Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 82 (2) :181-187, abr./jun. 1987

COMPARATIVE ELISA REAGENTS FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN (HBsAg)

I. F. CAMARGO*, A.M.C. GASPAR** & C.F.T. YOSHIDA**

*Bio-Manguinhos – FIOCRUZ and** Instituto Oswaldo Cruz, Departamento de Virologia, Caixa Postal 926, 20001, Rio de Janeiro, RJ, Brasil

Conjugates of goat anti-HBs IgG and horseradish peroxidase (HRP) prepared by two different methods, one using $NaIO_4$ and the other SPDP, were compared. Anti-HBs antibodies obtained from goat, rabbit and guinea-pig were tested as capture serum. The ELISA showed a sensitivity similar to RIA and a level of antigen captation ranging from 4.37 to 8.75 nanograms/ml was obtained when rabbit or guinea-pig captures were used combined with both $NaIO_4$ or SPDP conjugates.

Key words: ELISA – HBsAg

An effective control of hepatitis B depends upon the use of high sensitivity tests for HBsAg determination in blood banks. Third generation tests such as radioimmunoassay (RIA), passive reverse hemmaglutination (RPHA), and enzyme-linked immunosorbent assay (ELISA) have been recommended for routine screening of blood donors. The RIA, mainly utilized in laboratories with good resources, in spite of its high sensitivity, has serious disadvantages as short shelf-life and biohazard related to the use of radioisotopes. Another important limitation of this test is the high cost of reagents which are not produced in developing countries and often have to be imported from abroad. The RPHA tecnique has advantages of low cost, since the reagents are produced in Brazil with local technology. However, this method, is less sensitive than RIA (Caldwell & Barrett, 1977; Kimura et al., 1978). ELISA has a sensitivity close to RIA, is easy to perform, with stable reagents, without need of sophisticated equipment, since visual reading can dispense spectrophotometry. It has a low cost comparing with RIA, but up to now, the reagents are also imported. This paper describes the preparation and comparison of locally produced ELISA reagents for the detection of HBsAg and the criteria used in its evaluation.

MATERIAL AND METHODS

Hepatitis B surface antigen – HBsAg was obtained from positive blood donors plasma, purified in first step by affinity chromatography using Sepharose 4B (Pharmacia Fine Chemicals), activated with BrCN and coupled to hiperimmune anti-HBs rabbit serum (Porath et al., 1967; Houwen et al., 1975; Yoshida et al., 1984). Further purification was performed by three isopicnic centrifugations at 250000g in CsCl gradient during 24 hours, collecting the HBsAg in the fractions of density 1.20 (Bond & Hall, 1972).

