Direct estimation of oestrone sulphate in sow serum for a rapid pregnancy diagnosis test

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Summary. An antiserum raised against oestrone-3-glucuronide-BSA was used for the direct radioimmunoassay of oestrone sulphate in pig serum. The method has a minimum sensitivity of 0.2 ng/ml and is potentially an accurate pregnancy test in pigs. Serum oestrone sulphate concentrations were ≤ 0.5 ng/ml in non-pregnant cyclic sows and > 0.5 ng/ml in pregnant sows bled 26–29 days after service.

Introduction

Previous work on pigs had shown a marked increase in serum oestrone sulphate concentrations during early pregnancy, and the evidence indicated that this might form the basis of an early pregnancy diagnosis test (Robertson & King, 1974; Robertson, King & Dyck, 1978; Hattersley, Drane, Matthews, Wrathall & Saba, 1980). The procedures used by these authors to estimate oestrone sulphate are too elaborate for routine work, and a simple, rapid method is essential if the estimation of serum oestrone sulphate is to be a practical test for early diagnosis of pregnancy in pigs. This paper describes a method for the direct estimation of oestrone sulphate in pig serum and its application to samples taken 26–29 days after service when the steroid reaches maximum values.

Materials and Methods

Serum samples

Non-pregnant sows. Single blood samples were taken from the ear vein of 21 adult non-pregnant sows. Three of the sows were known to be in oestrus at the time of sampling but the state of the cycle when the other samples were taken is not known. The blood was collected into glass tubes and left at room temperature for 1 h and then at 4°C for 1 h. After centrifugation in the cold the serum was collected and stored at –20°C.

Mated sows. Blood was taken from the ear vein of 94 sows 26–29 days after service. These animals belonged to a commercial pig herd and the blood samples, which were sent by public transport, arrived at the laboratory 1–7 days after being taken. After centrifugation at 4°C, the serum was collected and stored at –20°C. Some of the sera had a definite pink colour indicative of slight haemolysis. Of these 94 sows, 87 subsequently farrowed and were therefore considered pregnant when the serum sample was taken; the 7 sows that returned to oestrus by Day 40 were classified as non-pregnant.

All the sera were assayed for oestrone sulphate.

Reagents

Phosphate-buffered saline pH 7.0 (PBS). This consisted of 0.05 M-phosphate, 0.9% (w/v) sodium chloride, 0.05% gelatin and 0.1% sodium azide.

Dry charcoal-agar disks. These were prepared by a modification of the procedure described by Boarer & Hattersley (1978). Wet disks with a diameter of 1.5 cm were prepared from a hot suspension of 10% charcoal and 5% magnetic iron oxide in 4% aqueous agar. After drying for 18 h at room temperature the disks had a mean $\pm$ s.d. weight of 64.1 ± 5.46 mg and a diameter of (approximately) 1.0 cm.

Steroids. The sodium glucuronide of oestrone and oestradiol-17 β , and oestradiol-17 β -3-sodium sulphate were obtained from Steraloid Ltd (Croydon, U.K.), all the other steroids were obtained from Sigma London (Poole, U.K.). Standard solutions of the steroids were prepared in pig serum which had undetectable levels of oestrone sulphate (< 25 pg/ml by Method 2, see below). The standard curve was based on serum containing 0.15, 0.25, 0.3, 0.5, 1.0, 2.0, 4.0 and 6.0 ng oestrone sulphate/ml.

Tritiated oestrone sulphate. [^3H]Oestrone sulphate (sp. act. 53 Ci/mmol) was obtained from New England Nuclear (Southampton, U.K.). Fresh solutions were always prepared in PBS to give 6.25 nCi/100 μl .

Antiserum. This was obtained from Dr W. F. Coulson and was raised in rabbits against oestrone-3-glucuronide-BSA; it was used at a final dilution of 1/70 000.

Oestrone sulphate assay

Method 1: direct assay in serum. The serum (20 μl) and PBS (280 μl) were dispensed into plastic tubes (Milli-6 vial, LKB Instruments Ltd, Croydon, U.K.) with a dispenser-diluter (Gilson, Dilugil V obtained from Anachem, Luton, U.K.). This was followed by 100 μl diluted antiserum, and then 100 μl [^3H]oestrone sulphate solution. The tubes were shaken gently and then left at room temperature for 15 min followed by 60 min at 4°C. One dried charcoal disk was then added to each tube and these were shaken for 2½ h. The disks were removed with a bar magnet, 4 ml scintillant (Aqualuma, LKB Instruments Ltd) were added and the counts measured (ICN/Tracerlab Corumatic 200, Tracerlab Ltd, Twickenham, U.K.).

