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Research Article

Online immunoaffinity assay-CE using magnetic nanobeads for the determination of anti-*Helicobacter pylori* IgG in human serum

About two-thirds of the world's population is infected with *Helicobacter pylori* (*H. pylori*). This Gram-negative bacterium is the most important etiological agent of chronic active type B gastritis and peptic ulcer diseases. Conventional methods such as gastric biopsy, ELISA and culture, require a long time for the determination of *H. pylori* infections. Moreover, the antibodies in human serum sample are capable to react immunologically with the purified *H. pylori* antigens immobilized on different kinds of support like magnetic nanobeads. In this study, we have developed an online immunoaffinity assay-CE to determine the concentration of anti-*H. pylori* IgG using magnetic nanobeads as a support of the immunological affinity ligands and an LIF as a detector. The separation was performed in 0.1 M glycine–HCl, pH 2, as the background electrolyte. The linear calibration curve to predict the concentration of *H. pylori*-specific immunoglobulin G antibodies in serum was produced within the range of 0.12–100 U/mL. The linear regression equation was $i = 492.86 + 96.03 \times C_{anti\text{-}H.\ pylori}$, with the linear regression coefficient $r^2 = 0.999$. The LOD calculated by fluorescence detection procedure was of 0.06 U/mL. The whole assay was done in no more than 35 min and it was entirely automatized. The development of immunoaffinity assay-CE in this study demonstrates that there is a large possibility to introduce nanotechnology in several fields with significant advantages over the classic methodologies. Our proposition comprises the diagnosis and screening field.

Keywords:
H. pylori / Human serum / Immunoaffinity assay-CE / LIF / Magnetic nanobeads
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1 Introduction

The Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) is the most important etiological agent of chronic active type B gastritis and peptic ulcer diseases. The infection produced by this microorganism is a risk factor in the development of gastric mucosa associated with lymphoid tissue lymphoma and adenocarcinoma [1]. In general, the infection does not indicate specific clinical symptoms and the negative impact of this illness over the Public Health is significant. Chronic gastritis and peptic ulcer are very common diseases across the world populations. Moreover, gastric cancer is in the second place among the causes of cancer death worldwide [2].

The goal of *H. pylori* treatment is the elimination of the microorganism for the human body. The combination of two or more antimicrobial agents is very important because they increase the percentage of people cured and reduce the risk of select resistant *H. pylori* strains. The most important antimicrobial agents used in the treatments for these infections are amoxicillin, clarithromycin, metronidazole and tetracycline [3]. During the lasts years, the concern about the diagnosis of this microorganism has been the subject of several reviews and numerous original articles that reported the use of the different invasive methods such as endoscopy, culture and a quick urease test. Otherwise, the noninvasive tests including serological tests and the urea breath test [4, 5]. Some of the serological tests are based on the determination of immunoglobulin G (IgG) against *H. pylori* in human serum. The anti-*H. pylori* IgG antibodies that can be found in the circulatory system have demonstrated to have a considerable value in the diagnosis of the active infections due to the reliable correlation between the

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Abbreviations: IA-CE, immunoaffinity assay-CE; **MNB,** magnetic nanobead

presence of the antibodies and the gastric mucosal colonization [6, 7]. Common serum IgG measurements are carried out using ELISA [8].

