is washed initially with 10 mL of methanol and the Sephadex allowed to swell for 1 hour or overnight (for convenience) in 7 mL methanol. Before use, the methanol is allowed to drain and the column is then washed with 20 mL of elution Solvent 1: hexane:methanol:chloroform:conc.ammonium hydroxide (9:1:1:0.01 vol/vol). The column remains useable for several months and gives clean blanks, provided it is well washed with and stored in methanol, as described. For routine analysis, 12 or more such columns can be set up as required.

Separation of estrogens and vitamin D metabolites on column

Immediately prior to chromatography, Solvent 1 is allowed to drain completely. Then the plasma extract obtained above is quantitatively transferred to the column with 3 × 0.1 mL of Solvent 1. After the last bit of liquid has entered the column, 2 mL more of Solvent 1 are added, allowed to drain completely, and the effluent discarded. Another 4 mL are added and the resulting effluent, containing the 25-OH-D_3 fraction, is collected. To elute the calcitriol fraction, 3 additional mL of Solvent 1 are added, discarding the resulting effluent (which contains 24,25-dihydroxyvitamin D_3), then another 7 mL are added, and the effluent containing the calcitriol fraction is collected. As soon as the column stops dripping, 2 mL of Solvent 2 (hexane:methanol:chloroform:conc.ammonium hydroxide (5:5:1:0.01) are added to displace Solvent 1, which is discarded. Then 3 mL more of Solvent 2 are added to elute the estradiol. If required, one can also elute the estrogen 3-sulfates (estrone sulfate and estradiol-3-sulfate) remaining on the column by adding 2 mL of methanol, discarding the effluent, and then adding 3 mL of methanol and collecting the effluent.

Estimation of procedural losses

To correct for procedural losses during extraction and chromatography, the fractions collected are evaporated to dryness under a stream of nitrogen at 45° C. and redissolved in 1 mL ethanol (vitamin D metabolites) or methanol (estrogen). A 0.1 mL aliquot of each fraction is then counted in a scintillation counter to determine the proportion of added counts recovered. The remainder is used for assaying.

Assays

Estradiol levels in this study were determined by radioimmunoassay using a specific antibody described by Robertson et al.[1] Calcitriol and 25-OH-D_3 were measured by specific receptor (Amersham, TRK870) and sheep serum binding protein (Amersham 860) assays, respectively.

Results

Efficiency of extraction

Recoveries of radiolabeled 25-OH-D_3, calcitriol, and estradiol after plasma extraction and column separation ranged from 79 to 96%, with coefficients of variation of 3 to 4% *(Table 1)*.

Table 1 Efficiency of extraction and intra- and inter-assay precision of system

Compound	Recovery of ^{3}H-tracer % (c.v.)	Pooled plasma A Intra-assay precision mean (c.v.) pg/mL	Pooled plasma B Inter-assay precision mean (c.v.) pg/mL
25-OH-D_3	96 (3%)	6695 (2%)	6345 (7%)
Calcitriol	79 (3%)	239 (11%)	196 (14%)
Estradiol	87 (4%)	237 (12%)	253 (6%)

Pooled plasma A and B were obtained at different times, and each was pooled from five or six young White Leghorn laying hens.
c.v.: coefficient of variation based on five or more replicate extractions.

Precision and accuracy of system

The intra-assay precision, based on assay of five independently extracted samples from the same pool of hen plasma ranges from 2 to 12% *(Table 1)*. The inter-assay precision (five assays) varies between 6 and 14% *(Table 1)*. The accuracy of measuring various known amounts of the three steroids added to plasma is shown in *Table 2*. In the case of 25-OH-D_3 and estradiol, the measured value was within 87 to 98% of the expected value (endogenous plus exogenous), whereas in the case of calcitriol there was a tendency toward overestimation by about 13%.