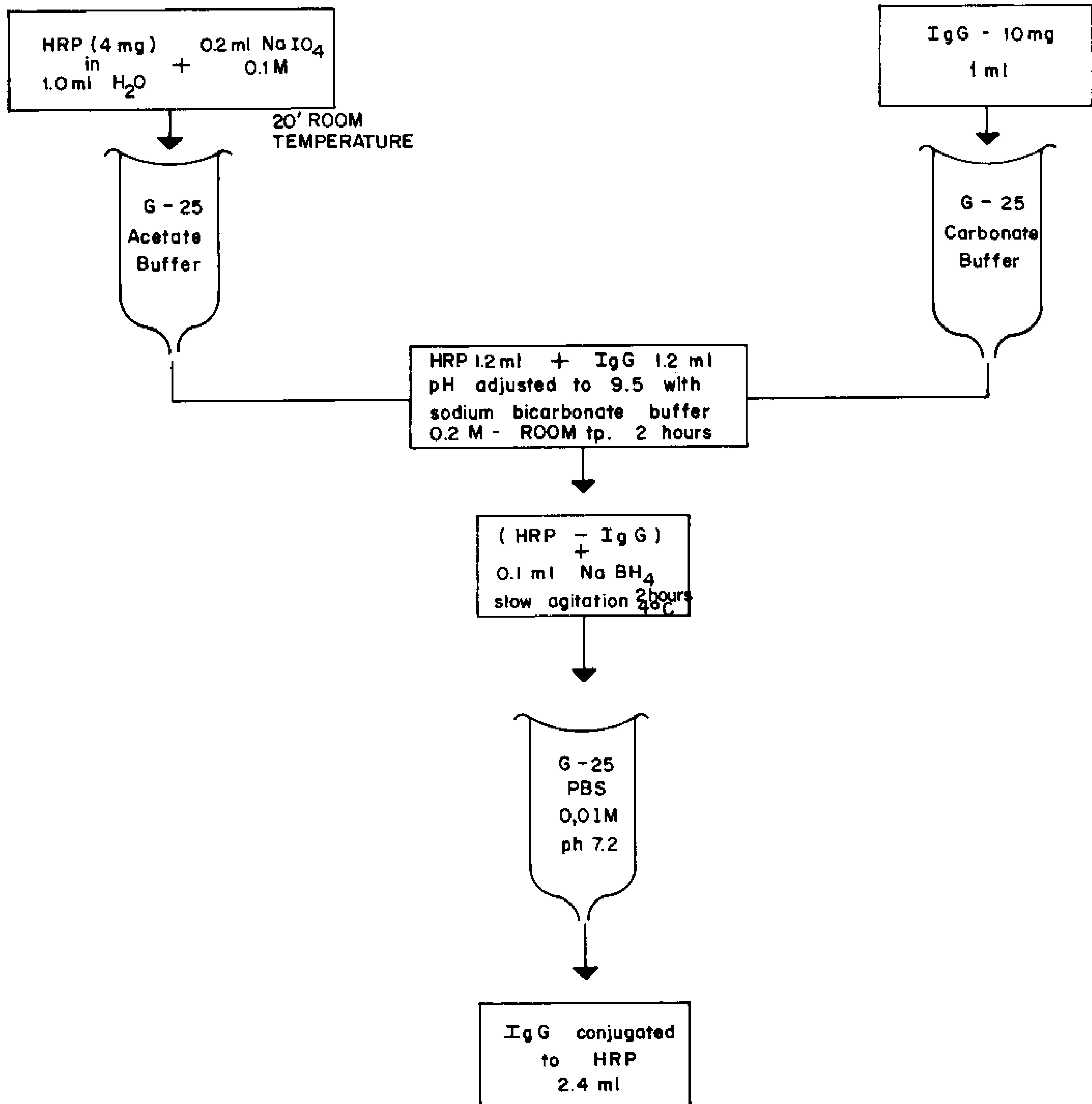
Anti-HBs immune sera – Goat, rabbit and guinea-pig immune sera were obtained after four intramuscular inoculations of purified HBsAg, at two weeks intervals. For each inoculation, 800, 80 and 40 μg of HBsAg were used for goat, rabbit and guinea-pig respectively. Antigen was emulsified in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant, for the three following injections. Immune sera were immunoadsorbed against normal human serum (NHS) coupled to BrCN activated Sepharose 4B.

Anti-HBs capture antibodies – Four different preparations were tested as capture antibodies: a) *C.97-4* – Goat anti-HBs gammaglobulin precipitated with ammonium sulphate, according to Hebert, Pelham & Pittman, 1973. This preparation had a protein concentration of 29,3 mg/ml and an anti-HBs titre of 1/1024 by counter-immunoelectrophoresis CIEP); b) *GT-102* – Goat anti-HBs IgG purified by precipitation with ammonium sulphate, followed by affinity chromatography with BrCN activated Sepharose 4B coupled to HBsAg. The CIEP titre of this preparation was of 1/128 and the protein concentration 1.4 (mg/ml; c) *R-104* – Rabbit anti-HBs IgG, purified in the same way as GT-102, showing a CIEP titre of 1/256 and protein concentration of 1.94 mg/ml; d) *GP-105* – Guinea-pig anti-HBs IgG purified in the same way as GT-102, with a CIEP titre of 1/8 and protein concentration of 0.351 mg/ml.