Immunoaffinity assays-CE (IA-CE) is a technique which combines immunocapture and CE separation [9–11]. In online IA-CE, a microextractor or a concentrator is introduced near the inlet of the capillary, which contains immunological affinity ligands that specifically retain the target analyte. Then a large volume of sample can be injected and the target analytes can be selectively captured. The washing and the cleaningup procedures are subsequently integrated online to remove the excess of the sample and the interferents which have been bonded in a nonspecific way. The bonded analytes are eluted and separated by CE and finally detected by a usual detection technique. Compared with the ELISA technique, IA-CE has significant advantages. Mainly, it is a miniaturized technique that combines speed, automation and low sample consumption. Moreover, the analyte concentrator at the forepart of the capillary is enable to capture the analyte from a sample. Depending on the concentration of the target analytes in the sample, volumes can be varied to obtain a suitable sensitivity. Compared with microliter wells commonly used in immunoassays, in IA-CE the reactive surface area *versus* solution volume ratio is large and the diffusion distances are reduced in capillaries and microchannels [12]. In addition, a large number of analytes are attached in a small volume, allowing a very sensitive detection [13, 14]. Additionally, the separation step decreases the probability of false-positive results as an additional parameter, electrophoretic mobility, could be used for increasing the identification of the nature of the peak. ELISA is prone to yield false-positive and false-negative data [15] and cannot be coupled to analytical separation instruments or mass spectrometry. An alternative technique to conventional ELISA is a microarray-based immunoassay termed ELISA microarray technology [16, 17]. This emerging multiplexed sandwich immunoassay technique can ovoid many of the drawbacks of conventional ELISA; however, it can still yield over-reporting (false positives) and under-reporting (false negatives) results, and it is not capable of being coupled to mass spectrometry for the corroboration of the data. So far, several applications of IA-CE have so far been described for peptides and/or proteins. The main difference between each approach lies in the way to develop the immunosorbent within the capillary.

Covalent or noncovalent binding of antibodies on the surface of the capillary [18, 19], functional particles or beads [20, 21] and monolithic materials [22, 23] are the mostly used. IA-CE has been used to separate analytes of interest from complex biological samples based on the selective binding of antibodies with their respective antigens [24]. The magnetic nanobeads (MNBs) are an interesting tool as solid supports to bond different types of analytes on them.

Fluorescence spectrometry is one of the main detection methods for CE. Furthermore, LIF detection is one of the most sensitive detection techniques in CE, which is capable to achieving LODs below 10–13 M [25, 26]. Due to the advantages of high sensitivity, rapid resolution, high separation efficiency and small sample size, the CE-LIF system has been demonstrated to be powerful for the determination of low concentration of different compounds in several kinds of biological samples.

In this study, we have developed an IA-CE with MNBs 3-aminopropyl-modified as a support phase. The purified antigens of *H. pylori* were immobilized on the surface of the particles. The completely process was developing into the capillary, even the modification of the particles and the immobilization procedure. The detection of the antibodies in the serum samples was achieving using a noncompetitive immunoassay. The recognised sensitivity of the LIF detector allowed a rapid and sensitive quantification of human serum IgG antibodies against *H. pylori*.

2 Materials and methods

2.1 Reagents and solutions

All reagents used were of analytical reagent grade. The second specific antibody labeled against human-chain was purchased from Sigma Chemical (St. Louis, MO, USA). Glutaraldehyde (25% aqueous solution) was purchased from Merck (Darmstadt). The MNBs 0.2 μm amino functionalized were purchased from Ademtech (France) and 1 μm amino functionalized were purchased from Sigma Chemical. All solutions were filtered through 0.45 μm nylon membrane filters (Titan Syringe Filters, Sun Sri, Rockwood, TN, USA) and thoroughly degassed under vacuum before use. All other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q system.

The ELISA test kit for the quantitative determination of *H. pylori*-specific IgG class antibodies was purchased from EQUIPAR Diagnostici (Rome, Italy) and was used in accordance with the manufacturer's instructions.

2.2 Instruments

The CE separations were performed in a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, CA, USA) equipped with an LIF detector. The excitation light from an argon ion laser (3 mW) was focused on the capillary window by means of a fiber-optic connection. Excitation was performed at 488 nm and the electropherograms were recorded by monitoring the emission intensity at 520 nm. The data-handling system comprising an IBM PC and P/ACE System MDQ Software (ESANCO) was used. Fused silica capillaries were obtained from MicroSolv Technology (Eatontown, NJ, USA) and were 30 cm in total length, 20 cm effective length, 50 μm id and 375 μm od. All solutions and reagents were conditioned to 37°C before the experiment, using a laboratory water bath Vicking Mason II (Vicking SRL, Argentina). The pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research, Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion

Research). Absorbance was determined with a Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 General UV/vis spectrophotometer.

