

London, 1964, p. 15.
 5. Nair, K. V. K., M.Sc thesis, Travancore Univeristy; unpublished, 1954.

6. Larsen and Poldervaart, A., *Miner. Mag.*, 1957, p. 545.
 7. Taylor, J. H., *Am. Miner.*, 1937, 22, 689.

AN EVALUATION OF RADIOIMMUNOASSAY OF SERUM TESTOSTERONE WITH AND WITHOUT EXTRACTION

S. P. AGARWAL, N. K. DIXIT, V. K. AGARWAL and P. K. DWARAKNATH
Department of Veterinary Physiology, Haryana Agricultural University, Hissar 125004, India.

ABSTRACT

The experiment was designed to evaluate the accuracy of radioimmunoassay (RIA) of testosterone without extraction of serum samples. The serum samples from cross-bred bulls were analysed for testosterone concentration with and without extraction. In addition, known amounts of testosterone were added to normal serum samples, serum blank and buffer blank and the recovery was estimated in extracted and non-extracted samples. On an average, the estimates of testosterone concentration in unextracted samples were only 28% of the values obtained in extracted serum samples and the recovery in unextracted samples was 20 to 50% as against 90 to 100% in extracted samples. It is concluded that RIA without extraction of serum is not an accurate method of testosterone estimation.

INTRODUCTION

IN steroid hormone assays, one of the cumbersome and time-consuming steps is the extraction of the hormone with a volatile organic solvent. In recent years, steroid hormones in unextracted samples of plasma and milk has successfully been estimated for progesterone¹⁻⁵, estradiol⁶ and estriol⁷ with the use of highly specific antisera and certain critical modifications in RIA procedure. Similar information on plasma or serum testosterone is not available. The purpose of the present study was to evaluate the accuracy of direct estimation of serum testosterone using common reagents.

MATERIALS AND METHODS

Blood samples were collected from 10 cross-bred bulls, serum was separated and stored at -20°C until analysed. A serum blank was prepared by treating blood serum from a 'one-month old' calf with activated charcoal.

The labelled hormone (1, 2, 6, 7, ³H testosterone) and testosterone antibody was obtained from New England Nuclear, Boston, USA and most of the chemicals and unlabelled hormones were procured

from M/s Sigma Chemicals, USA. Serum samples were analysed for testosterone by RIA techniques without chromatography as suggested by Smith and Hafs⁸ with minor modification. All samples were analysed in duplicates. The sensitivity of the assay was 10 pg/tube. The cross-reactivity of the antiserum was 100% for testosterone, 50% against dihydrotestosterone and 1% or less with other related steroid hormones. The assays were tested for parallelism and a correlation coefficient of 0.98 was found between the serum dilution rate and concentration of hormone estimated. The extraction and final recovery were found to be 95.7% and 91.2% respectively. The inter-assay and intra-assay coefficient of variation were 4% and 12%, respectively.

Estimation of testosterone in sera samples with extraction and without extraction.

A volume of 50 to 200 μl of sera from nine bulls was taken in two sets of duplicate tubes. One set was extracted with purified and distilled diethyl ether followed by evaporation of ether, dissolving of residue in phosphate buffer saline (PBS), addition of antibody and labelled antigen, incubation and separation of bound and free fractions with dextran-coated charcoal

(DCC). For the other set, the extraction step was skipped; otherwise the procedure was exactly similar and the same reagents were used.

Addition of known amounts of testosterone to sera and their estimation with extraction and without extraction.

Known amounts of testosterone (100, 250, 500 and 1000 pg/ml) were added to each of the four serum samples, serum blank and PBS. The samples were equilibrated for 4 hr at 4°C and a volume of 50 to 200 µl was analysed simultaneously with and without extraction.

The estimated values or the per cent recovery in extracted and unextracted samples were compared by paired *t* test⁹.

RESULTS

The data (table 1) revealed that on an average, the concentration in unextracted serum samples was only 28.1% and significantly ($P < 0.01$) lower to that of extracted samples.

The per cent recovery obtained in serum samples which were fortified with known amounts of testosterone is tabulated in table 2. It was found that the average recovery in extracted and unextracted samples varied from 90 to 100% and from 18 to 46% with an overall mean of 94.6 and 32.5%, respectively. The difference between two groups was statistically significant ($P < 0.01$).

The average recovery of testosterone in fortified serum blank samples was 58% in unextracted samples

Table 1 Testosterone levels in extracted and unextracted serum samples of crossbred bulls.

Serum samples No.	Quantity estimated ng/ml	
	With extraction	Without extraction
1	2.80	0.54
2	3.40	1.00
3	4.40	1.12
4	3.10	0.44
5	8.75	4.50
6	7.00	3.10
7	9.00	2.05
8	8.50	2.00
9	4.80	1.08
Mean	5.75	1.76*
± S.E.	±0.91	±0.47

* $P < 0.01$

as against 85.2% in the extracted samples (table 3) and was significantly ($P < 0.05$) lower when tested statistically. In contrast, the average recovery was 95% in extracted and 92% in non-extracted buffer samples containing different concentrations of known amounts of testosterone (table 3). The difference between two groups was not significant.

