

Technical Note: A Radioimmunoassay for Porcine Retinol Binding Protein

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ABSTRACT: This report describes a RIA for porcine retinol binding protein (RBP) that has been validated for measurement of RBP concentrations as low as 10 ng/mL in serum, follicular fluid, uterine flushings, allantoic fluid, and endometrial and uterine tissue culture medium. The increasing displacement of porcine [¹²⁵I]RBP resulting from decreasing dilutions of each sample was parallel to the standard curve, and exogenous RBP added to samples was accurately measured with the assay. To examine specificity, 1-mL samples of serum, uterine flushings, follicular fluid, and allantoic fluid were subjected to G-100 Sephadex chromatography, and the fractions were

assayed for RBP with the RIA and for fluorescence, which detects retinol bound to RBP. The RBP measured with the RIA and the fluorescent fractions coeluted. Samples of uterine flushings in which RBP was measured using immunoblotting and densitometry were also assayed with the RIA. The measurements using the two methods were correlated ($r = .84$, $P < .01$). These results are evidence that the assay is specific for RBP. The limit of detection of the assay was .46 ng. The RIA should facilitate the study of RBP function and the control of its secretion by various tissues in pigs.

Key Words: Vitamin A, Pregnancy, Uterus, Follicle

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Introduction

Retinol is required for the normal development of many tissue types (Vasois et al., 1989; Bryant and Gardiner, 1992; Covant and Hardy, 1990; Stellmach et al., 1991). Most of the retinol in plasma is bound to retinol binding protein (**RBP**). Several tissues including uterus of neonatal pigs (Vallet et al., 1992), endometrium during pregnancy (Stallings-Mann et al., 1993), and conceptus during pregnancy (Harney et al., 1990; Trout et al., 1991) secrete RBP or RBP-like proteins. The objective of this experiment was to establish a validated RIA for porcine retinol binding protein.

Materials and Methods

For standards, retinol binding protein was purified from allantoic fluid collected from pigs on d 60 of pregnancy (see Vallet, 1993). Generation of the antiserum used for this assay and the description of its specificity by immunoblotting are in Vallet (1993). Briefly, a rabbit was immunized with 100 μ g of

purified RBP in Freund's Complete Adjuvant followed by two booster immunizations of 50 μ g of RBP in Incomplete Freund's Adjuvant at 2-wk intervals. Two weeks after the last immunization, blood was collected and serum was obtained. Two-dimensional PAGE and immunoblotting indicated that the antibody specifically binds at least four isoelectric variants of RBP (not shown). The appropriate dilution of antiserum for RIA was determined from binding assays using a range of dilutions (1:100 to 1:10,000). Antibody dilution at 50% binding was 1:100. The dilution selected (1:1,000) was the greatest dilution that bound sufficient [¹²⁵I]RBP (approximately 13% specific binding) to generate a useful standard curve (nonspecific binding was 6.7%). This dilution is similar to another validated RIA for human RBP (Smith et al., 1970). Porcine RBP (4 μ g in 20 μ L of .5 M phosphate buffer, pH 7) was iodinated with 1 mCi of [¹²⁵I]NaI (in 25 μ L of phosphate buffer) for 60 s using chloramine-T (10 μ L of a 20 mg/mL solution). The iodination reaction was stopped with sodium metabisulfite (50 μ L of a 40 mg/mL solution). The [¹²⁵I]RBP was then mixed with .5 mL of assay buffer (50 mM Tris, pH 8.2, 5% TWEEN-20) and chromatographed on a 10-mL G-50 Sephadex column equilibrated in assay buffer. The [¹²⁵I]RBP was further chromatographed on a .5-cm \times 32-cm Ultrogel Aca54 (Pharmacia, LKB Biotech, Piscataway, NJ)