2.3 *H. pylori* specific IgG antibody immunoassay

A series of standards that covered the clinically relevant range (0.12–100 U/mL) were supplied with the ELISA test kit. A standard curve for the spectrophotometric procedure was produced by following the manufacturer's protocol [27]. Concentrations of *H. pylori*-specific IgG antibody were detected spectrophotometrically by measuring absorbance changes at 450 nm.

2.4 Preparation of the *H. pylori* antigens

The antigens were prepared from a sonicated *H. pylori* culture strain. *H. pylori* was grown on blood agar plates at 37°C or 3 days and then harvested, washed and resuspended in 20 mM PBS (pH 7.2). This preparation was subjected to sonication. The sonic amplitude level was set at 20, and the machine was operated using four cycles of 60 s regulated alternatively. The preparation was centrifuged at $1000 \times g$ for 10 min, and the supernatant was stored in the 20 mM PBS (pH 7.2), at −20°C between uses.

2.5 Procedure for the immunoaffinity reaction between the purified antigens of *H. pylori* with MNBs

Initially, the capillary was conditioned by flushing ultrapure water for 5 min, and with NaOH 1 M for 5 min, then water was flushed again for other 15 min. The integral process was developed online: the immobilization, the immunocapture and the separation. A suspension of 1.5 mg/mL of MNBs was flushed into the capillary and was retained in a fixed position into the capillary, near to the inlet of it by the action of a magnetic field generated by a removable external magnet which kept them in their place during the full experiment. Purified antigen of *H. pylori* was immobilized on MNBs modified with amino groups into the capillary. Once the particles were packed inside the capillary, they were rinsed with 20 mM PBS buffer, pH 7.2, for 15 at 0.5 psi and then with an aqueous solution of 5% w/w glutaraldehyde at pH 10.00 (200 mM carbonate) for 1 h at 0.5 psi. After 5 min, they were washed with PBS buffer, pH 7.2, to remove the excess of glutaraldehyde. Then, the capillary was rinsed with a solution 100 μg/mL of the antigen preparation, which was coupled to the residual aldehyde groups for 1 h. The MNBs with the antigen immobilized were finally washed with PBS buffer. The total process was accomplished at 37°C. The immobilized antibody preparation was perfectly stable for at least 1 month, whereas the capillary was stored at 4°C since it was not in use.

2.6 Procedure for the immunoaffinity reaction and separation

This method was applied in the determination of IgG antibodies to *H. pylori* in 38 human serum samples. An important problem in any immunoaffinity methodology system is the nonspecific absorption of proteins in the immunosorbent phase. This phenomenon usually decreases the selectivity of the methods, especially when complex samples, like serum, have to be analyzed. For this reason, it was very important to minimize these interactions as much as possible. Thus, the unspecific binding was blocked by rinsing with 1% of albumin in 20 mM PBS for 5 min at 0.5 psi, pH 7.2, and finally washing with 20 mM PBS buffer (pH 7.2) for 3 min at 0.5 psi. The serum samples were first diluted 100-fold with 20 mM PBS (pH 7.2) and then, they were injected at 0.5 psi for 10 min. The IgG-specific antibodies to *H. pylori* present in the serum sample reacted immunologically with antigens of *H. pylori* immobilized over MNBs. Then the capillary was washed with 20 mM PBS (pH 7.2) for 3 min at 0.5 psi to remove the excess of sample. The second specific antibodies against human IgG (dilution of 1/2000 in 20 mM PBS, pH 7.2) were rinsed at 0.5 psi during 5 min. Then, the capillary was rinsed with 20 mM PBS in order to wash the rest of the labeled-free antibodies that had not reacted with the complex for 5 min. Then the immune complex formed was washed with desorption buffer (0.1 M glycine–HCl, pH 2) at 1.5 min and the separation step was performed in the same buffer. Scheme 1 shows a representation of the reaction inside the capillary and the interaction between the molecules and the MNBs.

