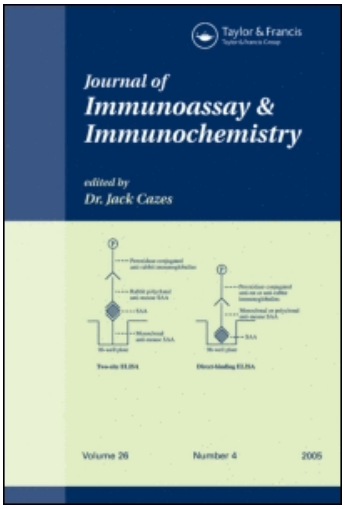

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Sol Particle Immunoassay (SPIA)

J. H. W. Leuvering[a]; P. J. H. M. Thal[a]; M. van der Waart[a]; A. H. W. M. Schuurs[a]
[a] Organon Scientific Development Group, The Netherlands

To cite this Article Leuvering, J. H. W. , Thal, P. J. H. M. , van der Waart, M. and Schuurs, A. H. W. M.(1980) 'Sol Particle Immunoassay (SPIA)', Journal of Immunoassay and Immunochemistry, 1: 1, 77 — 91
To link to this Article: DOI: 10.1080/01971528008055777
URL: http://dx.doi.org/10.1080/01971528008055777

JOURNAL OF IMMUNOASSAY, 1(1), 77-91 (1980)

SOL PARTICLE IMMUNOASSAY (SPIA)

J.H.W. Leuvering, P.J.H.M. Thal,
M. van der Waart, A.H.W.M. Schuurs
Organon Scientific Development Group
P.O. Box 20, 5340 BH OSS
The Netherlands

ABSTRACT

We describe the use of inorganic (metal) colloidal particles as a label for immunoassays. Dose-response curves for human placental lactogen (HPL) and human chorionic gonadotrophin (HCG) were obtained with sandwich immunoassays, using conjugates consisting of antibody-coated colloidal gold or silver particles.
Several techniques were used to measure the amount of bound conjugate, viz. colorimetry and carbon rod atomic absorption spectrophotometry (CRAAS). At higher antigen concentrations the results of the assay could be read by the naked eye.
Using gold particles as label and CRAAS as detection method, we found a detection limit for a sandwich HPL sol particle immunoassay (SPIA) of 1,4 pmol/l, which was equal to that of an optimalized competitive radioimmunoassay. When using a colorimeter the detection limit for HPL of this SPIA was 5,4 pmol/l, which was superior to that of a corresponding sandwich enzyme-immunoassay (EIA).
HPL and HCG were also simultaneously determined, using microtitration plates, coated with a mixture of anti-HPL and anti-HCG, and a mixture of silver particle anti-HPL conjugate and gold particle anti-HCG conjugate. CRAAS was used to measure the bound amount of silver and gold conjugate. This simultaneous assay requires more work in order to obtain better sensitivities.

INTRODUCTION

Although the radioimmunoassay (RIA), introduced by Yalow and Berson(1), has become a widely used and successful technique, much work has been done to apply labels other than radioisotopes in immunoassays during the past 10 years. These new labels should not only produce assays with sensitivities and specificities similar to or even better than those found with RIA but also lack disadvantages such as the need for special licences, short shelf life of the label and waste disposal problems. A successful method has been the enzyme-immunoassay (EIA) (2-4) in which an enzyme is used as label. Colloidal gold particles, on which antibodies are adsorbed, have been used as electron-dense markers for electron microscopical investigation of cell surfaces (5).

We report here on the use of colloidal particles, not necessarily gold particles, as labels for immunoassays, and propose the name sol particle immunoassay (SPIA). The aims of our investigations were:

a. to demonstrate that labelling with sol particles allows the development of quantitative and qualitative assays.
b. to show that several methods of detection can be used.
c. to compare the detection limit for human placental lactogen (HPL), in SPIA with that in RIA and EIA.
d. to demonstrate that this method of labelling allows simultaneous determination of two non cross-reacting antigens.

MATERIALS AND METHODS

Unless stated otherwise all reagents were of analytical grade and were dissolved in distilled water.

Gold Sols

Gold sol particles, with an average size of 60 nm as measured by electron microscopy, were prepared according to the method of Frens (6). After preparation the gold sol was diluted to an absorbance $A^{1\ cm}_{540\ nm} = 1,1$.