Standards were set up in quadruplicate and all other sera in duplicate.

For some samples, after removing the charcoal disk and before adding scintillation fluid, the RIA reaction mixture was bleached by adding 5 μl hydrogen peroxide (100 vols) and 45 μl 0.5 N-NaOH, mixing and then incubating at 40°C for 30 min. Haemolysed sera were bleached to a colourless or a very pale straw-coloured solution. The bleaching step was carried out only for testing the effect of haemolysis, otherwise the results are from unbleached assays.

Method 2: extraction and hydrolysis. The reagents and procedures were as previously described (Hattersley *et al.*, 1980). In this method free oestrogens were removed by a preliminary extraction with ether, and the oestrone sulphate was extracted into tetrahydrofuran and then hydrolysed with arylsulphatase at pH 7.3 to give free oestrone which was measured by RIA. Oestrone glucuronide is not hydrolysed under the conditions used and therefore does not contribute to the estimate of oestrone sulphate.

Results

Method 1

Effect of serum on residual unadsorbed steroid and binding of [^3H]oestrone sulphate by antiserum. The effect of increasing volumes of serum on binding of oestrone sulphate was tested by Method 1 with the modification that, to maintain a constant total volume, changes in serum volume were compensated for by altering the volume of PBS. By excluding antiserum from the

reaction mixture the residual unadsorbed steroid was determined; this was expressed as a percentage of total counts added, and measured the efficiency of disks to remove unbound oestrone sulphate. To correct for changes in residual unadsorbed steroid the antiserum-bound oestrone sulphate was calculated from the following equation:

$$C_B = (100 C_{OB} - pC_T)/100 - p$$

where C_B = counts bound to antiserum; C_T total counts added; C_{OB} , the observed 'bound counts'; p , the % residual unadsorbed steroid. The above equation was derived from the relationship $C_{OB} = C_B + C_F$, where C_F , the residual unadsorbed steroid, is equal to $p(C_T - C_B)/100$.

The results in Table 1 show that increasing the volume of pig serum in the RIA mixture resulted in a progressive and significant rise in residual unadsorbed steroid ($P < 0.025$ with 20 μ l serum and $P < 0.001$ with 40 μ l serum compared to PBS alone). The uncorrected results for antiserum-bound oestrone sulphate were unaffected by added pig serum, but correction for the unadsorbed steroid showed that there was a drop in bound oestrone sulphate for pig serum volumes ≥ 30 μ l ($P < 0.05$). All further studies were carried out using 20 μ l serum and the results were not corrected for unadsorbed steroid.

Table 1. Effect of different volumes of pig serum on residual unadsorbed and antiserum bound oestrone sulphate expressed as % of added [3 H]oestrone sulphate

Oestrone sulphate	Serum volume (μ l)				
	0	10	20	30	40
Residual unadsorbed	2.9 $\pm$ 0.82	3.4 $\pm$ 0.48	6.3 $\pm$ 0.72	6.5 $\pm$ 0.35	9.0 $\pm$ 0.37
Antiserum bound					
Uncorrected*	35.7 $\pm$ 1.25	35.8 $\pm$ 0.54	36.0 $\pm$ 0.19	34.9 $\pm$ 0.35	34.5 $\pm$ 0.65
Corrected*	33.8 $\pm$ 1.27	33.5 $\pm$ 0.56	31.7 $\pm$ 0.19	30.4 $\pm$ 0.36	28.0 $\pm$ 0.70

Values are mean $\pm$ s.e.m. of 4 replicates.

* In relation to residual unadsorbed oestrone sulphate (see text).

Standard curve and reproducibility. The standard curve (Text-fig. 1) had a linear regression of percentage bound (y) on log oestrone sulphate concentration (x) over the range of 0.25–6.0 ng/ml serum. The minimum sensitivity, defined as the amount of steroid corresponding to the B_0 counts $-2 \times$ s.d., corresponded to 89.5% binding. This was outside the linear portion of the curve but was within the concentration range of 0.15–0.25 ng oestrone sulphate/ml.

Tests with two serum samples containing 0.45 and 1.15 ng oestrone sulphate/ml gave respectively within-assay coefficients of variation of 8.1 and 11.2% (6 replicates) and between assay coefficients of variation of 12.7 and 4.6% (5 assays).