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column equilibrated in assay buffer. The fraction immediately preceding the peak RBP fraction, the peak fraction, and the two subsequent fractions were combined, aliquoted, and frozen at -70°C until used for the RIA. For RIA, 100 μL of a 1:50 dilution of bovine serum in assay buffer, 100 μL of .5% bovine gamma globulin in assay buffer, either 100 μL of standards (10 to 160 ng/ml) or samples diluted in assay buffer, 100 μL of porcine [^{125}I]RBP (approximately 40,000 cpm; specific activity approximately 25 $\mu\text{Ci}/\mu\text{g}$) in assay buffer, and 100 μL of 1:1,000 rabbit anti-porcine RBP serum in assay buffer were pipetted into 12-mm \times 75-mm borosilicate glass culture tubes. Following an overnight incubation at 4°C , bound RBP was separated from free RBP with .5 mL of 40% polyethylene glycol (PEG 8,000). Tubes were vortexed, incubated on ice for 20 min, then centrifuged at $1,800 \times g$ for 30 min. The supernatants were decanted, and the pellets were dissolved in .5 mL of assay buffer, reprecipitated with 40% PEG, and centrifuged. Bound RBP (i.e., ^{125}I in the pellet) was measured with a gamma counter.

For assay development and validation, allantoic fluid was collected from fetuses from two d-45 pregnant pigs. Blood was collected from two adult female pigs at slaughter, allowed to clot overnight at 4°C , and centrifuged to recover the serum. Cultures were prepared with endometrium collected from two d-60 pregnant pigs and uteri from two pigs that were 14 d of age. Endometrial or uterine tissues were cultured in Minimum Essential Medium modified to contain 5.25 $\mu\text{g}/\text{mL}$ of leucine (adult) or 1.49 $\mu\text{g}/\text{mL}$ of methionine (neonatal) plus 50 μCi of either [^3H]leucine (adult) or [^{35}S]methionine (neonatal) as described previously (Vallet and Christenson, 1993). Uterine flushings were collected from 22 d-12 pregnant pigs. Each uterine horn was flushed with 20 mL of .9% saline. Follicular fluid was collected from two sows 24 to 72 h after weaning.

To validate the RIA, dilutions of allantoic fluid (1:400 to 1:6,400), serum (1:200 to 1:3,200), uterine culture medium (1:4 to 1:32), endometrial culture medium (1:40 to 1:640), uterine flushings (1:1,600 to 1:128,000), and follicular fluid (1:200 to 1:3,200) in assay buffer were tested for parallelism to the standard curve. To determine assay accuracy, RBP ranging from 0 to 8 ng was added to diluted samples of allantoic fluid, serum, uterine culture medium, adult endometrial culture medium, uterine flushings, and follicular fluid, and the samples were assayed.

Vallet (1993) demonstrated that the antiserum used for this RIA was specific for porcine RBP in allantoic fluid and serum. To evaluate assay specificity for porcine RBP, the displacements of [^{125}I]RBP by dilutions of ovine and bovine serum and by 0 to 10 μg of bovine lactoglobulin (Sigma Chemical, St. Louis, MO) were compared to the displacements obtained with dilutions of porcine serum. Also, 1-mL samples of

porcine allantoic fluid, uterine flushings, serum, and follicular fluid were fractionated with G-100 Sephadex chromatography (1.5-cm \times 80-cm column), and the fractions were measured for fluorescence (330 nm excitation, 470 nm emission; Perkin Elmer, LS50, Perkin Elmer, Beaconsfield, Buckinghamshire, U.K.), which is characteristic of retinol bound to RBP, to identify fractions that contained retinol bound to RBP. Fractions were also assayed for RBP using the RIA. Several fluorescent peaks were detected for both serum and follicular fluid samples. To determine whether these peaks were due to the presence of RBP or another fluorescent compound, every other fraction from fraction 20 to 52 was dialyzed against distilled water, lyophilized, and subjected to SDS-PAGE and immunoblotting using anti-human RBP antiserum (Dako, Carpinteria, CA). This antiserum has been used previously for the detection of porcine RBP (Harney et al., 1990) and was used here because it is independent of the reagents used in the assay. Finally, uterine flushings from 22 d-12 pregnant pigs were assayed using both immunoblotting and RIA.

Statistical Analysis

Parallelism of the standard curve and various samples was tested using homogeneity of regression after log transformation of the dilutions of the various samples and standards and logit transformation of the resultant bound counts per minute. Accuracy of measurement of RBP added to the various samples was analyzed using regression analysis. The correlation between measurements of RBP in uterine flushings obtained using the RIA and those obtained using immunoblotting were calculated to compare the two methods.