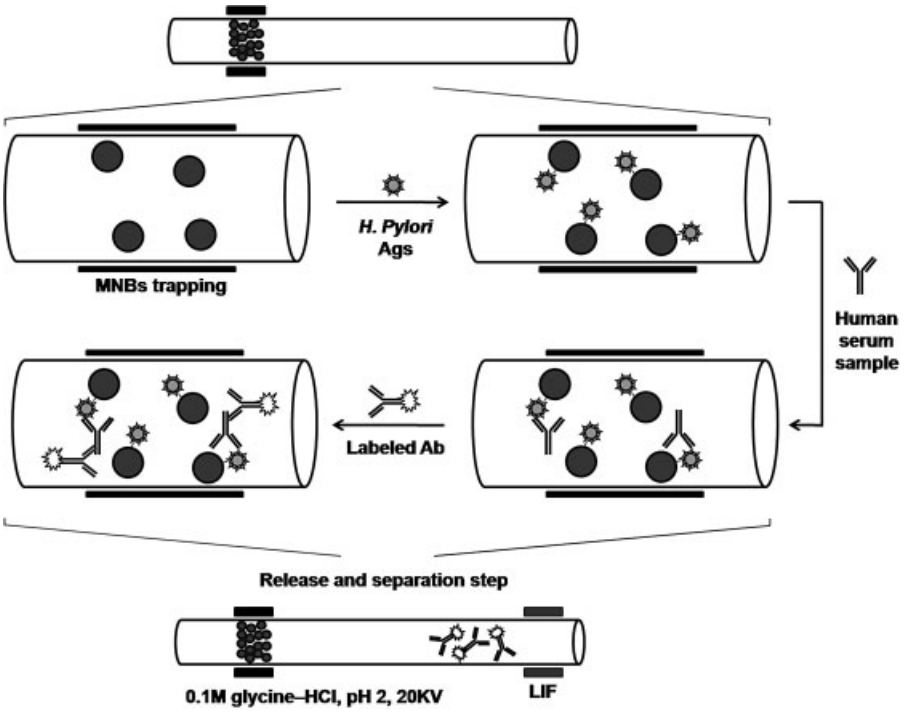
The determination of the second labeled antibody was followed using an LIF detector; the excitation was performed at 488 nm and the electropherograms were recorded by monitoring the emission intensity at 520 nm. After each separation, the capillary was washed with 20 mM PBS (pH 7.2). This system was developed to be used for at least 50 determinations without loss of sensitivity. All measurements were performed at 37°C.

A standard curve for the IA-CE with PMs method was built following our protocol with a series of standards that covered the clinically relevant range (0.12–100 U/mL). The proposed method was compared with the classical method (ELISA test kit).

3 Results and discussion

3.1 Retaining and stability of the MNBs in the capillary

MNBs with different diameters (1 and 0.2 μm) were investigated. Both of the different size particles could be used, but the smallest ones were chosen. This kind of beads stays as a uniform suspension for a considerable time, which is important in order to obtain a suitable reproducibility. Furthermore, the smallest ones provide a high surface area



Scheme 1. Schematic representation of the immunoaffinity assay-CE.