Discussion

The advantage of the present method is that tetrahydrofuran-ethyl acetate (THFE) extracts both the major estrogen and the vitamin D_3 metabolites efficiently. The extraction of 25-OH-D_3, calcitriol, and estradiol is virtually quantitative using 2 $\times$ 4 mL THFE to extract 1 mL of plasma. When a large number of samples is to be processed, one can extract with 4 mL THFE without significantly reducing recovery (approximately 10% less). The extracted steroids are separated on a Sephadex-LH 20 column using relatively simple and inexpensive solvent systems. The individual steroids are then measured by the specific assays. The use of concentrated ammonium hydroxide in the elution solvent is important to prevent hydrolysis of estrone sulfate and estradiol-3-sulfate, which would cause overestimation of free estradiol and estrone. In the laying hen, the concentration of estradiol-3-sulfate in plasma is three to five times that of free estradiol,[2] whereas in human beings,[3] pregnant sheep,[4] and the pregnant cow[5] estrone sulfate can be more than 10 times that of free estrone. The specificity of the method depends mainly on the specificity of the respective antibody, receptor, or binding protein and is further improved by purification, that is, separation using a Sephadex LH-20 column.

During the column separation, estrone is co-eluted with calcitriol. However, estrone does not cross-react significantly with the calcitriol receptor and therefore does not interfere with the measurement of calcitriol. We are not particularly concerned with estrone in our present study because it is relatively inactive in the regulation of calcium metabolism. However, a specific antibody for its measurement is available if there is such a need (e.g., in clinical studies of patients taking estrone sulfate as estrogen replacement therapy). Likewise, if estrogen monosulfates are of interest, tritiated labeled estrone sulfate and/or estradiol-3-sulfate should be added to the plasma prior to extraction to correct for procedural losses. The recovery of the estrogen sulfates should be close to 90%. The estrogen sulfates eluted from the column can be hydrolyzed using 20% acetic acid in methanol,[6] and the free estrone and estradiol released are separated on column as described above.

In the laying hen, plasmal levels of estradiol and calcitriol vary greatly during the laying cycle. However, the time of blood collection in relation to oviposition was not recorded in the present work. Nevertheless, the levels of estradiol determined in this study (231 to 287 pg/mL) are in general agreement with reported values in the literature[7–9] obtained by different methods of extraction and purification. Calcitriol levels (132 to 259 pg/mL) are similar to those reported by Soares and Ottinger[10]

Table 2 Accuracy of method

Compound	Amount added (pg)	Plasma base value (pg/mL)	Expected value (plasma & added) (pg/mL)	Measured value (plasma & added) (pg/mL)	Accuracy %	Accuracy Mean (c.v.)
25-OH-D	4000	6728	10728	9585	89	
	4000	9905	13905	13006	94	
	4000	4332	8332	7631	92	
	4000	6495	10495	10570	101	
	4000	3555	7555	8654	115	98 (10%)
	8000	6728	14728	11483	78	
	8000	9905	17905	11853	83	
	8000	4332	12332	11438	93	
	8000	6495	14495	14224	98	
	8000	3555	11555	9559	83	87 (9%)
Calcitriol	200	239	439	605	138	
	200	259	459	612	133	
	200	134	334	299	90	
	200	281	481	457	95	
	200	132	332	343	103	112 (20%)
	500	239	739	1018	138	
	500	259	759	841	111	
	500	134	634	633	100	
	500	281	781	810	104	
	500	132	632	740	117	114 (13%)
Estradiol	200	255	455	440	97	
	200	386	586	605	103	
	200	244	444	438	99	
	200	387	587	542	92	
	200	231	431	394	91	96 (5%)
	500	255	755	686	91	
	500	386	886	841	95	
	500	244	744	699	94	
	500	387	887	749	84	
	500	231	731	724	99	93 (6%)

Plasma base value means endogenous value that was predetermined or determined simultaneously.
Expected value = sum of plasma base value (3rd column) + amount added (2nd column).

$$\text{Accuracy} = \frac{\text{measured value}}{\text{expected value}} \times 100.$$

c.v.: coefficient of variation.

and Frost and Rolland.[11] The plasma level of 25-OH-D_3 is greatly affected by the dietary concentration of dietary vitamin D_3. Levels of 25-OH-D_3 (3.6 to 9.9 ng/mL) determined by this method are considerably lower than those reported by Abe et al.[12] but are similar to values measured by Tsang and Grunder[13] using acetone nitrile as the extraction solvent and Sep-Pak C_{18} and Silica cartridges for separation by the method of Reinhardt et al.[14] In that study, we found that the plasma level of 25-OH-D_3 increased almost twofold (from approximately 7 to 12 ng/mL) when the dietary concentration of vitamin D_3 was doubled (from 27.5 to 50 μg/kg). The procedures described here should prove useful to researchers who would like to determine estrogens and the vitamin D_3 metabolites in a single plasma or tissue sample.

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