Results

The increasing displacement of [^{125}I]RBP that resulted from decreasing dilutions of serum, allantoic fluid, uterine culture medium, endometrial culture medium, uterine flushings, and follicular fluid were parallel to the standard curve (Figure 1), giving evidence that different dilutions of each sample were measured accurately. The regression lines comparing RBP added to the various samples vs the amount of RBP measured with the assay were $y = .85x + .32$ ($r^2 = .99$) for serum, $y = .91x + .14$ ($r^2 = .99$) for allantoic fluid, $y = 1.06x + .54$ ($r^2 = .98$) for endometrial culture medium, $y = .97x + .86$ ($r^2 = .97$) for uterine culture medium, $y = 1.0x + 1.4$ ($r^2 = .95$) for uterine flushings, and $y = .99x + .92$ ($r^2 = .94$) for follicular fluid. The slopes of these lines indicate that the assay accurately measured RBP when it was added to samples. Finally, intra- and interassay CV from nine assays of a pool of allantoic fluid measured in duplicate were 11.0 and 10.4%.

Relative to porcine serum, ovine and bovine serum (Figure 2) were only 2 and 7% as effective, respectively, at displacing porcine [125 I]RBP in the RIA (50% displacement). Because serum retinol is similar

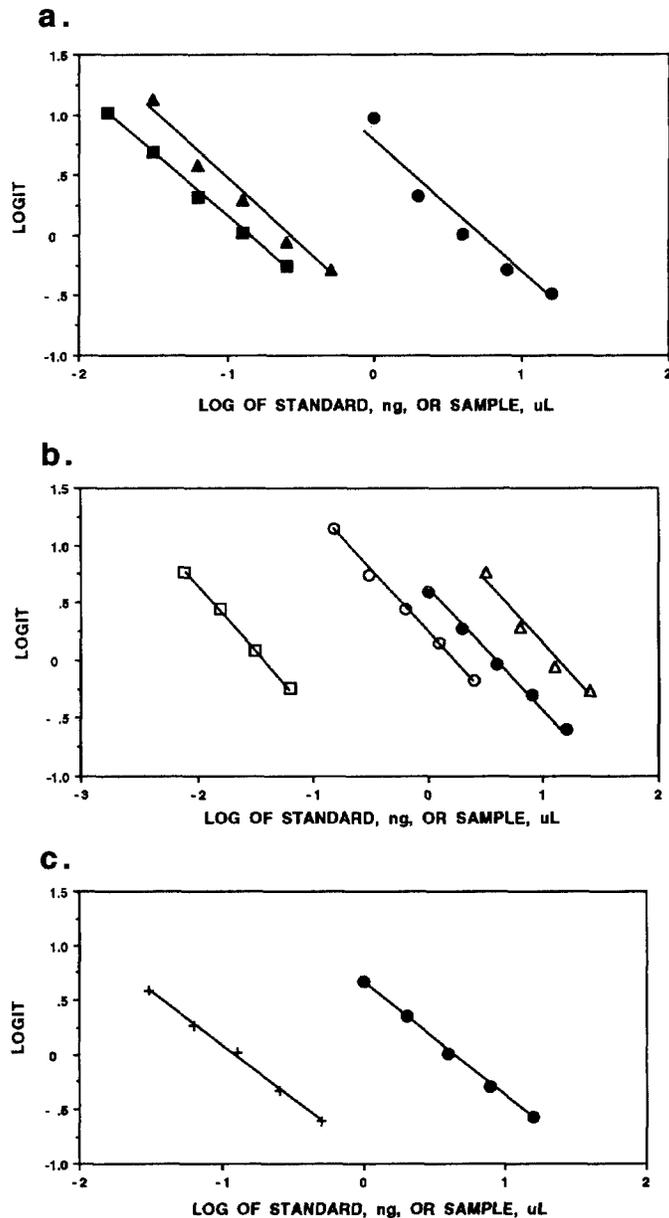


Figure 1. (a) Comparison of the displacement resulting from different amounts of porcine serum (▲) and allantoic fluid (■) with the standard curve (●). (b) Comparison of the displacement resulting from different amounts of medium from culture of endometrium from a d-60 pregnant pig (○), medium from culture of uterus from a 14-d-old pig (△) and uterine flushing from a d-12 pregnant pig (□) with the standard curve (●). (c) Comparison of the displacement resulting from different amounts of porcine follicular fluid (+) with the standard curve (●). Volume of sample or amount of standard was log-transformed, and bound porcine [125 I]retinol binding protein was logit-transformed.

