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A NEW METHOD FOR THE PRODUCTION OF A MAGNETIC IMMUNOSORBENT USED IN RADIOIMMUNOASSAY AND IMMUNORADIOMETRIC ASSAY*

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A method has been developed enabling the direct coupling of first or second antibody to finely dispersed magnetite (Fe_3O_4). The immunosorbent thus produced was applied in various radioimmunoassay systems (T3, T4, TSH, Cortisol) for the separation of bound and free antigens. The elimination of the need for "precoating" the magnetic particles with a polymer has several advantages. One of them lies in the ease of one-step production of the immunosorbent and other is the high antibody/magnetite ratio. The influence of the concentration of the immunosorbent and detergent (TWEEN 20 or TRITON X-100) on the assay parameters (Bo, NSB, etc.) has been systematically investigated and the optimum concentration of the magnetizable particles and detergent has been determined. The reliability of magnetic separation has been validated by comparing it with the conventional PEG separation method.

Solid phase techniques are widely used to separate free and bound antigen (hapten) in radioimmunoassay (RIA) or as a carrier of one of the two antibodies used in the immunoradiometric assay (IRMA). If specific antibodies are coupled to the magnetizable particles, these participate in the initial binding reaction. The magnetizable particle to which the second antibody is attached, are often used as separation reagent added after completion of the antigen-antibody interaction to separate the bound from the free fraction.

A few solid phase methods along with their advantages and drawbacks are listed in Table 1. Of the methods presented, there are only three which eliminate the need for the centrifugation step. i. e., the coated tube, the coated plastic ball and the coated magnetizable particle method (magnetic immunosorbent).

In the case of large scale production both the coated tube and the coated plastic ball methods require highly sophisticated technology. Especially when the first antibody is used for coating it is rather difficult to ensure uniform coating. On the other hand, the

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Table 1
Comparison of solid phase techniques

Solid phase method	Advantage	Drawback
Coated tube	No centrifugation low NSB	Limited reproducibility limited surface capacity
Coated plastic ball	No centrifugation low NSB	Limited surface capacity
Sedimentable plastic beads	Good reproducibility	Mixing during incubation centrifugation
Non-sedimentable plastic beads	Good reproducibility	Centrifugation
Coated magnetizable particles	No centrifugation good reproducibility	

coating of magnetizable, e.g., megnetite ($FeOFe_2O_3$) particles can be performed even in a moderately well equipped laboratory.

The production cost of solid phase antibodies consists of the cost of antibody used for coupling to a solid phase and of the cost of the solid phase itself. As for the former, it may depend on the coupling efficiency too. It is usually the solid phase itself which has a substantial impact on the production cost. Considering the fact that the cost of the home made magnetizable Fe_3O_4 particles per tube is essentially lower than that of the plastic tube or plastic ball, the application of the antibody coated magnetizable particles seems to be more economical as compared to the other solid phase methods.

Experimental

Magnetizable particles: The magnetizable particles most frequently used in the RIA and IRMA techniques are ferromagnetic nickel,[1] ferrimagnetic magnetite (Fe_3O_4)[2-5] and barium-ferrite ($BaO \cdot 6Fe_3O_4$).[6] Sometimes γ-Fe_2O_3 particles are also used for production of immunosorbents.[7]

The magnetic particles used as immunosorbents are to meet the following criteria: (a) particle size and density should be small enough to prevent spontaneous sedimentation during incubation, (b) high surface capacity, and (c) high magnetic response, i.e., high magnetic susceptibility.

In the case of larger particles, especially with high density, the reaction mixture must be either shaken or rotated in order to keep the solid phase particles in suspension. If the sedimentation obeys the Stokes law, particles up to about 1 μm diameter and 3 g/cm^3

density remain in suspension, particles of 3 μm diameter of the same density sediment slowly during incubation.[7] As the density of magnetite (Fe_3O_4) is 5.18 g/cm^3, the density of the antibody coated particles will be approximately 3 g/cm^3 if the magnetite/antibody ratio is equal to 1. The magnetic response, i.e., attractive force (F) acting on the magnetic particle by a permanent magnet plate is given by

$$F = k \cdot H \cdot V \cdot \mathrm{d}H/\mathrm{d}X \quad (1)$$

where H stands for the strength of the magnetic field, expressed in oersted (Oe), k for the magnetic susceptibility, dH/dX for the heterogeneity of the magnetic field and V for the volume of the magnetic core of the particles. According to Eq.(1), the attractive force exerted on the magnetite particle is proportional to the core size of the particles. On the other hand, the rate of spontaneous sedimentation is proportional to the mass of the particles. Thus the very slow spontaneous sedimentation rate (i.e. very stable suspension) and fast sedimentation on a magnetic plate are incompatible.

