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DIRECT RADIOIMMUNOASSAY FOR DETERMINATION OF BETAMETHASONE 17-BENZOATE

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ABSTRACT

A direct radioimmunoassay for the determination of betamethasone 17-benzoate (I) in serum was developed. In this assay, a highly specific antibody is used to bind the ^{125}I-labeled antigen. Polyethylene glycol is used for the separation of the free from bound antigen. A blocking agent (ANS) was used to inhibit binding of the steroid onto serum proteins. The minimum detectable concentration was 15 pg/ml. The intra and inter assay coefficients of variation were 7.4% and 10.2%, respectively. This assay required 0.1 ml of serum and it did not require extraction or chromatographic separation of the steroid.

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INTRODUCTION

Several radioimmunoassays have been developed for the determination of endogenous steroids in plasma or serum. Due to the lack of highly specific antiserum, most of these assays required extraction of the steroids by organic solvents from the serum and then, separation of the interfering steroids by paper, column or thin layer chromatography before incubation with the antiserum was attempted.[1,2] These assays were very tedious to perform and, therefore, their employment for analyzing large numbers of serum samples was impractical. However, in recent years several radioimmunoassays have been developed which make use of highly specific antisera for the direct determination of certain steroids in serum or in urine and thus, eliminating the time consuming steps of extraction and chromatographic separation. Furthermore, the replacement of the ^{3}H-labeled tracers by ^{125}I-labeled tracer has greatly simplified the counting procedure. This paper describes the development of a direct radioimmunoassay for the determination of an experimental steroid in serum samples. This assay has adequate sensitivity, is highly specific and extremely simplified.

MATERIALS AND METHODS

Reagents: Steroid (I) was synthesized according to a previously described procedure.[3] Bovine serum albumin was obtained from Miles Co., complete Freund's adjuvant from Difco, polyethylene glycol 6000 (PEG) from Sigma, $Na^{125}I$ (17 Ci/mg) from ICN, and 8-anilino-1-naphthalene-sulfonic acid (ANS) from Eastam Kodak C.

Preparation of Immunogen and Immunization: The 3-oxime derivative of I was prepared and conjugated with bovine serum albumin using a modification of a method described by McKenzie and Clements.[4] The ratio of I to protein was 24:1 as determined by U.V. spectrophotometry. Four albino New Zealand male rabbits

were immunized according to a method previously described.[5] Each rabbit was injected with an initial dose of approximately 150 µg of I-bovine-serum albumin conjugate emulsified with Freund's complete adjuvant. Five booster injections each of 200 µg were injected at monthly intervals. The animals were bleeded two weeks after the final booster injection.

^{125}I-I Solution: Iodine 125-labeled-I was prepared by coupling a previously iodinated histamine through a 3-oxime bond to I according to a procedure previously described.[6,7] The labeled steroid was isolated from the reaction mixture by preparative thin layer chromatography (silica gel, benzene: ethanol 7:3). The spot with the labeled steroid was eluted with ethanol. The specific activity of the ^{125}I-I ranged from 1000 mCi/mg to 1950 mCi/mg. The ethanolic solution of the labeled steroid was diluted with a buffer solution consisting of 350 ml 0.1 M citrate and 650 ml 0.2M Na_2HPO_4, pH 6.0, containing 0.2% bovine gamma globulin to an activity of 200,000 cpm per ml. The solution was dispensed in siliconized vials and kept at -20 C.

Stripped Calf Serum: Calf serum (300 ml) was passed through a charcoal column (1.5 cm x 40 cm) which was previously washed with distilled water. The first 50 ml of the eluate were discarded and the rest was collected and used as steroid-free calf serum.

Radioimmunoassay: Standards of I were prepared by dilution of a 0.2 mg/ml stock solution in ethanol with stripped calf serum. The following standards were prepared 0, 200, 400, 800, 1600 pg/ml. The antiserum was diluted (1:5,000) with 0.1 M borate buffer, pH 8.6, containing ANS (950 µg/ml). The radioiodinated antigen was diluted with 0.1 M borate buffer, pH 8.6, so that a 0.1 ml aliquot had activity of 20,000 cpm. The radioimmunoassay protocol is shown in Table 1. The procedure was as follows. The standards or the unknowns (0.1 ml) were mixed with 0.1 ml

Table 1. Radioimmunoassay Protocol

Tube No.	Description	Standard Volume ml	Buffer 0.1 M Borate ml	Labeled Steroid ml	Anti-Serum ml	Total Volume ml
1,2	0 pg standard	0.1	-	0.1	0.1	0.3
3,4	200 pg standard	0.1	-	0.1	0.1	0.3
5,6	400 pg standard	0.1	-	0.1	0.1	0.3
7,8	800 pg standard	0.1	-	0.1	0.1	0.3
9,10	1600 pg standard	0.1	-	0.1	0.1	0.3
11,12	Total count	-	0.2	0.1	-	0.3
13,14	Nonspecific biding[a]	0.1	0.1	0.1	-	0.3
15,16	Unknowns	0.1	-	0.1	0.1	0.3

[a]stripped serum

of the ^{125}I-I solution and 0.1 ml of antiserum. After incubation of 5 hours at room temperature, 1 ml of 25% polyethylene glycol in 0.1 M borate buffer, pH 8.6, was added. All tubes were votrexed for 10 sec. and centrifuged at room temperature at 2000 xg for 20 min. The supernatant layer was aspirated and the precipitate was counted.