Silver Sols

8,5 ml of a solution containing 58,9 mmol/l $AgNO_3$ was mixed with 5 ml of a solution containing 34 mmol/l sodium citrate $2H_2O$ and diluted to 500 ml. Under vigorous stirring, 40 ml 10 ml/l hydrazine hydrate (80% Baker grade) was added and the mixture was stirred for 15 min. The polydisperse sol was fractionated by repeated centrifugation twice at 80 000 N/kg for 20 min and once at 3 000 N/kg for 5 min; the resulting fraction contained particles between 40 and 60 nm measured by electron microscopy. This fraction was stored in a solution containing 0,34 mmol/l sodium citrate $2H_2O$ at pH = 7,5 and diluted to $A^{1\ cm}_{420\ nm} = 1,0$.

Antisera

Anti-HPL sera were prepared according to van Hell et al. (7). Anti-HCG sera were prepared by immunizing rabbits (8), using HCG preparations reported by van Hell et al. (9). The antisera were freeze-dried and stored at -25°C until required for use. Antibody solutions were prepared from the freeze-dried antisera, dissolved in 0,15 mol/l

NaCl pH 7,0 by precipitation of the antibody fraction after addition of Na_2SO_4 (final concentration 1,27 mol/l) as described by Kekwick (10).

The precipitate was dissolved in a 0,15 mol/l NaCl solution pH 7,0, and dialysed thoroughly against 5 mmol/l NaCl pH 7,0 or 0,15 mol/l NaCl pH 7,0. Antibody solutions, dialysed against 5 mmol/l NaCl pH 7,0 were used for conjugate preparation and those dialysed against 0,15 mol/l NaCl pH 7,0 were used for coating of microtitration plates. Precipitated euglobulins were removed by centrifugation at 200000 N/kg for 30 min. The stock solutions were adjusted to a protein concentration of 13 mg/ml and stored in 1 ml aliquots at $-20^{\circ}C$.

Gold Particle Antibody Conjugates

The pH of the gold sol was adjusted to 7,0 by addition of 0,2 mol/l K_2CO_3. A diluted antibody solution (2 ml), containing 250 µg protein/ml, was added to 100 ml of neutralized gold sol ($A^{1\ cm}_{540\ nm} = 1,0$). After 10 minutes incubation at room temperature 2 ml of a solution containing 10 g/l Carbowax 20M (Union Carbide) and 5 mmol/l NaCl, pH 7,0 was added. After 1 hour incubation at room temperature the gold particle antibody conjugates were centrifuged at 50 000 N/kg for 20 minutes. The dark red pellet was redispersed in a medium consisting of 5 mmol/l NaCl, 0,2 g/l Carbowax 20M and 0,1 g/l thiomersal pH 7,0. The final volume was adjusted so that $A^{1\ cm}_{540\ nm}$ of the conjugate was approx. 1,1.

Silver Particle Antibody Conjugates

A diluted antibody solution (2 ml), containing 1300 µg protein/ml, was added to 100 ml of the silver sol (pH 7,5; $A^{1\ cm}_{420\ nm}$ = 1,0). After 10 min incubation at room temperature 2 ml of a solution containing 10 g/l Carbowax 20M and 5 mmol/l NaCl, pH 7,0 was added. After 1 h incubation at room temperature the conjugate was centrifuged at 125 000 N/kg for 20 minutes. The dark brown pellet was redispersed in a solution of 5 mmol/l NaCl, 0,2 g/l Carbowax 20M and 0,1 g/l thiomersal, pH 7,0. The final volume was adjusted so that $A^{1\ cm}_{420\ nm}$ was approx. 1,1. The conjugate was stored in the dark at 0-4°C.

Coating of Microtitration Plates

Wells of polystyrene microtitration plates (Greiner, specially produced for Organon, the Netherlands), were coated either with 0,1 ml of an anti-HPL solution or with a 1:1 mixture of anti-HPL and anti-HCG solutions according to Wolters et al. (11).

HRP-labelled anti-HPL Conjugates

Horse-radish peroxidase (HRP) (Miles; RZ 3,0) was coupled to rabbit anti-HPL according to the method of Wolters et al. (11).

^{125}I-HPL

^{125}I-labelled HPL was prepared according to Greenwood et al. (12).

Preparation of HRP-Substrate Solution

The HRP-substrate solution consisted of 0,1 mol/l sodium phosphate-citric acid buffer pH 5,0 containing 0,002 mol/l ortho-phenylenediamine di-HCl and 0,1 mol/l H_2O_2.