A compromise is to be found concerning the particle size which enables to keep the particles in suspension without mixing during the incubation period and ensures sedimentation on the magnetic plate within 5–10 minutes.

As for the magnetic properties (e.g., magnetic susceptibility) Fe_3O_4, Fe_2O_3, $BaO6Fe_2O_3$ and Ni can be used as matrixes for the magnetic immunosorbent. The advantage which speaks for magnetite lies in the fact that it can be prepared by precipitation of ferric and ferrous salts from solution,[8,9] which makes the control of the required particle size easier than in the case of other ferromagnetic materials, which can be produced either by oxide sintering, thermal decomposition or by other sophisticated methods.[10] On the other hand, the magnetite prepared by precipitation of iron salts adsorbs proteins (e.g., IgG) with high efficiency.

The antibody is usually coupled to the magnetic or magnetizable matrix by covalant linkage; the matrix particles are covered with cellulose, 3-aminopropyltriethoxysilane, agarose, polyacrylamide, polymerized bovine serum albumin,[1] polyacrolein,[5] p-aminophenyltrimethoxysilane[2] and the antibody is conjugated to the particles via the active groups (e.g., amino groups). The advantage of the method presented here lies in the fact that the antibody can be coupled to the magnetite particles directly, thus eliminating the need of coating the surface of the particles either with cellulose or any other polymer as well as the need of cyanogen bromide activation.

Preparation of magnetite suspension: A slightly modified version of the method of ELMORE[8] was used to prepare the magnetite particles. To an aqueous solution of ferrous and ferric chloride, a NaOH solution was added under continuous stirring. In

Reaction (2) a brownish black precipitate was formed:

$$2FeCl_3 + FeCl_2 + 8NaOH = FeOFe_2O_3 + 8NaCl + 4H_2O \quad (2)$$

The precipitate was decanted and washed with distilled water. The suspension stored in a stoppered bottle proved to be stable for one year or more.

Magnetite has been studied intensively as a prototype of ferrites in the thirties. It has an inverse spinel structure, the unit cell contains 32 oxygen atoms in an almost cubic

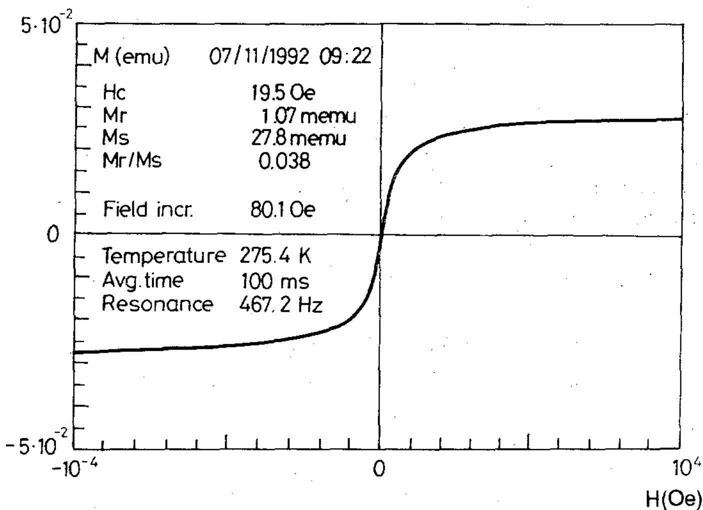

Fig. 1. Magnetization curve of the magnetite particles. (The evaluation of the magnetite particles was kindly performed by Anthony J. Cumbo Princeton Measurement Corporation) on a MicroMag 2900 Alternating Gradient Magnetometer)

close-packed arrangement, the iron ions occupy 8 tetrahedral and 16 octahedral interstices in this oxygen lattice.[11] In the inverse spinel structure both di-and trivalent cations occur in the octahedral sites. The Fe^{2+} ions are all in octahedral position while the Fe^{3+} ions are equally divided between octahedral and tetrahedral positions. The Fe^{3+} ions in tetrahedral positions lie antiparallel to those in octahedral positions thus their net magnetic moment is zero. Only the Fe^{2+} ions contribute to the magnetic moment of magnetite. The permeability and coercive force of magnetite vary considerably with its source and particle size. GOTTSCHALK[12] observed that the coercive force increased as the particle size diminished both in the case of natural and artificial magnetite.