in these three species, these results indicate that the assay is specific for porcine RBP compared with RBP from other species. No displacement of porcine [125 I]RBP with bovine lactoglobulin was detected (data not shown). When 1-mL samples of allantoic fluid and uterine flushings were chromatographed using G-100 Sephadex, fractions that contained RBP as measured with RIA were the same fractions as those that contained fluorescence (Figure 3). When serum and follicular fluid were chromatographed, several fluorescent peaks were detected (Figure 3c,d). Using immunoblotting, RBP was detected only in fractions that contained RBP based on the RIA (Figure 4). Finally, the correlation between RBP measured in uterine flushings using immunoblotting and RBP measured with the RIA was .84 ($n = 35$; $P < .01$). Taken together these results indicate that the assay is specific for RBP.

Mean sensitivity of the assay (measured as the concentration of unlabeled RBP that would result in binding of porcine [125 I]RBP equal to two standard deviations less than the amount of [125 I]RBP bound in the presence of no unlabeled RBP) was .46 ng, which was less than the lowest standard. Because the lowest standard was 1 ng in 100 μ L, the assay is capable of detecting RBP concentrations as low as 10 ng/mL using the standards described here.

Discussion

The results indicate that the RIA for porcine RBP is sufficiently accurate, specific, and sensitive to measure RBP in a variety of biological fluids in which RBP

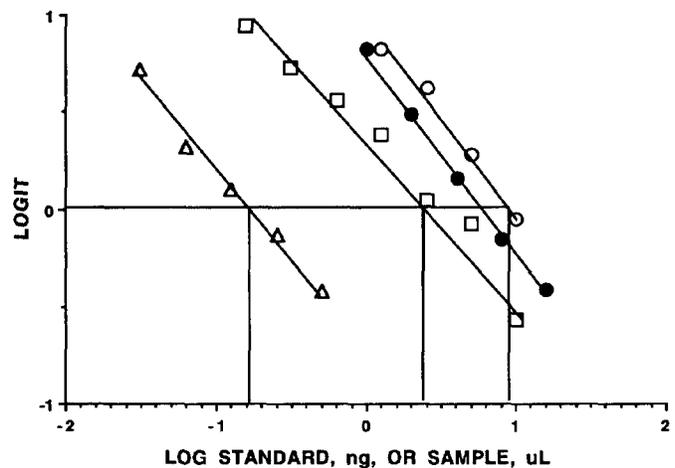


Figure 2. Comparison of the displacement resulting from different amounts of sheep (□), bovine (○), and porcine (△) serum with the standard curve (●). Serum volume or standard amount was log-transformed, and bound [125 I]retinol binding protein was logit-transformed. Lines indicate log dilution at 50% displacement.