Sandwich SPIA for HPL

A 0,1 ml sample (HPL standard or blank), dissolved in 0,02 mol/l sodium phosphate buffer pH 7,4, containing 0,15 mol/l sodium chloride and 1 g/l bovine serum albumin (BSA), was pipetted into a well of a microtitration plate coated with anti-HPL, and incubated for 2 h at 37°C. Gold particle anti-HPL conjugates (0,1 ml), diluted with TRIS buffer (1 mol/l TRIS, 1 mol/l NaCl, 1 g/l BSA adjusted to pH 7,4 with 4 mol/l HCl) to a final absorbance of $A^{1\ cm}_{540\ nm} = 1,0$ was added and the reaction mixture was incubated overnight at 37°C. The wells were aspirated and washed 6 times with 0,3 ml 0,1 mol/l TRIS buffer containing 0,5 g/l Tween 20, to remove the unbound conjugate. Finally, 0,11 ml 0,1 mol/l HCl was added to dissociate the bound immune complex. After 5 min the content of each well was homogenized. Either the colour intensity of the dispersions obtained was inspected visually or the metal concentration was measured by means of a colorimeter at 540 nm or a CRAAS apparatus at 242,8 nm.

Simultaneous Sandwich SPIA for HPL and HCG

A 0,1 ml sample containing known amounts of both HPL and HCG (or a blank), dissolved in the 0,02 mol/l sodium phosphate buffer (see above), was pipetted into a well of a microtitration plate, coated

with anti-HPL and anti-HCG, and incubated for 2 h at 37°C. A mixture (0,1 ml) containing equal volumes of gold particle anti-HCG conjugate ($A^{1\ cm}_{540\ nm}$ = 1,0) and silver particle anti-HPL conjugate ($A^{1\ cm}_{420\ nm}$ = 1,0) in the TRIS buffer, was added and the reaction was incubated overnight at 37°C. The wells were aspirated and washed 6 times with 0,3 ml 0,1 mol/l TRIS buffer containing 0,5 g/l Tween 20, to remove the unbound conjugate. Finally, 0,11 ml 0,1 mol/l HCl was added to dissociate the bound immune complexes. After 5 min the content of the well was homogenized. Using 2 µl aliquots, the atomic absorption due to the presence of gold and silver was sequentially measured by CRAAS. Atomic absorption due to gold was measured at 242,8 nm and related to the concentration of HCG. Atomic adsorption due to silver was measured at 338,4 nm and related to the concentration of HPL.

Sandwich EIA for HPL

A 0,1 ml sample (HPL standard solution or blank) dissolved in the phosphate buffer described in the SPIA method, was pipetted into a microtitration plate well, which was coated with the same antiserum as for SPIA, and incubated for 2 h at 37°C. HRP-labelled anti-HPL conjugate (0,1 ml) was added to each well and incubated overnight at 37°C. The wells were aspirated and washed 6 times with 0,1 mol/l TRIS buffer pH 7,4, containing 0,5 g/l Tween 20. The enzyme substrate solution (0,1 ml) was added to each well and incubated in the dark for 50 min at room temperature. The enzyme substrate reaction was stopped by adding 0,05 ml of a 4 mol/l sulphuric acid solution. Either the colour was observed by the naked eye or the absorbance was measured at 492 nm, using a colorimeter.

Competitive RIA for HPL

The sodium phosphate buffer described in the SPIA method was used throughout the assay. The (whole) antiserum, the same as used to prepare anti-HPL antibody solutions for SPIA and EIA, was diluted to such an extent that about 50% of the added ^{125}I-HPL was bound in the absence of unlabelled HPL. The HPL used for the standard solutions was pipetted into a tube together with 0,1 ml of the antiserum diluted with the sodium phosphate buffer (1:160 000 for the normal RIA (RIA-1) and 1:400 000 for the very sensitive RIA (RIA-2) and 0,05 ml of the buffer containing ^{125}I-HPL (10 000 cpm for RIA-1 and 1000 cpm for RIA-2). This reaction mixture was incubated overnight at room temperature. Separation of antibody-bound and free labelled HPL was carried out by incubating the reaction mixture with 0,8 ml of a 1 g/l Dasp ^{R} anti-rabbit suspension (Organon Teknika, the Netherlands) for 2 h in a shaking bath. The tubes were centrifuged for 3 min at 20 000 N/kg. The pellet was washed three times with the sodium phosphate buffer (without BSA) and the radioactivity counted for 5 min in a well type gamma counter (LKB/Wallac).