Antiserum specificity. The cross-reactivity was defined as the amount of steroid giving a 50% drop in the binding of [3 H]oestrone sulphate. Each steroid was tested at three levels and in quadruplicate at each level. Cross-reactivities were calculated from the linear regression equation of percentage bound on log steroid concentration. As shown in Table 2, only oestrone, oestrone glucuronide and oestrone 3-methylether gave displacement of the tritiated tracer comparable to that of oestrone sulphate. Androstenedione, dehydroepiandrosterone sulphate, testosterone, progesterone and 11 α -hydroxyprogesterone did not show any cross-reaction at 100 ng/tube.

Recovery of oestrone sulphate added to pig serum. Standard amounts of oestrone sulphate (0.25–2.00 ng/ml) were added to three serum samples and the steroid content was assayed (Table 3). The mean ($\pm$ s.e.m.) recovery was 104 ($\pm$ 2.11)%.

Stability of oestrone sulphate in whole blood left at room temperature. Fresh blood from 4 sows was divided into aliquots and after standing at room temperature for 3 h, 7 h, 3 days and 7 days, the serum was separated and stored at -20°C . Oestrone sulphate was also added to

Text-fig. 1. Standard curve for serum oestrone sulphate measurements in 3 assays. The regression of % bound (y) on log concentration (x) gave a linear equation $y = -40.27x + 65.27$ over the range 0.25–6.0 ng/ml; the standard error of the slope was ± 0.571 .

Table 2. Specificity of the oestrone-3-glucuronide antiserum defined as the amount of steroid producing 50% reduction in binding of [^3H]oestrone sulphate

Steroid	pg/tube
Oestrone	37
Oestrone sulphate	40
Oestrone-3-methylether	51
Oestrone-3-glucuronide	85
Oestradiol-17 α	12 000
Oestradiol-17 β	19 000
Oestradiol-17 β , 3-glucuronide	5000
Oestradiol-17 β , 3-methylether	14 000
Oestradiol-17 β , 3-sulphate	25 000
Oestradiol-17 β , 3-benzoate	36 000
Epi-oestradiol-16 β	20 000
Oestriol	>20 000
Oestriol-3-methylether	>20 000
Oestriol-3-sulphate	>20 000

Table 3. Recovery (ng/ml) of oestrone sulphate added to pig serum

Serum sample	Oestrone sulphate added (ng/ml)				
	0	0.25	0.5	1.0	2.0
1	0.25 $\pm$ 0.03	0.56 $\pm$ 0.04	0.76 $\pm$ 0.09	1.2 $\pm$ 0.12	—
2	1.7 $\pm$ 0.10	—	2.3 $\pm$ 0.11	2.8 $\pm$ 0.10	3.9 $\pm$ 0.25
3	3.1 $\pm$ 0.25	—	—	4.6 $\pm$ 0.22	5.0 $\pm$ 0.25

Values are means $\pm$ s.e.m. of 4 replicates.

aliquots of fresh whole blood (about 2 ng/ml) from 3 of the sows and serum was separated after standing at room temperature for the specified periods. Oestrone sulphate concentrations were measured by Method 1.

There was no evidence of loss of immunoreactive material even after 7 days at room temperature (Table 4). The result of Pig 3 serum with added steroid (3 days) is lower than expected but in general the variations are within the error of the method.

Table 4. Stability of oestrone sulphate in pig blood at room temperature

Serum sample	Oestrone sulphate (2 ng)	Time at room temperature			
		3 h	7 h	3 days	7 days
1	—	0.2	0.2	0.2	0.2
1	+	2.3	2.3	2.5	2.6
2	—	0.5	0.5	0.5	0.6
2	+	3.0	3.1	3.0	2.6
3	—	0.5	0.4	0.35	0.5
3	+	2.7	3.1	2.1	2.7
4	—	0.4	0.4	0.4	0.45

Values are ng oestrone sulphate/ml serum.

Comparison of Methods 1 and 2

The oestrone sulphate concentrations of 31 pig sera were determined by both methods. The results by the two methods were highly correlated ($r = 0.96$) and the mean $\pm$ s.e.m. oestrone sulphate concentration was 2.04 ± 0.19 ng/ml by Method 1 and 1.88 ± 0.18 ng/ml by Method 2. The mean $\pm$ s.e.m. difference between Method 1 and 2 was 0.158 ± 0.054 ng/ml, indicating that the mean concentration by Method 1 was significantly higher than that given by Method 2 ($P < 0.01$, paired t test).

