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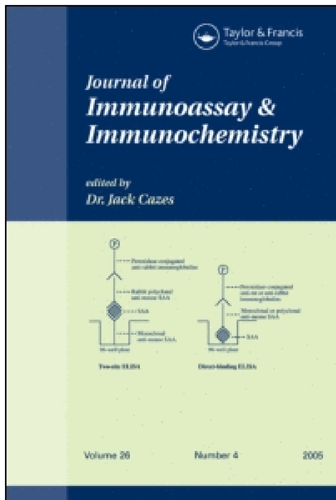

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Enzyme Immunoassay to Measure Low Levels of Haptoglobin in Biological Fluids

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ENZYME IMMUNOASSAY TO MEASURE LOW LEVELS OF HAPTOGLOBIN IN BIOLOGICAL FLUIDS*

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ABSTRACT

A novel immunoenzymatic assay is described for the quantitation of human haptoglobin (Hp). Two binding sites on the Hp molecule, namely for hemoglobin (Hb) and for the specific antibody, are involved in the reaction. Hb adsorbed onto the polystyrene microplate binds Hp present in any biological fluid. The formed Hp-Hb complex is detected with horse-radish peroxidase conjugated with anti-Hp antibody. By means of this ELISA, Hp may be measured in the range of 5 to 150 µg/L. Comparison of the Hp-ELISA with two other methods of Hp determination resulted in correlation coefficients of 0.97 to 0.99. Intra- and inter-assay coefficients of variation ranged from 4.7 to 6.7 %. Hp levels were measured in urine, cord serum, cerebrospinal fluid, amniotic fluid and saliva.
(KEY WORDS: Haptoglobin, ELISA)

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Abbreviations used: Hp, haptoglobin; Hb, hemoglobin; anti-Hp, antibody directed against human haptoglobin; HRP, horse-radish peroxidase; PEG, polyethylene glycol 6000; PBS, phosphate buffered saline; T, Tween 20.

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INTRODUCTION

Haptoglobin (Hp) is an α_2-glycoprotein, present in biological fluids of many mammalian species. The most characteristic property of Hp is to bind hemoglobin (Hb) in one-to-one ratio, yielding a relatively high molecular weight complex, which is rapidly removed from circulation by the liver. Hp belongs to the acute phase glycoproteins, thus its serum level is markedly increased in malignancy, infections, trauma, and inflammations. On the other hand, Hp level is significantly decreased or Hp is absent in neonatal serum and following intravascular hemolysis (for review see ref.1).

Serum Hp may be measured by peroxidase activity of Hp-Hb complex (2), electrophoresis (3), immunodiffusion (4), differential acid denaturation of Hb and Hp-Hb complex (5), immunonephelometry (6), etc. However, all of the methods are not sufficiently precise, when Hp is present in low amounts.

The aim of our work has been to create an enzyme-linked immunosorbent assay for quantitative estimation of human Hp in biological fluids of low Hp content.

MATERIALS AND METHODS

Materials

Cerebrospinal fluids were obtained from Clinic of Neurology, pleural effusions from Clinic of Pulmonology,

samples of saliva from Clinic of Preventive Dentistry, cord sera from Clinic of Neonatology, Medical Academy, respectively; amniotic fluids were from the City General Hospital; urines were obtained from healthy volunteers and athletes from Academy of Physical Training.

Human Hp type 2-1 was isolated from ascitic fluid of a patient with ovarian carcinoma (7); the preparation formed one peak in crossed immunoelectrophoresis.

Hb was prepared from horse blood (8).

Antiserum directed against human Hp, produced in goat, and antibody against Hp linked with horse-radish peroxidase (anti-Hp-HRP), were prepared as described previously (9). HRP was from Calbiochem (USA).

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Hp-Enzyme-Linked-Immunosorbent Assay (Hp-ELISA).

Principle. The Hp-ELISA is based on the specific interactions of two different binding sites on the Hp molecule, namely for Hb, and for anti-Hp antibody, resp. Horse Hb, adsorbed on the polystyrene microplate binds quantitatively Hp present in any biological fluid. The formed Hp-Hb complex is detected by means of the anti-Hp-HRP conjugate.

Reagents

1) PBS; phosphate buffered saline, pH 7.3 containing 0.2 g/L Thiomersal (BDH, England).