The magnetization curve of the magnetite particles produced by the use of the method coutlined above is shown in Fig. 1.. As can be seen, the coercive force is almost

negligible, the magnetization curve is practically anhysteretic. The consequence of this is that the particles will have no magnetic moment in the absence of magnetostatic field and do not stick together. The advantage of this magnetic property is that a magnetic plate can be used to speed up the sedimentation in the course of the washing or coating procedure. Before coating the particles with IgG the suspension was placed in a beaker on a magnetic plate. After sedimentation of the particles the distilled water supernatant was aspirated and the proper amount of coupling buffer was added, the suspension was homogenized either by shaking or by sonicating. The suspension thus obtained was ready for coupling.

Separation of the IgG fraction from the second antiserum: It has been found by preliminary experiments that the ratio of IgG bound to the magnetic particles and remaining in solution decreases when the concentration of the antiserum in the coupling reaction mixture increases. This finding, although not investigated in details, can be attributed to the lower coupling reactivity of IgG as compared to the other serum proteins. For this reason IgG fraction instead of the whole antiserum has been coupled to the magnetizable particles.

The second antibody was separated from the anti-rabbit goat antiserum by the use of DEAE-Sephacell (Pharmacia). In a column (diameter 50 mm, length 1000 mm) 1600 ml gel was filled. 400 ml of anti-rabbit goat antiserum was poured on the top of the column. and was allowed to soak in. The IgG fraction was eluted with 0.05M Tris-HCl buffer (pH 8).

The elution was monitored with a UV monitor (Pharmacia dual path UV monitor) at 280 nm. After elution of the IgG fraction the albumin and the other proteins were washed out with 0.05M TRIS-HCl buffer containing 0.5M NaCl (pH 8). The column was regenerated with 6000 ml 0.05M TRIS-HCl buffer (pH 8.0).

The IgG fraction was precipitated from the effluent (2000 ml) with an equal volume of saturated ammonium sulfate. Then the precipitate was suspended and dialized against distilled water at 4 °C for 48 hours and against 0.05M phosphate buffer (pH 6.3) at 4 °C for a few days. From 400 ml anti-rabbit goat antiserum, 7–8 g IgG has been separated.

Coating the Fe_3O_4 *particles with the second antibody:* Preliminary experiments showed that freshly prepared magnetite particles can efficiently be coated with immunoglobulins by the use of the carbodiimide method. The coating procedure was as follows:

(1) To the suspension of magnetite particles (1–2 mg/ml) the solution of the IgG fraction separated from the second antiserum was added (0.5 mg IgG per 1 mg magnetite) in 0.003M phosphate buffer (pH 6.3). The mixture was incubated for 1 hour at 4 °C.

(2) To this mixture 2 mg EDAC (1-ethyl-3{3-dimethyl-aminopropyl} carbodiimide) was added per 1 mg IgG. The reaction mixture was incubated overnight at 4 °C.

(3) After incubation the suspension was washed three times with 0.01M potassium dihydrogen phosphate + 0.15M NaCl, (pH 7.2).

(4) After having resuspended in 0.01M phosphate buffer (pH 7.4) the suspension was ready to use.

The coupling efficiency was checked by controlling the IgG concentration remaining in the supernatant by UV spectrophotometry. The coupling yield was between 60 and 75%.

Results

In the commercially available T4, T3 and cortisol RIA kits produced by the Institute of Isotopes Co., LTD Budapest, the PEG 6000 separation reagent was replaced by magnetic immunosorbent to which anti-rabbit goat IgG was coupled. The assay protocols are presented in Table 2.