Effect of bleaching on the estimate of oestrone sulphate concentration

Two sets of serum samples were used to test the effect of bleaching: Set A ($n = 28$) had undergone slight haemolysis and were pale red in colour while Set B ($n = 42$) showed no evidence of haemolysis. The mean $\pm$ s.e.m. oestrone sulphate concentrations for Set A were 2.0 ± 0.25 ng/ml (unbleached) and 1.88 ± 0.23 ng/ml (bleached); the corresponding values for Set B were 2.25 ± 0.22 ng/ml and 2.19 ± 0.20 ng/ml. The mean $\pm$ s.e.m. difference between unbleached and bleached assays was 0.123 ± 0.076 ng/ml for Set A and 0.06 ± 0.055 ng/ml for Set B; these differences were not significant. If the comparison is restricted to serum samples with oestrone sulphate concentrations < 1 ng/ml, then the mean $\pm$ s.e.m. difference between unbleached and bleached was -0.017 ± 0.032 ng/ml ($P > 0.05$) for 9 unhaemolysed sera and 0.054 ± 0.013 ng/ml ($P < 0.005$) for 8 haemolysed sera. This suggests that the presence of blood pigments may result in a small but significant overestimate of the serum oestrone sulphate concentration.

Oestrone sulphate concentration in pig serum

Non-pregnant, cyclic sows. The oestrone sulphate concentration was < 0.3 ng/ml in 15 serum samples, and 0.3 – 0.5 ng/ml in 6 samples, 3 of which were taken on the day of oestrus.

Mated sows. The mean $\pm$ s.d. oestrone sulphate concentration for serum from 87 pregnant sows was 2.7 ± 1.52 ng/ml; 76 samples contained > 1 ng/ml, 11 were in the range 0.5 – 1.0 ng/ml and none of the samples had < 0.5 ng/ml.

Of the 7 sows that did not conceive, 6 had undetectable levels of serum oestrone sulphate and the other 0.34 ng/ml.

Discussion

Previous methods for estimating oestrone sulphate in serum have involved solvent extraction

followed by (1) hydrolysis and estimation of the free oestrone (Robertson *et al.*, 1978; Hattersley *et al.*, 1980), or (2) the direct estimation of oestrone sulphate in the extract with an antiserum raised against oestrone glucuronide-bovine thyroglobulin (Wright, Collins, Musey & Preedy, 1978). The method described in this paper is applied directly to serum and results in considerable simplification of the assay.

The close agreement between results obtained by the direct assay (Method 1) and one involving extraction and hydrolysis (Method 2), and the satisfactory measurement of oestrone sulphate added to pig serum are strong arguments for the validity of the direct method, particularly when applied in early pregnancy when free oestrone is very low (Robertson & King, 1974). However, the slightly higher mean oestrone sulphate concentration (0.15 ng/ml) given by the direct method is an indication that non-specific factors contributed to the oestrone sulphate measurement. The results of the bleaching experiments suggest that haem pigments may lead to an overestimate equivalent to 0.05 ng oestrone sulphate/ml; the capacity of the antiserum to cross-react with oestrone and its derivatives implies that these compounds might also contribute to an overestimate of oestrone sulphate. However, the data from non-pregnant, cyclic sows indicate that this source of error is confined to about 30% of these animals (6 out of 21), and it may be significant that 3 of these sows were in oestrus at the time of sampling.

Despite the presence of the small non-specific component the results from pregnant pigs show that the direct assay of serum oestrone sulphate is a potentially accurate pregnancy diagnosis test. All serum samples from pregnant sows (87) had oestrone sulphate concentrations >0.5 ng/ml whereas the 28 samples from non-pregnant animals contained ≤ 0.5 ng/ml; in fact, the samples from 25 sows not in oestrus had oestrone sulphate concentrations ≤ 0.4 ng/ml. The above evidence suggests that sows are (1) pregnant if serum oestrone sulphate is >0.5 ng/ml, (2) not pregnant if the concentration is ≤ 0.4 ng/ml, and (3) of uncertain status if serum values are 0.4–0.5 ng/ml. Tests underway with a large-scale field trial may lead to greater precision in the definition of criteria 1 and 2 and elimination of the third.

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