Instrumentation

Absorbance was measured by a Vitatron DCP small sample volume colorimeter.

CRAAS measurements for gold and silver were performed with a Varian AA6 atomic absorption spectrophotometer, equipped with a CRA 63 carbon rod and a BC 6 background corrector. For each measurement 2 µl aliquots were pipetted into the carbon rod. If the absorbance exceeded 1,0 the sample was diluted and the measured absorbance was multiplied by the appropriate dilution factor.

Detection Limits

The detection limit for HPL was determined according to the following definition: the detection limit for HPL is equal to the concentration of HPL giving a response that differs twice the standard deviation from the average response of the blanks.
This definition was applied to all described HPL assays except those involving visual inspection, in which the responses were classified as either positive or negative.

RESULTS

Dose-response curves for HPL were obtained using gold particle rabbit anti-HPL conjugates and the SPIA method. For measurements with the colorimeter and CRAAS, every point on the dose-response curve is the average of three wells and the standard error is also indicated.
After incubation with 0,1 ml aliquots of solutions containing 170 pmol HPL/l or more, the wells displayed a visible red colour and were classified "positive" (+) in Figure 1. Using a colorimeter to measure the absorbance of the contents of the wells, the detection limit for HPL was 5,4 pmol/l (Figure 1). Using CRAAS to measure the absorbance due to gold the detection limit for HPL in SPIA was 1,4 pmol/l (Figure 1).

We determined the detection limits of the sandwich EIA and of two RIAs for HPL using the same anti-HPL serum and HPL standard. The detection limits for HPL in these assays and in SPIA for HPL are listed in Table 1. Dose-response curves for the simultaneous assay of HPL and HCG are shown in Figure 2. The detection limits for HPL and HCG were approx. 3 pmol/l and approx. 1 IU/l respectively.

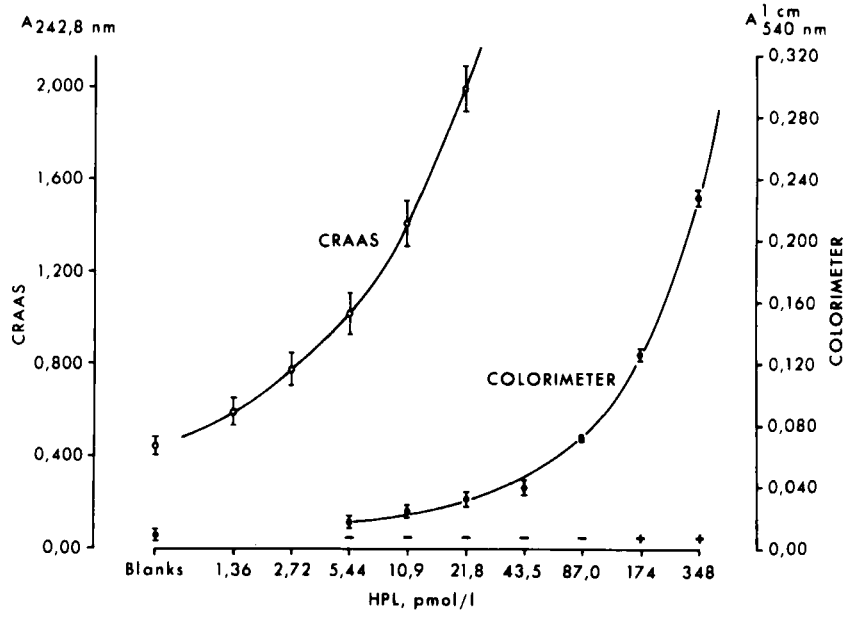

Figure 1 : Calibration curves for HPL in 0,02 mol/l phosphate buffered saline pH = 7,4 containing 1 g/l BSA using visual inspection colorimetry and CRAAS for detection.

●——● : Results of measurements using a Vitatron DCP to measure $A^{1\,cm}_{540\,nm}$.

o——o : Results of the measurements at 242,8 nm of 2 µl samples with a Varian AA6 atomic absorption spectrophotometer equiped with a CRA 63 carbon rod and a BC6 background corrector

Results of readings with the naked eye: ----- : negative readings
+++++ : positive readings.