Table 2
Protocol of the T4, T3 and cortisol radioimmunoassay
(volumes in microlitres)

Radioimmunoassay	T4	T3	Cortisol	
Sample, standard or control	10	100	10	
Tracer	200	100	100	
Antiserum	100	100	100	
Incubation at	37 °C	37 °C	rt	for 2 hours
PEG 6000 solution	1000	500	500	
Centrifuge at 40 °C and 1500 g for 20 minutes				
Aspirate the supernatant				
Count the tubes				

Table 3
Protocol of the TSH radioimmunoassay (volumes in microlitres)

Sample, standard or control	200
Antiserum	100
Incubation at room temperature for 16–24 hours	
125-I-TSH	100
Incubation at room temperature for 16–24 hours	
DAB/PEG 6000 solution	1000
Incubation at room temperature for 15 minutes	
Centrifugation at 4 °C and 2000–2500 g for 15 minutes	
Aspirate or decant the supernatant	
Count the tubes	

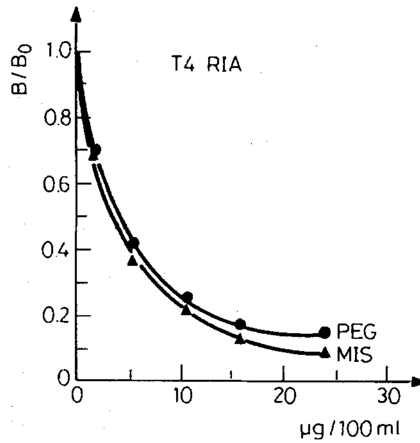

Fig. 2. T4 standard curves obtained with PEG 6000 and magnetic immunosorbent (MIS) separation, (MIS 0.4 mg/ml, 025%, TWEEN-20)

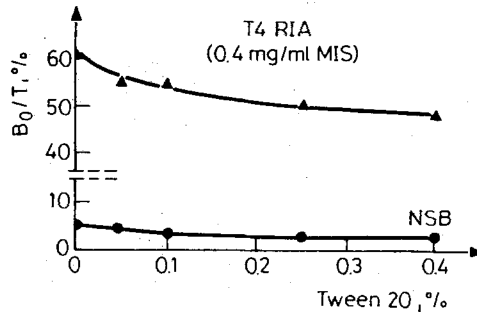

Fig. 3. The zero binding (B_0/T) and the NSB as a function of the TWEEN-20 concentration in the T4 RIA

The protocol of the TSH radioimmunoassay is shown in Table 3.

In the case of the second antibody magnetic immunosorbent the assay protocol was the same till the end of the incubation period, thereafter instead of PEG 6000 solution 0.5 ml immunosorbent (magnetite coated with anti-rabbit goat IgG) was added to the tubes. After 15 minutes incubation at room temperature the tubes were attached in a rack on to a magnetic plate (Amersham plc). After 15 minutes without removing the rack from the magnetic plate the supernatant was poured off and discarded keeping the magnetic plate inverted. Typical standard curves obtained by the use of the PEG 6000 separation method and the magnetic immunosorbent are shown in Figs 2,6,13,16.

To optimize the assay parameters, the zero binding (B_0) and NSB have been determined at different concentrations of the immunosorbent and detergent

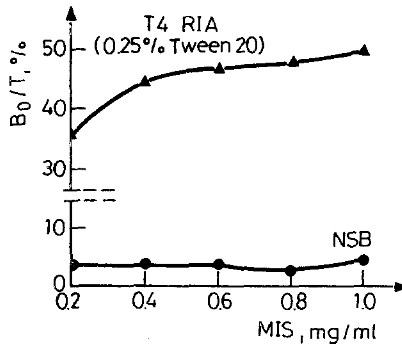

Fig. 4. The zero binding (B_0/T) and the NSB as a function of the magnetic immunosorbent (MIS) concentration in the T4 RIA

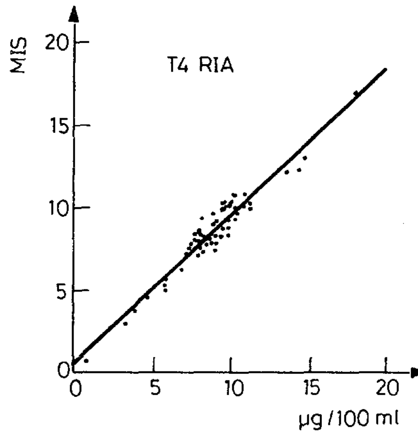

Fig. 5. Correlation of T4 levels of human sera obtained with PEG 6000 and magnetic immunosorbent (MIS) separation ($y = 0.53 + 0.9x$, $r = 0.97$, $n = 59$, $\bar{x} = 8.73$, $\bar{y} = 8.37$)