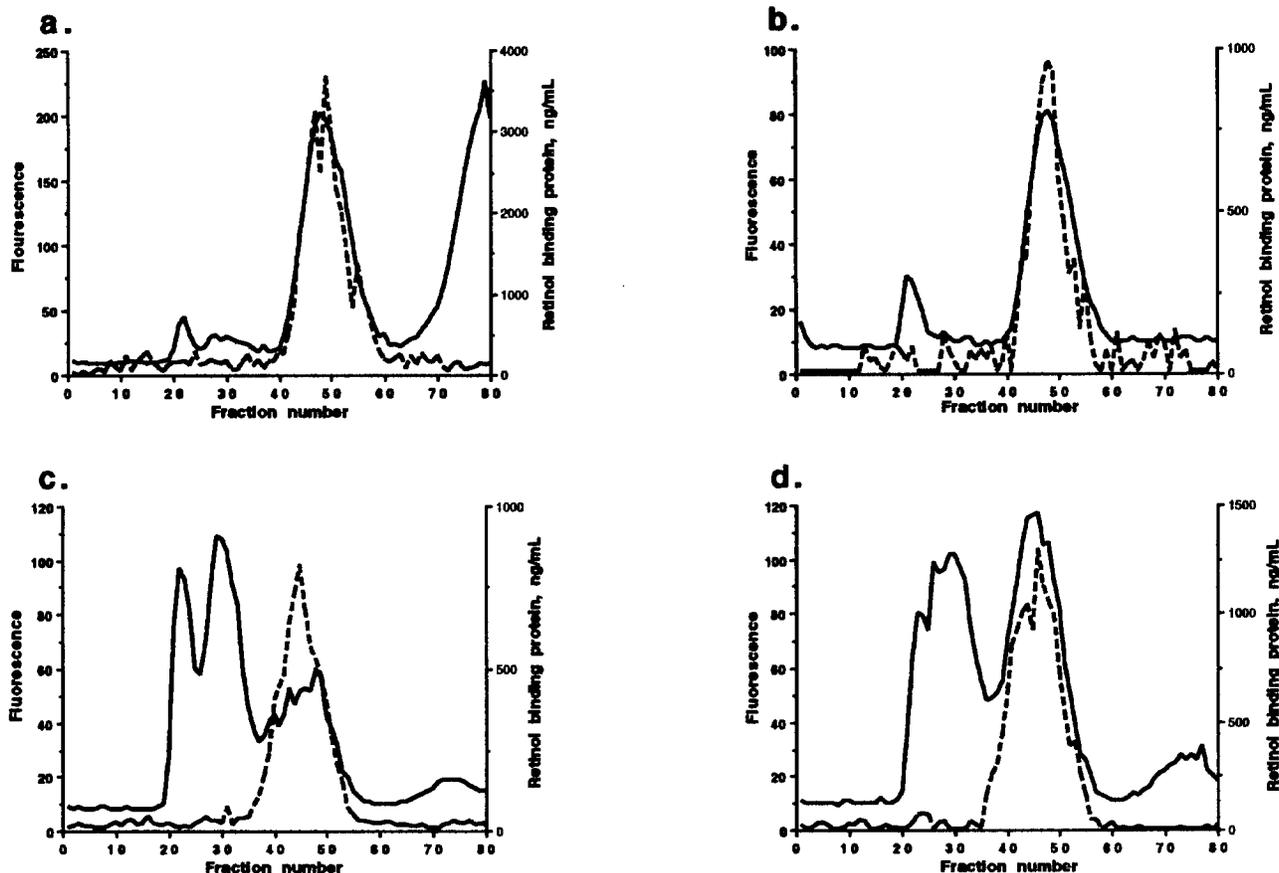


Figure 3. Comparison of the fluorescence (solid line) and RBP values (generated with RIA, dashed line) when 1 mL of allantoic fluid (a), uterine flushings from a d-12 pregnant pig (b), pig serum (c), and porcine follicular fluid (d) were fractionated with G-100 Sephadex chromatography.

is present and will allow study of the control of concentrations of this protein in those fluids.

Ovine and bovine sera were only 2 and 7% as effective as porcine serum, respectively, at displacing porcine [125 I]RBP in the assay. Assuming that the actual concentrations of RBP are similar in these three species, this result indicates that the RIA is specific for porcine RBP. This assumption is logical because retinol concentrations in these three species are similar (Webb et al., 1968; Chew et al., 1984; Bruns and Webb, 1990) and most retinol in plasma is bound to RBP. Bovine lactoglobulin, a protein related to RBP (Ali and Clark, 1988), also did not displace [125 I]RBP in the RIA. The specificity of the assay is surprising because RBP from different species are very similar (Sundelin et al., 1985; Trout et al., 1991). These results make it unlikely that other less-related proteins would cross-react in the RIA.

Several peaks in fluorescence were detected when serum and follicular fluid were chromatographed on G-100 Sephadex. However, using either immunoblotting or RIA, only one fluorescent peak in each chromatogram was determined to be due to RBP. These results provide further evidence that the assay is specific for RBP in each sample.

The results of measurement of RBP in uterine flushings using immunoblotting and the RIA were correlated, confirming that the RIA is specific for RBP. The RIA has several advantages over the immunoblotting technique. These include the following: 1) the RIA measures RBP in micrograms/milliliter of biological fluid, whereas immunoblotting gives only relative concentrations between groups; 2) the RIA is likely to be more accurate than immunoblotting because immunoblotting is a more complicated procedure with numerous steps that may introduce variation into the measurement; and 3) the RIA provides the measurements faster and with less expense than immunoblotting. One disadvantage is that the RIA does not distinguish between RBP bound to retinol and RBP not bound to retinol; however, other methods may be used to determine the degree of saturation of RBP. Another disadvantage is that it is not known whether the assay measures all the isoforms of RBP equally. Several isoelectric variants of RBP are present in neonatal uterine cultures (Vallet et al., 1992), adult endometrial tissue cultures (Stallings-Mann et al., 1993), uterine flushings (Stallings-Mann et al., 1993), and allantoic fluid (Vallet, 1993). The antibody used in this assay seems to bind to the different