Conjugates – Two different methods were used to conjugate goat affinity chromatography purified anti-HBs IgG to horseradish peroxidase (Sigma). One lot was prepared using sodium metaperiodate ($NaIO_4$) by the method described

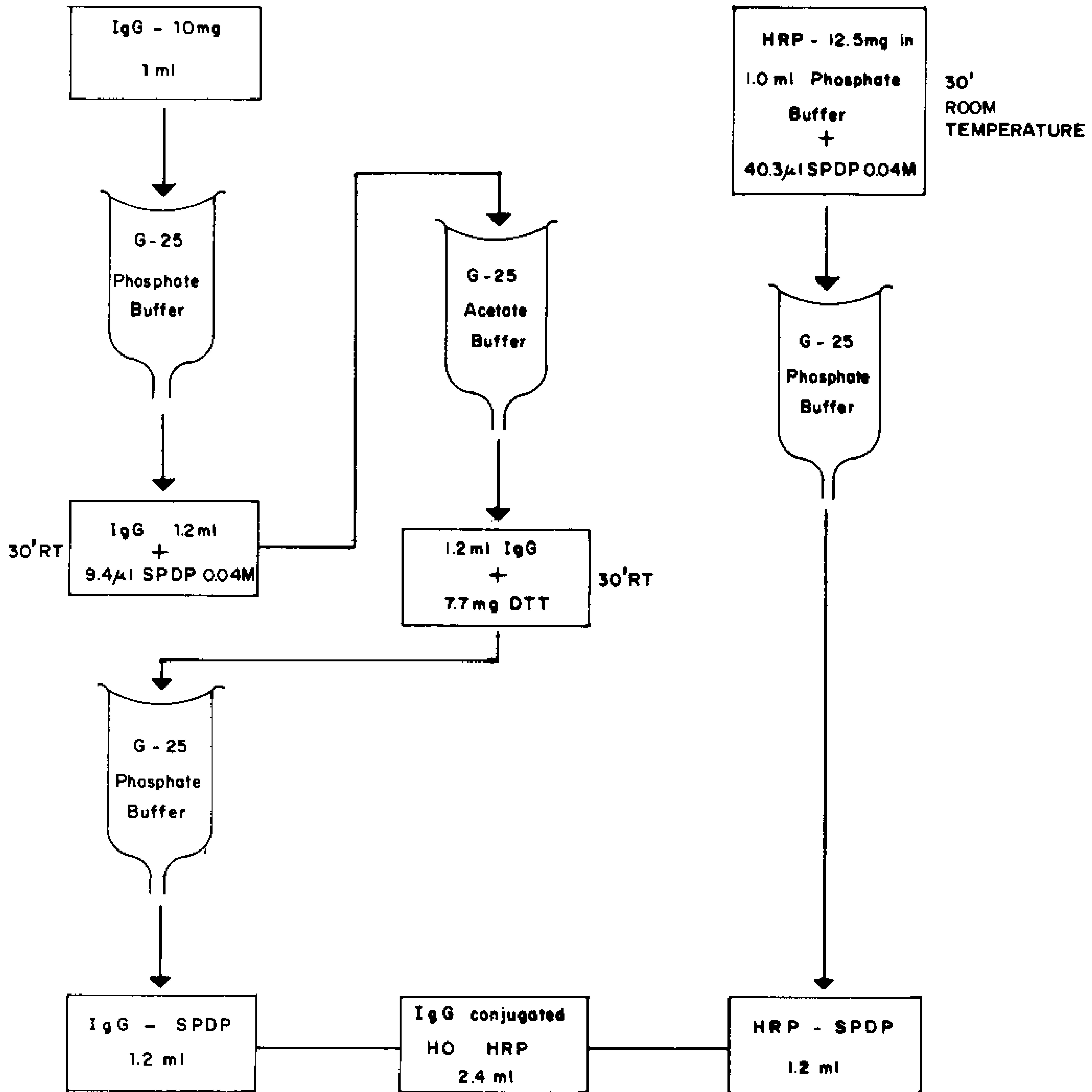
Received August 19, 1986.
Accepted September 24, 1986.

by Nakane & Kawaoi (1974) and another lot was prepared using N-succinimidyl 3 (2-pyridyldithio) propionate (SPDP), according to Nilsson et al. (1981). Both methods were used with modifications consisting in the use of Sephadex G-25 PD-10 colums instead of dialysis and concentration as shown schematically in Figs. 1 and 2.