DISCUSSION

We have demonstrated that colloidal gold and silver particles can be used as labels for immunoassays. The detection limit for an HPL-SPIA compared favourably with that for EIA and RIA. The detection limit depends on the detection method applied. With visual inspection,

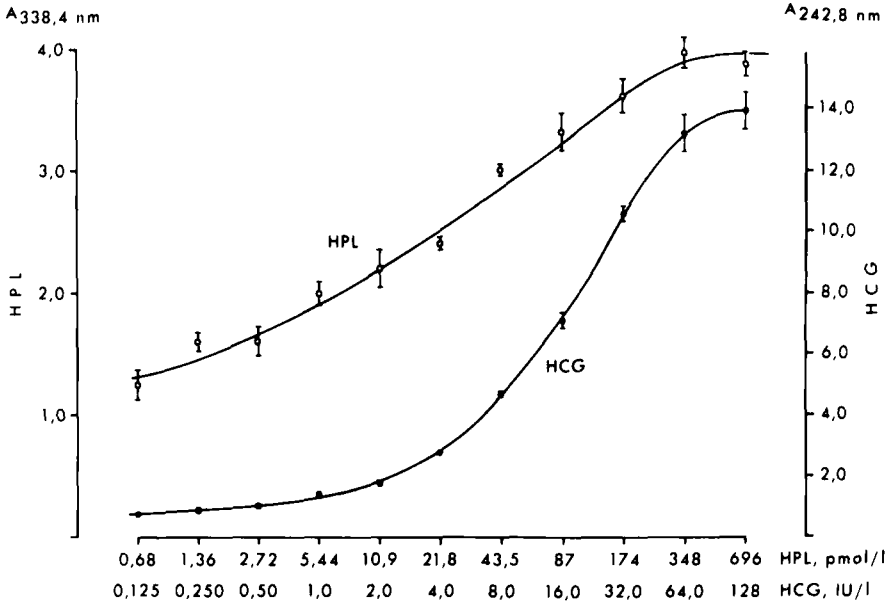

Figure 2 : Calibration curves for simultaneous determination of HPL (o——o) and HCG (●——●) using CRAAS for sequential determination of the atomic absorption of silver (338,4 nm) and gold (242,8 nm) in 2 µl samples.
(1 IU purified HCG ≈ 140 ng ≈ 4,7 pmol).

EIA has a detection limit of 110 pmol/l which is lower than that of SPIA (170 pmol/l). Using a colorimeter, the detection limit for HPL in SPIA (5,4 pmol/l) is lower than that of EIA (90 pmol/l). Using the very sensitive detection method for metals, CRAAS, the detection limit for HPL in SPIA (1,4 pmol/l) is equal to that of the very sensitive RIA-2; however, the sample volume of the SPIA was half of that of RIA. The higher cost of the CRAAS apparatus in comparison with γ-counters, is a drawback, but this may be compensated by even lower detection limits for SPIA after further optimalization.

Metal atoms, in the form or organometallic or co-ordination

TABLE 1

Detection limits of different HPL-assays

type of assay	method of detection or quantification	detection limit pmol/l	detection limit fmol/test tube
SPIA	naked eye	170	17,0
SPIA	colorimeter	5,4	0,54
SPIA	CRAAS	1,4	0,14
EIA	naked eye	110	11
EIA	colorimeter	90	9
RIA-1	γ-counter	6,5	1,3
RIA-2	γ-counter	1,4	0,28

complexes, as labels for haptens or macromolecular antigens have been described by Cais et al (13). They obtained a detection limit for steroids of about 50 nmol/l using a competitive assay and CRAAS for detection. In their system only one metal atom (or a number < 10) was bound in every bound labelled-steroid antibody complex. However, if a sol particle is used a a label as in SPIA, one sol particle consisting of some millions of atoms is bound in every bound antibody-antigen-labelled antibody complex. This explains the difference between detection limits in the metalloimmunoassay (MIA) and SPIA.

Specificity, another important feature of an immunoassay, is mainly determined by the quality of the antiserum. As a result of the chemical changes that take place during the labelling of antibodies,

the specificity can be affected. The method described for labelling with sol particles consists of physical adsorption of antibodies on to the sol particles. Because of this mild procedure the specificity of SPIA is likely to be comparable with that of EIA and RIA. Preliminary data (as yet unpublished) appear to confirm this.

Several examples of simultaneous RIAs have been described previously (14-16). We have demonstrated that simultaneous determination of HPL and HCG using a sandwich SPIA is also possible. If a quantitative multi-element analysis technique, e.g. plasma emission spectrophotometery or X-ray fluorescence, is used instead of CRAAS, sequential determination of the bound amount of the labels can be avoided.