DISCUSSION

The testosterone estimates were much lower and the recovery was very poor, if the extraction step was

Table 2 Percent recovery of testosterone in extracted and unextracted serum samples after addition of known amount of testosterone.

Testosterone added in serum samples pg/ml	Percent recovery in serum samples@							
	Serum samples ¹		Serum samples ²		Serum sample ³		Serum sample ⁴	
	Extr.	Unextr.	Extr.	Unextr.	Extr.	Unextr.	Extr.	Unextr.
100	95.5	50.0	95.1	63.4	104.6	41.5	95.2	22.6
250	78.4	35.1	76.1	47.9	96.4	35.7	105.3	19.3
500	88.7	27.4	99.2	43.0	101.2	25.9	92.7	17.1
1000	98.2	25.0	90.5	31.7	99.2	19.9	98.5	15.2
Mean	90.2*	34.4	90.2*	46.5	100.4*	30.8	97.9*	18.6
± S.E.	±5.1	±6.5	±5.8	±7.6	±2.0	±5.6	±3.2	±1.8
C.V.	9.8	32.8	11.2	28.3	3.4	31.5	5.6	17.3

Extr. – Extracted; Unextr. – Unextracted.

@ The serum samples No. 1, 2, 3 and 4 contained endogenous concentration of 120, 110, 310 and 320 pg/ml testosterone, respectively.

* $P < 0.01$

Table 3 Percent recovery of testosterone in serum blank and buffer blank with and without extraction after addition of known quantities of hormone.

Testosterone added pg/ml	Blank serum		Buffer	
	Recovery %		Recovery %	
	Extr.	Unextr.	Extr.	Unextr.
0	0	0	0	0
100	85	72	94	100
250	64	40	68	72
500	92	58	110	96
1000	100	62	110	100
Mean	85.25*	58.00	95.50	92.00
±S.E.	±8.91	±7.72	±11.58	±7.77
C.V.	18.11	23.05	20.75	14.64

* $P < 0.05$; Extr.—Extracted; Unextr.—Unextracted

avoided. This is in contrast to the direct assays of estradiol⁶ and estriol⁷ which have been precisely estimated in unextracted plasma samples using highly specific antisera. Close correlations between direct and extracted assays have been reported for plasma progesterone by limiting the volume of plasma aliquote to 20 μ l to avoid large blank effects and non specific interferences³. In the present study serum volumes varying from 50–200 μ l were used and antisera possessed 50% cross-reactivity against dihydrotestosterone. It is not known if these factors, in any way, affected the recovery in direct assays. It is well established that most circulating steroid hormones are bound to plasma proteins with varying degree of affinity. Therefore, for any method of measuring these hormones, all protein bound fractions of hormone should be liberated. It has been suggested that the preliminary extraction with an organic solvent breaks the weak binding of steroid hormones with plasma protein¹⁰. In direct assays of testosterone in this study, the protein bound testosterone had no opportunity to be freed completely and to compete for the antibody sites in RIA system. Thus, it appeared that in unextracted samples, only the free hormone was es-

timated. Alternatively, the poor recovery in direct assays may be due to the presence of some inhibiting factors in the serum which interfered with the antigen-antibody reaction in the RIA system. These postulates are supported by the observations that exogenously added testosterone also showed a poor recovery in unextracted assays and that the testosterone added to the phosphate buffer solution yielded similar values with and without extraction.

The results of this investigation indicate that the extraction of serum samples for testosterone is an indispensable step as the values obtained in unextracted samples are much lower than those in extracted samples.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support from International Atomic Energy Agency, Vienna.

30 May 1983; Revised 9 August 1983.

1. Booth, W. D., Newcomb, R., Strange, H., Rowson, L. E. and Sacher, H. B., *Vet. Record.*, 1975, 97, 366.
2. Sugden, E. A., *Can. J. Comp. Med.*, 1978, 42, 229.
3. Holdsworth, R. J., *Brit. Vet. J.*, 1980, 136, 135.
4. Heap, R. B., Laing, M. J. A. and Walters, D. E., *J. Agric. Sci. (Camb.)*, 1973, 81, 151.
5. Dobson, H., Midmer, S. E. and Fitzpatrick, R. J., *Vet. Record*, 1975, 96, 222.
6. Jurjenes, H., Pratt, J. J. and Woldring, M. G., *J. Clin. Endocrinol. Metab.*, 1975, 40, 19.
7. Soares, J. R., Gross, S. J. and Bashore, R. A., *J. Clin. Endocrinol. Metab.*, 1975, 40, 970.
8. Smith, O. W. and Hafs, H. D., *Proc. Soc. Exp. Biol. Med.*, 1973, 142, 804.
9. Snedecor, G. W. and Cockran, W. G., *Statistical methods*, Indian Edition, Oxford and IBH Publishing Co., Bombay, 1968.
10. Read, M. J. and Murray, A. F., In *Hormones in blood* (eds) C. H. Gray and V. H. T. Jame, Vol. 3, Academic Press London, 1979, 304.