(TWEEN-20). The results are plotted in Figs 3,4,7,8,10,11,14,15. As can be seen from Fig. 3, at constant magnetic immunosorbent concentration (0.4 mg/ml) both the zero binding and the NSB decrease with increasing TWEEN-20 concentration, however the decrease of NSB is higher than that of the zero binding. The optimum TWEEN-20 concentration proved to be 0.25%. The increase of the magnetic immunosorbent concentration results in the increase of both the zero binding and NSB (Fig. 4). The optimum concentration proved to be 0.4 mg/ml, i.e., 0.2 mg/tube. In the case of T3 RIA the TWEEN-20 concentration and in the case of TSH RIA the Triton X-100 concentration had only a moderate effect on zero binding and on NSB (Figs 10, 11), while in the case of cortisol RIA the TWEEN-20 drastically reduced both the zero binding and the NSB (Fig. 15).

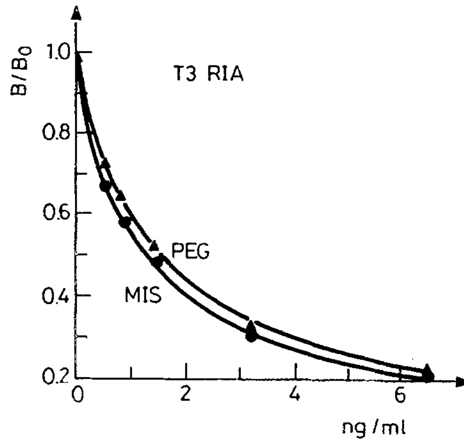

Fig. 6. T3 RIA standard curves obtained with PEG 6000 and magnetic immunosorbent (MIS) separation (MIS 0.6 mg/ml, 0.5% TWEEN-20)

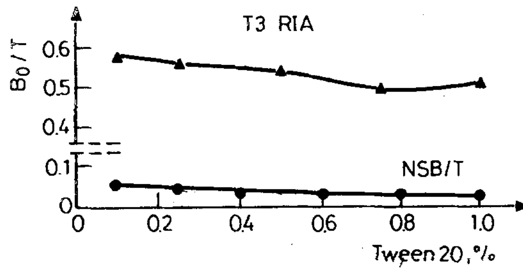

Fig. 7. The zero binding (B_0/T) and NSB as a function of the TWEEN-20 concentration in the T3 RIA

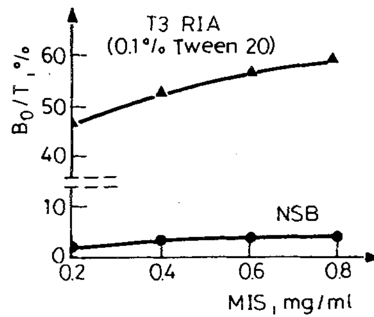

Fig. 8. The zero binding (B_0/T) and NSB as a function of the magnetic immunosorbent (MIS) concentration in the T3 RIA

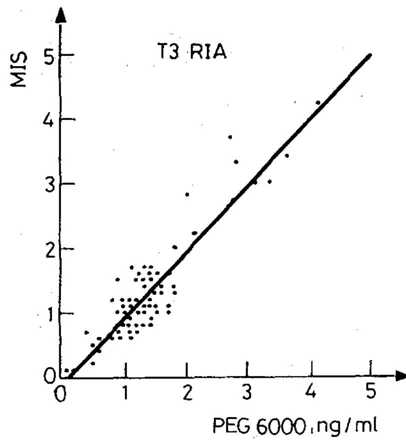

Fig. 9. Correlation of T3 levels of human sera obtained with PEG 6000 and magnetic immunosorbent (MIS) separation ($y = 0.11 + 1.01x$, $n = 91$, $r = 0.92$, $n = 93$, $\bar{x} = 1.36$ ng/ml, $\bar{y} = 1.26$ ng/ml)