2) PBS-T; PBS containing 0.5 ml/L Tween 20 (Schuchard, FRG).
3) PBS-T-PEG; PBS-T containing 20 g/L polyethylene glycol 6000.
4) 0.5% horse Hb in PBS; stock solution (~ 100 g/L) with 0.2% Thiomersal was stored in small aliquots at -20^oC and diluted immediately before use.
5) Hp standard; 2-40 ng of pure Hp in PBS-T per well. Properly diluted serum containing Hp may be used as the standard.
6) Anti-Hp-HRP; stock solution of the conjugate (5.8 mg/ml) was stored at -20^oC in PBS buffer, pH 7.3 , containing 50% glycerol, 0.2 g/L Thiomersal, 10 g/L bovine serum albumin. Stock solution of anti-Hp-HRP was diluted 2000 times with PBS-T-PEG before use.

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Test procedure

1) 200 μl of 0.5% Hb in PBS was added to the wells of microtiter plate, incubated for 60 min at 37^oC, and overnight at 4^oC, followed by 5 washing cycles with PBS-T: once fast, and 4 times with 3 min intervals between.
2) For the construction of the calibration curve, 200 μl of standard Hp solution or respective dilution of standard human serum in PBS, control samples, and

unknown unhemolysed samples in at least two dilutions, were added, incubated for 30 min at 37°C, followed by 5 cycles of washing with PBS-T-PEG.

Calibration curve was prepared for each series of analyses. As a rule, samples giving an amount of Hp per well higher than 40 ng, should be diluted. Sera of adults, cancer and pleural effusions had to be freshly diluted 10 000 times with PBS-T and 50 µl of such diluted samples was taken for the test. Other biological fluids, like cord serum, urine, saliva, amniotic fluid, cerebrospinal fluid, were taken usually without predilution.

We recommend to put into the wells cold solutions of standards and samples as well as to use cold washing solutions.

3) 200 µl of anti-Hp-HRP in PBS-T-PEG was added to each well and incubated at 37°C for 30 min, followed by 5 cycles of washing with PBS-T-PEG.

4) 200 µl of substrate solution (0.5 mg o-phenylenediamine in 1 ml of citrate buffer, pH 5.0) was added to each well. After incubation in dark at room temperature for 30 min, 50 µl of 12.5% H_2SO_4 was added and the absorbance was measured at 492 nm in Stripreader Microelisa System (Organon Teknika, Holland).

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All analyses were carried out in triplicate.

Hp concentration was determined besides by Hp-ELISA, by the peroxidase method of Jayle (2), and by rocket immunoelectrophoresis (10).

RESULTS AND DISCUSSION

I. Assay conditions and precision.

Incubation of microtiter plate with 0.5% Hb in PBS for 60 min at 37°C, and overnight at 4°C, was established as optimum conditions for coating. The blocking step is missing, because Hb binds to Hp very quickly and strongly. The association constant for the reaction of Hb binding to Hp is higher than 10^{15} (1). There was no Hp binding to the surface of microtiter plate. The binding of Hp to immobilized Hb achieved a maximum after 30 min incubation at 37°C (Fig. 1).

The values of control absorbances repeated on different plates on 30 days were either 0.123 ± 0.03 in the absence of serum, or 0.126 ± 0.025 in the presence of serum or biological fluids not containing Hp.

Calibration curve is shown in Fig. 2. Similar curves were obtained either with purified Hp preparations or with human standard serum. Hp concentrations read from the standard curve in the range of 5 to 150 µg/L, were

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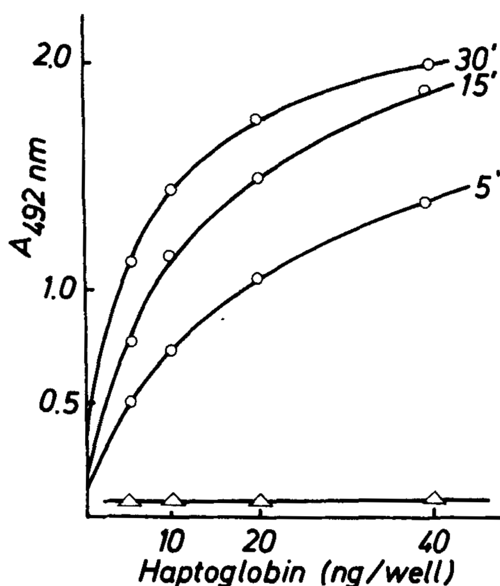

FIGURE 1. Time dependent interaction of Hb with Hp o—o. In the control Δ—Δ , Hp was incubated in PBS-T with the plate without immobilized Hb. All values are means out of 4 independent measurements. Experimental details as in Methods.