RESULTS

Figure 1 shows the standard curve of the assay. The sensitivity of the assay was 1.5 pg with 95% confidence. The measurable range of the assay was 1.5 to 160 pg and the nonspecific binding was less than 5%. The cross reactivity of the antiserum with various steroids was tested and expressed according to a previously described method.[8] The results are shown in Table 2.

The results of dilution study is shown in Figure 2. Two serum samples spiked with different amounts of I were serially diluted with charcoal treated calf serum and assayed. A linear

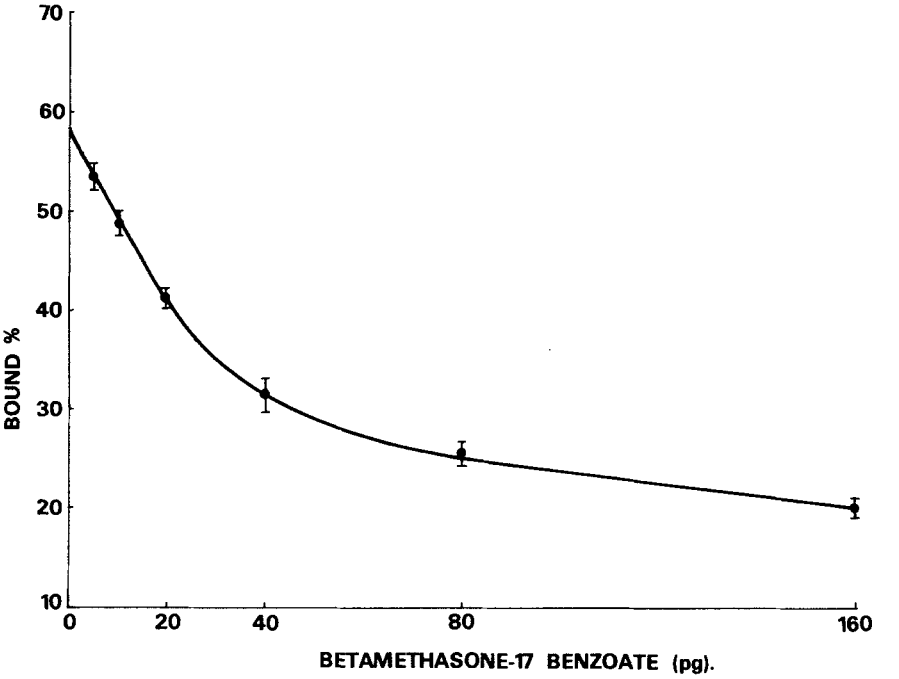

Figure 1. Standard curve of the radioimmunoassay. Each point is the averge of 10 replications. The bars indicate one standard deviation.

relationship was obtained between the samples dilution and the concentration of I in each sample.

The accuracy of the assay was evaluated by recovering various amounts of I from a series of controlled sera of I. On the average, the percent recovery was 101.2 $\pm$ 8.3%. The correlation coefficient, r, between recovered I and added I was 0.99, (Fig. 3). The precision, as expressed by the intra-assay and inter-assay reproducibility, was examined by assaying a series of controlled sera. The results are summarzied in Table 3.

TABLE 2. Cross-reactivity of the Antiserum With Various Steroids

Steroid	Cross-reaction%	Steroid	Cross-reaction%
Betamethasone-17-benzoate	100	Testosterone	< 0.00031
Cortisol	< 0.0001	Progesterone	< 0.0001
Cortisone	< 0.00009	Corticosterone	< 0.0015
		Aldosterone	< 0.0003