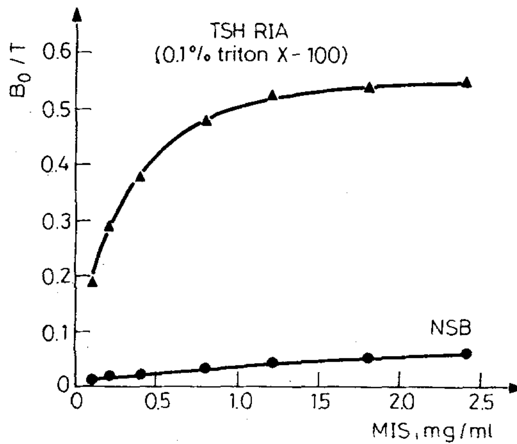

Fig. 10. The zero binding (B_0 /T) and NSB as a function of the magnetic immunosorbent (MIS) concentration in the TSH RIA

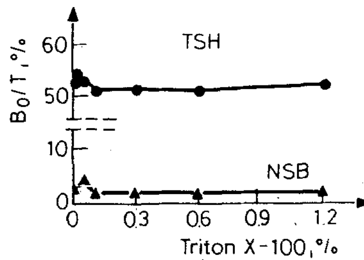

Fig. 11. The zero binding and NSB as a function of the TRITON X-100 concentration in the TSH RIA

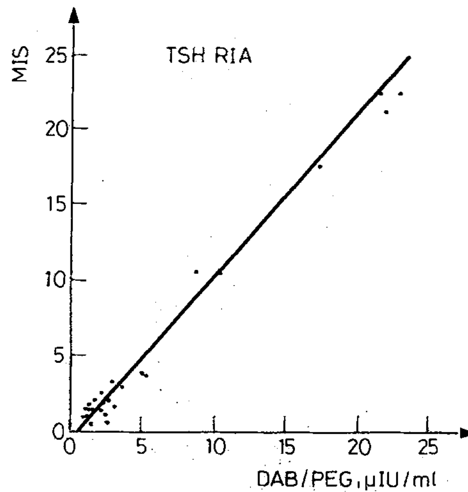

Fig. 12. Coorelation of TSH levels obtained with DAB/PEG and magnetic immunosorbent (MIS) separation ($y = -0.59 + 1.09x$, $r = 0.98$, $n = 33$, $x = 5.45.1$, $y = 5.38$)

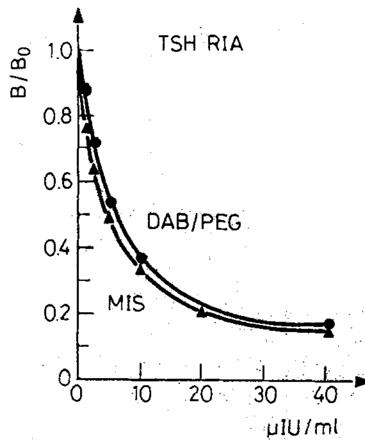

Fig. 13. TSH standard curves obtained with PEG 6000 and magnetic immunosorbent (MIS) separation (MIS 0.8 mg/ml, 0.1% TRITON X-100)

At fixed TWEEN-20 or Triton X-100 concentration both parameters (zero binding and NSB) increased with increasing magnetic immunosorbent concentration (Figs 4,8,10,14). The influence of the magnetic immunosorbent concentration proved to be the most definite in the case of the TSH RIA (Fig. 10).

The reliability of the magnetic separation method was checked in such a way that different human sera were assessed with the original RIA kits based on the PEG 6000 (or in the case of TSH on the DAB/PEG) separation method on one hand, and with the

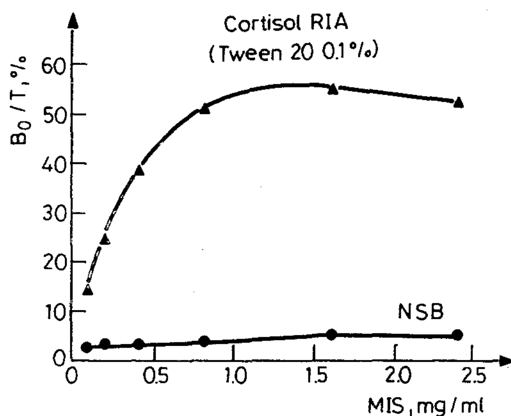

Fig. 14. The zero binding (B_0/T) and NSB as a function of the magnetic immunosorbent (MIS) concentration in the cortisol RIA