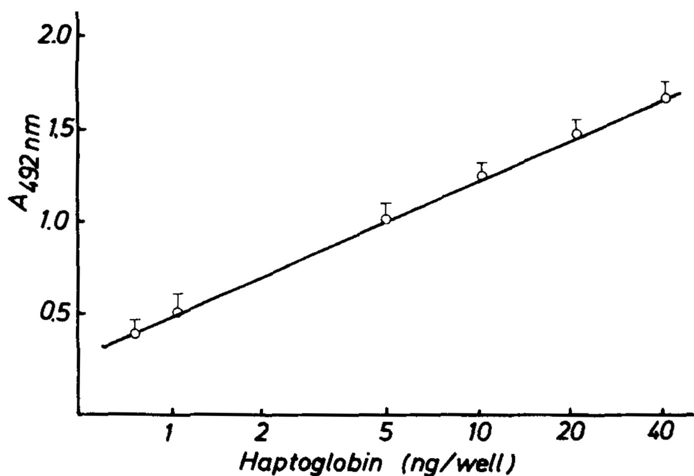

FIGURE 2. Calibration curve prepared by the use of purified Hp. Each point represents mean value ± SD out of 10 experiments, repeated with different microtiter plates. Experimental details as in Methods.

TABLE 1. Precision of the Hp-ELISA.

Sample	n	Hp concentration mg/L Within day*	Between-day**
Cerebrospinal fluid	10	0.494 ± 0.03 (5.5)	0.48 ± 0.03 (6.5)
Urine	10	3.8 ± 0.18 (4.7)	3.81 ± 0.19 (4.9)
Serum	10	434.5 ± 27.0 (6.3)	442.8 ± 30.0 (6.7)

Values are means ± SD. In parentheses coefficients of variation in percentage, are given.
*Within-day precision was calculated from repeated assays on the same day, with different microtiter plates.
**Between-day precision was calculated from repeated assays with different microtiter plates on several days, each time with an own standard curve.

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used. The minimum detectable concentration calculated as 3 SD of the mean out of 30 determinations of blank (PBS-T), was 4.0 µg/L.

The maximum of "pseudoperoxidase" activity of Hb, and "true" peroxidase activity of Hp-Hb complex, are manifested at pH range of 4.0 to 4.4 (ref.1). In our test the reaction of Hp binding to Hb and that of anti-Hp-HRP to Hp-Hb complex, is performed at pH 7.3. Peroxidase activity revealed in the Hp-ELISA is developed at pH 5.0, therefore it belongs mainly to anti-Hp-HRP (value of

control absorbance was twice lower than the lowest point of the curve).

Serum, urine and cerebrospinal fluid were analysed repeatedly for the within-day and between day precision (Table 1). Variation coefficients obtained by the Hp--ELISA were 4.7 - 6.7%.

II. Comparison of the Hp-ELISA with other methods.

Results of Hp determinations (0.05 to 8.0 g/L),carried out by Hp-ELISA were compared to those obtained either by electroimmunoassay (10) or by the method of Jayle (2) (Fig.3-A,B). There were excellent correlations (r= 0.99 and 0.98) between Hp-ELISA and both of the methods.

III. Determination of Hp levels in biological fluids.

Hp concentrations in biological fluids were determined by the Hp-ELISA (Table 2). Distribution of Hp levels depended on the origin of a fluid and patho-physiological state of subjects. In urines of healthy adults Hp concentrations were mainly in the range of 4 to 8 µg/L. After strenuous exercise Hp levels were higher than 9 µg/L in almost half of the cases, but in the same time, in 13 out of 33 cases, Hp was undetectable, probably as a symptom of individual stress reaction. In urines from diabetics one could observe Hp levels of 10 to 999 µg/L.