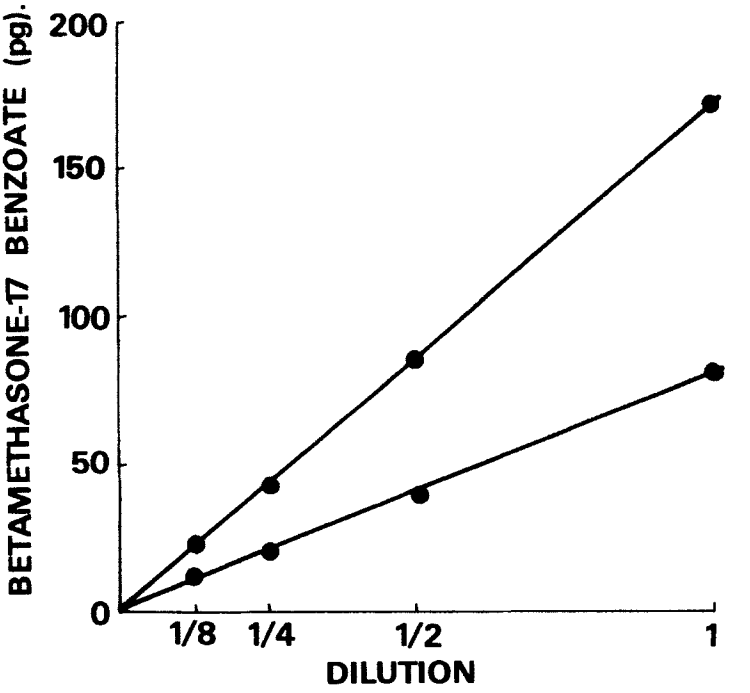

Figure 2. A linear relationship exists between concentration of I in serum and sample dilution.

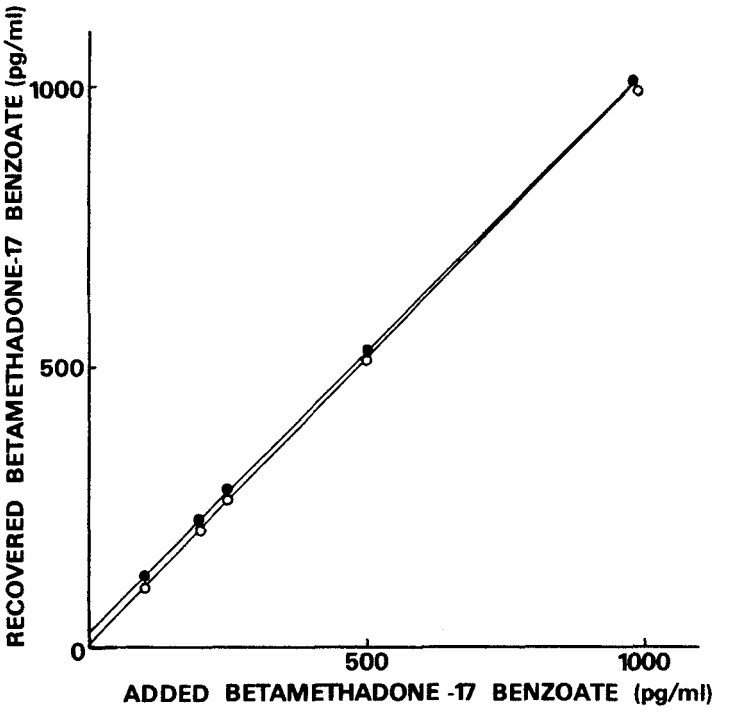

Figure 3. Recovery of I added to two different serum pools.

DISCUSSION

The highly specific antibody made it possible to measure the concentration of I directly in serum without extraction. In producing such highly specific antiserum, the following suggestions of previous investigators were taken into consideration: a) the hapten was conjugated through a 3-oxime bond to the protein; b) the hapten was carefully purified before coupling to the carrier protein; c) the animals were immunized using small doses of immunogen according to a previously described method.[5] As it is shown in Table 2, no substantial cross-reactivity of the antiserum with endogenous steroids was observed.

TABLE 3. Precision of Radioimmunoassay

	Concentration of I (pg/ml)			
	Intra-assay (n = 8)		Inter-assay (n = 5)	
	Mean ± SD	C.V. (%)	Mean ± SD	C.V. (%)
1	77.4 ± 8.2	10.6	85.5 ± 15.0	17.5
2	237.2 ± 19.6	8.3	258.6 ± 21.4	8.3
3	550.8 ± 30.3	5.5	567.6 ± 41.8	7.4
4	1102.5 ± 56.2	5.1	1162.4 ± 88.6	7.6
	Mean	7.4	Mean	10.2

Because of the relatively extensive specific and nonspecific binding of steroids on plasma proteins, several investigators used compounds which blocked the binding of the steroids and did not cross-react with the antibody. Agents such as testosterone, ANS, and sodium salicylate have been used to minimize interference of plasma binding proteins in several steroid radioimmunoassays.[9-11] It was found that ANS was useful in minimizing the specific and non-specific binding of I on plasma proteins.

The accuracy, precision, and reproducibility of the assay were satisfactory. Furthermore, the use of the ^{125}I-I as a tracer has eliminated the need of liquid scintillation counting, while the use of polyethylene glycol as a method to separate the bound from free antigen has made it more convenient to handle many samples simultaneously since the time of incubation and its temperaure dependency have been reduced.

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