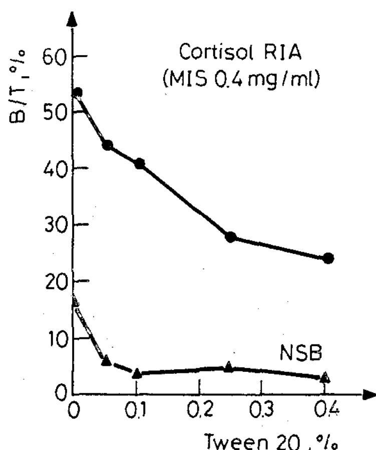

Fig. 15. The zero binding (B_0) and NSB as a function of the TWEEN-20 concentration in the cortisol RIA

modified kits in which the PEG 6000 (or DAB/PEG) separation reagent was substituted by the magnetic immunosorbent. The correlation of the T4, T3, Cortisol and TSH levels thus obtained are shown in Figs 5,9,12,17.

Short and long term stability trials with TSH, T4, T3 and Cortisol RIA kits have been conducted for an 8–10 week and a 9 month period to assess the shelf life of the magnetic immunosorbent stored at 4 °C. The stability trials were performed with kit components (including magnetic immunosorbent) of the same lot with the exception of the tracer which was freshly prepared every month.

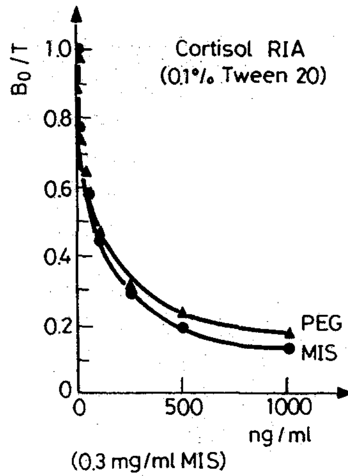

Fig. 16. Cortisol standard curves obtained with PEG 6000 and magnetic immunosorbent (MIS) separation (MIS concentration 0.8 mg/ml, 0.1% TWEEN-20)

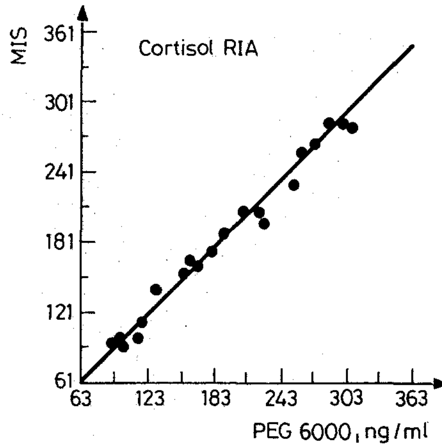

Fig. 17. Correlation of the cortisol levels of human sera obtained by the use of PEG 6000 and magnetic immunosorbent (MIS) separation (y = 12.90.g x,r = 0.99, n = 22, $\bar{x}$ = 194.1, $\bar{y}$ = 186.8)

The assay parameters thus obtained and the levels of the control serum provided with the kit are enlisted in Tables 4–7.

To follow the variation of the cortisol levels during the 8 week period of the short term stability trial, the same human serum samples have been assessed every week

Table 4
Variation of the T4 assay parameters with the age of the magnetic immunosorbent (MIS) (0.4 mg/ml MIS, 0.25% TWEEN 20)

Age, day	B/T, %	B/B_0 (50%), µg/100 ml	B/B_0 (80%), µg/100 ml	Control serum µg/100 ml
7	48	3.0	1.3	8.1
14	47	2.9	1.3	7.3
26	44	3.0	1.2	8.4
40	42	3.4	1.3	6.7
54	43	3.0	1.2	7.8
67	39	3.2	1.0	8.9

Table 5
Variation of the T3 assay parameters with the age of the magnetic immunosorbent (MIS) (0.6 mg/ml MIS, 0.5% TWEEN 20)