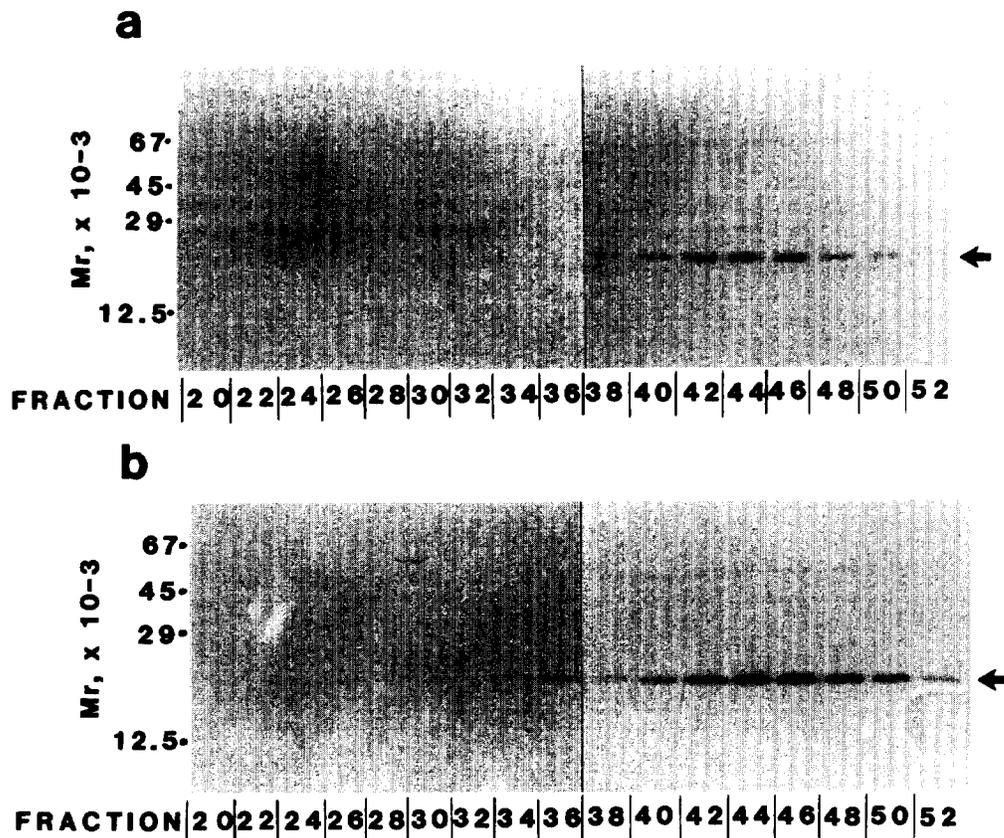


Figure 4. Immunoblots of every other fraction from G-100 Sephadex chromatography of serum (a) and follicular fluid (b) using anti-human retinol binding protein (RBP) antiserum. Arrows indicate the position of porcine RBP. Standards for M_r determination were bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (29,000), and cytochrome C (12,500). Note the lack of the 20,000 M_r band (RBP) in fractions 20 to 36 indicating that the fluorescence in these fractions (Figure 3) is not due to RBP.

isoforms with approximately equal affinity when two-dimensional PAGE gels, which are either stained with Coomassie Brilliant Blue or processed for immunoblotting, are compared (Vallet, 1993). This conclusion is also supported by the fact that parallelism could be demonstrated between dilutions of serum, which seems to contain only a single variant, and the standard curve, which contains all the variants found in allantoic fluid (Vallet, 1993). It is therefore likely that the assay measures each isoform equally; however, a technique that reliably separates the variants of RBP is not available, so this question cannot be satisfactorily addressed. Therefore, results from this assay should be considered immunoreactive RBP. This consideration is similar to that for LH and FSH RIA, where multiple isoelectric variants are present in most samples.

Implications

An assay for porcine retinol binding protein has been developed that can reliably measure the amount

of immunoreactive retinol binding protein in uterine flushings, allantoic fluid, serum, follicular fluid, and endometrial and neonatal uterine tissue culture medium. This assay will be useful in studies of factors that influence secretion of retinol binding protein as well as in experiments to determine the role of retinol binding protein in physiological processes.

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