- PHOSPHATE BUFFER 0,01 M pH 7.5
- SODIUM BICARBONATE BUFFER 0.2 M pH 9.5
- SODIUM CARBONATE BUFFER 0.01 M pH 9.5
- G-25 SEPHADEX PD-10 COLUMN (PHARMACIA FINE CHEMICALS)
- SODIUM BOROHYDRATE Na BH_4 0.4 % (SIGMA)
- ACETATE BUFFER 1 mM pH 4.4
- SODIUM M-PERIODATE $NaIO_4$ 0,1 M (SIGMA)

Fig. 1: Schematic representation of anti-HBs IgG conjugation to HRP by $NaIO_4$.



— PHOSPHATE BUFFER 0.1M pH 7.5
— SPDP HETEROBIFUNCTIONAL REAGENT (PHARMACIA FINE CHEMICALS)
0.04 M IN ABSOLUTE ETHANOL
— ACETATE BUFFER 0.1 M pH 4.5
— SEPHADEX G-25 - PD-10 COLUMN (PHARMACIA FINE CHEMICALS)
— DTT (-) 1.4 DITHIO - L - THREITOL GRADE II (SIGMA)
— HRP - HORSERADISH PEROXIDASE RZ > 3.0 25.000 UNITS (SIGMA)

Fig. 2: Schematic representation of anti-HBs IgG conjugation to HRP by SPDP.

Test performance – Microplate wells (Dynatech) were coated with 100μl of anti-HBs IgG (capture antibody) diluted in 0.2M carbonate buffer (C.B.) pH 9.6. After 18 hours at 4°C the wells were aspirated and washed three times (Dynawasher) with phosphate buffered saline (PBS) containing 0.05% of Tween 20 (PBST). A volume of 100μl of each sample was dispensed into each well. Two wells received 100μl of positive control and five wells received 100μl of negative control (pool of normal human sera). The microplate was incubated at 37°C during 2 hours, the wells were washed three times with PBST and 100μl of the conjugate diluted in PBS with 25% of normal goat serum and 25% of normal human serum was

pipetted into all wells, except the two substrate control wells. After 2 hours of incubation at 37°C and washing three times in PBST, 200μl of freshly prepared substrate [O-Phenylenediamine (OPD) 0.4 mg/ml and H_2O_2 0.15% in Phosphate-Citrate Buffer pH 5.0] was added to each well and incubated in the dark for 10 minutes, at room temperature. The enzyme reaction was stopped by adding 50μl of H_2SO_4 2M. The enzyme reaction product shows a yellow colour and both spectrophotometric and visual reading were performed. Eye reading was against a diffuse white light underneath the microplate and samples with more colour than the negative controls were considered to be positive. Spectrophotometric reading was performed at 492nm (Titertek) and samples showing absorbance at least twice as high as the average of five readings of negative control sera (cut-off ratio of 2.1), were considered positive.

Checkerboard titration – To establish the optimal conditions of capture antibody and conjugate dilutions, a checkerboard titration was performed using a boarderline HBsAg positive control and normal human serum (NHS) as negative control. Each combination of capture and conjugate was tested in different dilutions with the positive and negative controls, and the best was considered to be the one showing the highest absorbance for the positive control and the lowest absorbance for the negative control, i.e., the highest P/N value.

Sensitivity and specificity – A reference panel, MPR-Bio-Manguinhos, consisting of 85 lyophilized plasma samples, containing strongly positive, boarderline positive and HBsAg negative samples presented in code, was used to determine the sensitivity and specificity of the test, comparing with RIA (Austria II, Abbott Lab), which was considered as a standard condition.

Antigen captation – Serial two fold dilutions of purified HBsAg in normal human serum, were used to determine antigen captation for both ad and ay subtypes.

RESULTS

Table I illustrates the checkerboard titration of anti-HBs capture sera obtained from different animal species against goat anti-HBs IgG conjugated to HRP by either $NaIO_4$ or SPDP. The optimal dilutions of capture and conjugate to be used in the test were chosen with basis on the highest P/N rate obtained from each combination of both. P/N values obtained when goat affinity chromatography purified anti-HBs IgG (GT-102) was used as capture, were noticiably lower than the ones obtained with capture antibodies from guinea-pig and rabbit. Non-affinity chromatography purified goat gammaglobulin capture (C.97-4) yielded lower P/N values than guinea-pig and rabbit captures only when used with $NaIO_4$ conjugate in the same test.