Besides the advantages already mentioned above, viz. sensitivity and simultaneous assays, SPIA has some additional advantages.

Because the sol particle labelled conjugates have an intense colour, there is no enzymesubstrate reaction necessary to produce a colour like in EIA. SPIA avoids the use of radioactive materials and therefore the shelf-life of reagents for SPIA is likely to be longer than for RIA. Preliminary investigations (unpublished) have shown that conjugates in liquid form can be stored at 4°C for 6 months. We have managed to prepare freeze-dried sol particle labelled conjugates and this is likely to result in much longer shelf-lives.

The use of sol particles offers more flexibility with regard to the method of assay and method of detection. Besides the described sandwich immunoassay competitive assays are also possible.

Recently, we have developed a homogeneous assay. In this kind of assay the antibody-coated sol particle acts like the antibody-coated, latex particle in latex agglutination tests or antibody coated erythro-

cyte in haemagglutination tests (to be published elsewhere).

ACKNOWLEDGEMENTS

We thank Dr. Anneke Bosch for optimizing and performing the RIA. Dr. Hans van Hell and Mr. Jo Brands for their expert advice concerning the sandwich EIA and Dr. Sander Stols (University of Nijmegen) for his excellent help by the electron microscopical investigations of gold and silver sol preparations. We also thank Dr. H. Berkeley for carefully reading the manuscript.

Requests for reprints should be addressed to J. H. W. Leuvering, Organon Scientific Development Group, P.O. Box 20, 5340 BH OSS, the Netherlands.

REFERENCES

1. Yalow RS, Berson SA. Assay of Plasma Insulin in Human Subjects by Immunological Methods. Nature (London) 1959; 184: 1648-9.

2. Engvall E, Perlmann P. Enzyme-linked Immunosorbent Assay (ELISA). Quantitative assay of Immunoglobulin G. Immunochemistry 1971; 8: 871-4.

3. Van Weemen BK, Schuurs AHWM. Immunoassay using Antigen-Enzyme Conjugates. FEBS Lett 1971; 15: 232-6.

4. Schuurs AHWM, van Weemen BK. Enzyme-immunoassay. Clin Chim Acta 1977; 81: 1-40.

5. Horisberger M, Rosset J, Bauer H. Colloidal Gold Granules as Markers for Cell Surface Receptors in the Scanning Electron Microscope. Experientia 1975; 31: 1147-9.

6. Frens G. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. Nature (London), Phys Sci 1973; 241: 20-22.

7. Van Hell H, Brands JAM, Schuurs AHWM. Enzyme-immunoassay of Human Placental Lactogen. Clin Chim Acta 1979; 91: 309-16.

8. Schuurs AHWM, de Jager E, Homan JDH. Studies on Human Chorionic Gonadotrophin. III Immunochemical characterisation. Acta Endocrinol (Copenhagen) 1968; 59: 120-138.

9. Van Hell H, Matthijsen R, Homan JDH. Studies on Human Chorionic Gonadotrophin. I Purification and some physicochemical properties. Acta Endocrinol (Copenhagen) 1968; 59: 89-104.

10. Kekwick RA. The Serum Proteins in Multiple Myelomatosis. Biochem J 1940; 34: 1248-57.

11. Wolters G, Kuypers L, Kacaki J, Schuurs A. Solid-phase enzyme-immunoassay for detection of Hepatitis B Surface Antigen. J Clin Path 1976; 29: 873-9.

12. Greenwood FC, Hunter WM, Glover JS. The Preparation of ^{131}I-labelled Human Growth Hormone of High Specific Radioactivity. Biochem J 1963; 89: 114-23.

13. Cais M, Dani S, Eden Y, et al. Metalloimmunoassay. Nature (London) 1977; 270: 534-5.

14. Bluett MK, Reiter EO, Duckett GE, Root AW. Simultaneous Radioimmunoassay of Thyrotrophin and Thyroxine in Human Serum. Clin Chem 1977; 23: 1644-7.

15. Kaul B, Davidow B, Millian SJ. Preparation of Polyvalent Antibodies for Use in Simultaneous Multiple Drug Radioimmunoassay. J Anal Toxicol 1977; 1: 14-19.

16. Gruss AD, Spier-Michl IB, Gotschlich EC. A Method for a Radioimmunoassay using Microtiter Plates allowing simultaneous Determination of Antibodies to two non cross-reactive Antigens. Immunochemistry 1978; 15: 777-80.