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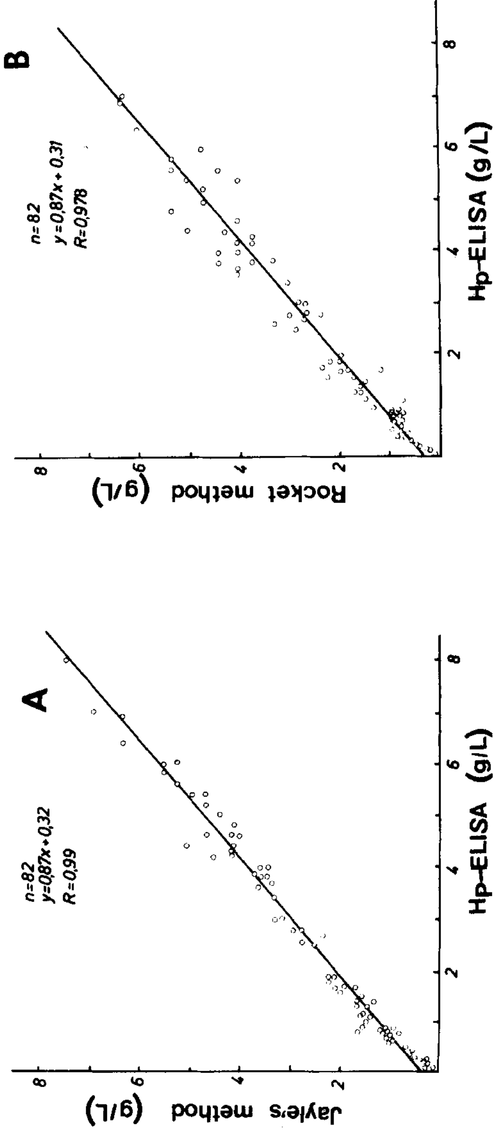

FIGURE 3. Correlation of Hp values in serum of 82 persons, as measured by the method of Jayle (2) - A, and by rocket electroimmunoassay (10) - B.

TABLE 2. Distribution of Hp concentrations in biological fluids

Biological fluid	n	Percentage distribution of samples according to Hp concentration in µg/L				
		0	4-8	10-99	100-999	≥1000
I. URINE						
Normal adults	18	5.6	94.5	0	0	0
Adults - strenuous exercise	33	39.4	12.1	21.2	21.2	6.1
Diabetes mellitus	27	0	59.3	33.3	7.4	0
II. CEREBRO-SPINAL FLUID						
Aneurysm	33	87.8	3.0	6.2	0	3.0
Protrusion of pulpy nucleus	19	42.0	5.3	15.8	21.1	15.8
Cancer of the brain	15	53.3	6.7	0	33.3	6.7
Hydrocephalus	3	0	0	0	0	100.0
III. CORD SERUM	71	88.8	5.6	0	2.8	2.8
IV. AMNIOTIC FLUID	15	6.7	73.3	0	20.0	0
V. SALIVA	74	31.1	12.2	16.2	16.2	24.3

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From 42 to 88% cerebrospinal fluids of patients with non-imflammatory diseases (protrusion of pulpy nucleus, aneurysm) had no detectable Hp. In cancer of the brain rather high levels of Hp were found in 40% of cases, while in 3 cases of hydrocephalus (100%), Hp concentrations ranged from 1000 to 10 000 µg/L.

Almost 90% cord sera did not contain Hp at all; 73% samples of amniotic fluid showed low level of Hp (1 to 8 µg/L). Most of these samples with Hp > 100 µg/L derived from complicated pregnancies. It is known that human neonatal sera either do not contain Hp, or contain it at very low levels (1). Raam et al. (11) reported, that neonatal Hp differs in some biological and physico-chemical properties as compared with normal adult serum Hp. Nevertheless, neonatal Hp is able to bind Hb, therefore in our test, this Hp (if any) is bound to immobilized Hb. Neonatal Hp does not show immunological identity with normal adult Hp, but demonstrates some similarities in antigenic determinants to tumor associated Hp from malignant tissues and fluids (12,13). We prepared anti-Hp antibodies after immunization of the goat with Hp, derived from the ovarian cancer ascitic fluid (9). Thus, we might expect that our antibodies would react with neonatal Hp.

The range of Hp levels in saliva was very wide, from 0 to 22 000 µg/L. The samples of saliva were taken from

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the people of different age and various states of the oral cavity, thus further characteristics of samples is necessary, in order to draw out eventual relationship between Hp level and pathology in dentistry.

Marsden and Simmonds (14) developed an ELISA for rat and mouse serum Hp, using different conditions of the test (buffers, coating and binding, mouse Hp and Hb, detection with alkaline phosphatase). However, they obtained no clear reactivity with human serum nor did purified human Hp gave a titratable response from 10 μg/ml.

We believe, that besides above shown examples, our Hp-ELISA could be of special significance in measurements of Hp levels in cord sera and amniotic fluids (diagnosis of intrauterine fetal infections), effusions of different etiology (discrimination of transudative vs. exudative ascites), adult sera of low Hp content (hemolytic anemias), in diagnosis of true anhaptoglobinemia, respectively.

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