TABLE I

Positive/Negative (P/N) values obtained in the checkerboard titrations of different conjugates and captures

Capture dilution (μg/ml)			Conjugate dilution: $NaIO_4$ 1:1000	$NaIO_4$ 1:2000	$NaIO_4$ 1:5000	SPDP 1:2000	SPDP 1:5000	SPDP 1:8000
GT-102	1:500	(2.8)	1.4	1.7	1.8	3.3	2.4	4.4
	1:1000	(1.4)	1.5	2.3*	1.9	4.2	6.8*	3.9
C.97-4	1:5000	(1.4)	5.3*	4.0	4.7	13.0	11.9	12.9
	1:10000	(2.9)	3.8	4.6	3.7	17.15*	11.5	9.7
R–104	1:500	(3.8)	9.8	12.1*	11.7	17.9	16.8	12.2
	1:1000	(1.9)	8.5	8.7	11.0	15.8	16.6	19.4*
GP–105	1:100	(3.5)	8.1	8.6	8.4	9.9	7.8	10.7
	1:250	(1.4)	8.7	10.9*	10.3	11.2	9.83	12.0*

* Highest P/N

The results of sensitivity and specificity obtained using the reference panel MPR-Bio-Manguinhos are illustrated in Table II. All positive samples by RIA were positive by ELISA when guinea-pig or rabbit capture were used combined with SPDP conjugate. The same sensitivity of 100% was obtained with guinea-pig capture and $NaIO_4$ conjugates, while with rabbit capture and $NaIO_4$ conjugate sensitivity was of 98.5%. A sensitivity of 98.5% was achieved when both GT-102 and C.97-4 goat captures were used with SPDP conjugate. The lowest sensitivity, 97% (two false negative) was obtained when goat affinity chromatography purified anti-HBs capture was used with $NaIO_4$ conjugate. None of the combinations of capture and conjugate gave false positive results, except C.97-4 goat capture (non-affinity chromotography purified) with $NaIO_4$ conjugate that showed two false negative results, with a specificity of 94.7%.

Table III shows the antigen captation obtained with the different combinations of capture and conjugate sera. For subtype ad, the highest captation (4.37 nanograms/ml) was achieved with guinea-pig capture used combined with both SPDP or $NaIO_4$ conjugate. For ay subtype, antigen captation was higher (4.37 ng/ml) with guinea-pig capture and SPDP conjugate or rabbit capture, with $NaIO_4$ conjugate. The lowest captation for both ad and ay subtypes was achieved with the twó goat captures used combined with $NaIO_4$ conjugate.

DISCUSSION

The sensitivity and specificity of an ELISA system for antigen/antibody detection depend upon all the components involved in the test. In the direct method for HBsAg determination, important factors that contribute to the overall sensitivity are: the solid phase used for coating, the capture antibody, the antibody used for conjugation, the enzyme, the conjugation procedure, and the substrate, besides pH, temperature and incubation time. Most of these parameters are well established already. In the present work, our effort was mainly directed towards establishing a good animal source of anti-HBs to be used as capture and a simple and efficient conjugation procedure. Goat anti-HBs IgG was chosen for the linkage to horseradish peroxidase, since goat has the advantage of

TABLE II

Sensitivity and specificity of the different preparations of captures and conjugates used in the optimal dilution tested with the reference panel MPR-Bio-Manguinhos

Conjugate	Capture	TP	TN	FP	FN	Sensitivity %	Specificity %
$NaIO_4$	GT-102	65	18	0	2	97.0	100
	C.97-4	66	17	1	1	98.5	94.7
	R-104	66	18	0	1	98.5	100
	GP-105	67	18	0	0	100	100
SPDP	GP-102	66	18	0	1	98.05	100
	C.97-4	66	18	0	1	98.05	100
	R-104	67	18	0	0	100	100
	GP-105	67	18	0	0	100	100

TP – True Positive; TN – True Negative; FP – False Positive; FN – False Negative.