Age, day	Bo/T, %	B/B_0 (50%), ng/ml	B/B_0 (80%), ng/ml%	NSB/T, %	Control serum, ng/ml
7	44	1.16	0.20	2.8	1.60
21	44	1.12	0.25		1.25
27	45	1.17	0.20	2.3	1.46
37	48	1.17	0.26	1.8	1.48
42	47	1.23	0.18	2.3	1.62
49	49	1.14	0.15	2.5	1.47
56	47	1.34	0.20	2.3	1.44
63	49	1.20	0.23	2.4	1.45

Table 6
Variation of the TSH assay parameters with the age of the magnetic immunosorbent (MIS) (1.6 mg/ml MIS, 0.1% Triton X-100)

Age, day	B_0/T, %	B/B_0 (50%), µgU/ml	B/B_0 (80%), µgU/ml	NSB/T, %	Control serum, µgU/ml
0	54	5.5	1.0	1.8	9.0
8	48	7.7	1.0	1.5	10.5
14	43	5.0	1.0	2.0	10.5
23	42	4.5	0.8	1.8	10.0
28	36	5.3	1.2	1.8	9.5
35	35	4.4	0.8	1.8	8.8

Table 7
Variation of the Cortisol assay parameters with the age of the magnetic immunosorbent (MIS) (0.8 mg/ml MIS, 0.1% TWEEN 20)

Age, day	B_0T, %	B/B_0 (50%), ng/ml	B/B_0 (80%), ng/ml	NSB/T, %	Control serum, ng/ml
7	50	78	21	4.3	94.4
14	56	81	19	4.0	100.0
21	57	75	32	3.8	84.3
28	53	106	25	4.5	102.7
35	61	94	23	4.5	99.8
49	62	107	24	5.2	95.3
56	60	123	28	5.6	207.4

Table 8
Correlation of the cortisol levels obtained with magnetic separation in the 8-week period, (the levels obtained on the first week were compared in turn with the levels assessed on the following weeks)

Time of assay run	r
1 week	–
2 weeks	0.99
3 weeks	0.97
4 weeks	0.98
5 weeks	0.97
7 weeks	0.98
8 weeks	0.97

Table 9
Long term stability data of the magnetic immunosorbent used in T4 RIA kit

Age of MIS, day	B/T, %	NSB/T, %	B/B_0 (50%), μg/100 ml	B/B_0 (80%), μg/100 ml	Control serum, μg/100 ml
8	53	3.2	3.99	1.14	7.4
37	47	2.6	4.42	1.56	7.4
64	48	2.5	4.47	1.36	8.2
97	46	2,1	3,18	1.11	8.0
146	46	2.5	3.07	1.13	7.0
150	46	1.9	4.26	1.26	8.8
279	41	3.4	3.62	1.10	7.3

using the magnetic immunosorbent separation. As it turns out from. Table 8, excellent correlation (r) has been found between the data of the first week and those in turn of the following weeks. The results of the long term stability trial are listed in Table 9.

Conclusions

The data, i.e., the assay parameters, the correlation of the hormone levels obtained with PEG and magnetic immunosorbent separation method as well as the short and long term stability data indicate that the magnetite particles coated with the IgG fraction of the second antiserum can efficiently be used to separate the free and bound fractions of the antigen (hapten) in T3, T4, TSH and Cortisol radioimmunoassay.

Inspection of the short term stability trial standard curves reveals a slight shift of the 50 and 80% intercepts towards higher doses, however, it does not influence the levels obtained for the control serum. A shift of similar direction and extent was observed in the case of PEG or DAB/PEG separation too, consequently it can rather be attributed to the ageing of the tracer than to that of the immunosorbent.

This conclusion is supported by the long term stability trial too, in which every month a freshly prepared tracer was used for the six months trial period. In this case no shift of any assay parameters was observed at all.

In the scope of the short term stability trial very good correlation has been found between the levels of T3, T4, TSH and cortisol obtained with PEG (or DAB/PEG) and magnetic separation method.

The long term stability data indicate that at least in the nine months trial period no change in the performance of the coated magnetic particles can be observed. Experimental data not reported here indicate that the second antibody magnetic immunosorbent can be efficiently used in testosterone, urinary albumin and several Prostaglandin radioimmunoassays too.

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