TABLE III

Antigen captation (nanograms/ml) for subtypes ad and ay with different combinations of captures and conjugates

	Subtype ad		Subtype ay	
Capture \ Conjugate	$NaIO_4$	SPDP	$NaIO_4$	SPDP
GT-102	70.0	8.75	70.0	35.0
C.97-4	70.0	8.75	35.0	8.75
R-104	8.75	8.75	4.37	8.75
GP-105	4.37	4.37	8.75	4.37

providing large volumes of immune serum with high antibodies concentration. Horseradish peroxidase has been widely used in ELISA systems and has advantages over alkaline phosphatase. It is less expensive, it is a glycoprotein and so can be linked to the antibody by means of the carbohydrate portion, thus leading to less interference with enzyme function. The use of O-phenylenediamine (OPD) as substrate produces an intense yellow colour that provides more definite visual determination (Yolken, 1982).

To establish the best conditions for the test, a checkerboard titration was performed to determine the equilibrium point of capture and conjugate concentrations. The theoretical optimal dilutions yield the maximum value of P/N and this was the criterion used to formulate the combinations of each pair of capture and conjugate. However, for C.97-4 capture serum and its combination with both conjugates, this criterion was not used since visual reading did not permit clear discrimination, either due to a colored negative or a very weakly colored positive, in the highest values of P/N.

The use of bifunctional reagents such as N succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or aldehyde generating reagents as sodium metaperiodate ($NaIO_4$) has been described for the covalent linkage of enzymes to IgG molecules. The conjugation procedures are usually laborious and time consuming. The use of Sephadex G-25 PD-10 colums substitutes dyalisis and concentration reducing to about 3 hours the time required for conjugation of IgG to horseradish peroxidase, whether using SPDP or $NaIO_4$. When using guinea-pig or rabbit anti-HBs IgG as capture, both conjugates showed no remarkable difference in sensitivity or antigen captation. The antigen captation ranged from 4.37 to 8.75 nanograms/ml. These results are in accordance with data found in the literature for commercially available kits (Lostia et al., 1983).

The use of capture and conjugate antibodies from the same animal source, in this case goat, decreased the test quality when $NaIO_4$ conjugate was used. P/N values were lower and there was some background making the visual reading less reliable. Using affinity chromatography purified goat anti-HBs IgG (GT-102) as capture, we observed the same contradiction described by Fields et al. (1983). This purified preparation that one should expect to increase the test sensitivity since it has high specificity towards HBsAg, yielded less sensitive results (97%) than $(NH_4)_2SO_4$, precipitated goat gammaglobulin (98.5%) when used combined with $NaIO_4$ conjugate to test the panel MPR-Bio-Manguinhos, although specificity was higher. With SPDP conjugate, both GT-102 and C.97-4 goat anti-HBs captures showed the same sensitivity and specificity of 98.5% and 100% respectively. The antigen captation for ad subtypes was also the same (8.75 nanograms/ml), however, ay antigen captation was of 35.0 nanograms/ml with GT-102 and of 8.75 nanograms/ml with C.97-4.

These results lead us to recommended the use of guinea-pig or rabbit as source of capture anti-HBs IgG if goat anti-HBs is used for linkage to enzyme. Comparison of conjugates prepared by both procedures showed similar results concerning the parameters evaluated. Data shown in the literature demonstrate that SPDP method allows greater recovery of anti-HBs and HRP activities than $NaIO_4$ method (Sito & Mackeen, 1982). This allows the use of higher dilutions of conjugate, which is economically advantageous.

With optimized reagents, ELISA answers the requirements for a highly sensitive method for HBsAg determination in blood from donors and patients, and is an efficient alternative method to RIA for wide scale application.

RESUMO

Comparação de reativos para o teste de ELISA na evidenciação do antígeno de superfície da hepatite B – Conjugados de IgG anti-HBs de cabra e peroxidase, preparados por dois métodos diferentes, um usando $NaIO_4$ e o outro SPDP foram comparados. Anticorpos anti-HBs obtidos de cabra, coelho e cobaio foram testados como captura. O ELISA apresentou sensibilidade similar ao RIA e o nível de captação antigênica foi de 4,37 a 8,75 nanogramas/ml quando utilizados captura de coelho ou cobaio combinados tanto com o conjugado pelo $NaIO_4$ como com o conjugado pelo SPDP.

ACKNOWLEDGEMENTS

We are very grateful to Dr. H.G. Pereira for comments, suggestions and review of the manuscript.

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