to volume ratio, which not only reduces the diffusion distance for all steps but also increases the density of binding sites for immobilization of antigens. It is important to mind that the magnetic force of the field which retains the MNBs must be stronger than the other competitive forces [27], which are pressure and electric field in the system. In our study, the MNBs were exposed to 1 and 0.5 psi. At 1 psi, the particles could not be retained by the magnet (Fig. 1); moreover, when the flow was decreased, the interaction showed to be better. Hence, we decided to inject and rinse the capillary with 0.5 psi. Another important factor was the stability of the MNBs under electric field and their behavior through several procedures. A range between 15 and 25 kV was studied. The capillary was rinsed and the different voltages were applied during 20 min after the injection of the MNBs in the capillary. Then, the MNBs were removed by applying high pressure. As shown in Fig. 2, no significant amount of particles was loss because the MNBs removed at the end of each analysis were similar. The following electrophoresis experiments were performed at 20 kV. Figure 3 shows the sensitivity of the system with different lengths of the injected plug of MNBs in the capillary. Considering the results, we decided to work with 4 cm of the capillary fill with particles.

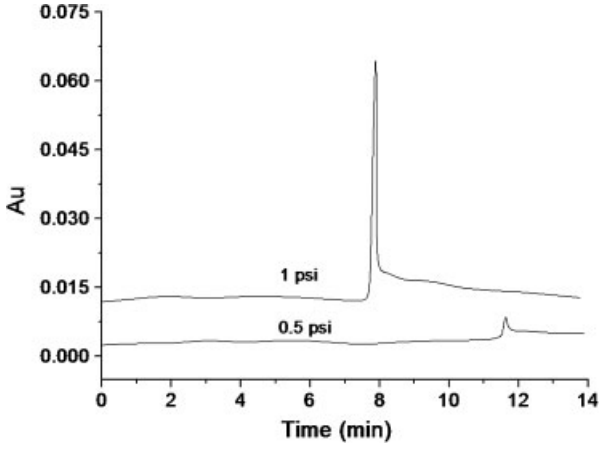


Figure 1. Stability of retained MNBs applying different pressures (0.5 and 1 psi). Capillary: total/effective length, 30/20 cm; UV absorbance at 210 nm in 20 mM PBS (pH 7.2).

3.2 Quantitative test for the detection of *H. pylori*-specific IgG antibodies with the proposed method

As shown in Fig. 4, the sensitivity can be linearly improved by increasing the sample injection time from 1 to 10 min, whereas the binding amount reached a saturation level after 10 min injection (Fig. 4). Thus, depending on the sample to be studied, as different sensitivity levels could be achieved, the experimental conditions can be tuned to fulfil the requirements. Under the conditions described above, the measurement of the realized labeled antibody was proportional to the concentration of *H. pylori*-specific IgG antibodies in serum. The overall time consumed for the IA-CE method was approximately 34 min once the particles were stabilized in the capillary. The migration time of the labeled antibodies was found at 2.45 min. Figure 5 shows an electropherogram of one serum sample. The same sample

was used to optimize the rest of the system parameters. A linear calibration curve to predict the concentration of *H. pylori*-specific IgG antibodies in serum was made within the range of 0.12–100 U/mL. The linear regression equation was $i = 492.86 + 96.03 \times C_{anti\text{-}H.\ pylori}$, with the linear regression coefficient $r^2 = 0.999$. The CV for the determination of 20 U/mL *H. pylori*-specific antibody was below 2.7% (six replicates). These values demonstrate that our system could be used to quantify the amount of *H. pylori*-specific IgG antibodies in unknown samples. The ELISA procedure was also carried out; absorbance changes were plotted against the corresponding *H. pylori*-specific IgG antibody' concentration and a calibration curve was constructed. The linear regression equation was $A = 0.137 + 0.029 \times C_{anti\text{-}H.\ pylori}$, with the linear relation coefficient $r^2 = 0.985$; the CV for the determination of 20 U/mL *H. pylori*-specific antibodies was 4.8% (six replicates). The LOD was considered to be the concentration that gives a signal three times the SD of the blank. The LOD for our proposed method and EIA procedure were 0.06 and 3.6 U/mL, respectively. This result shows a great advantage in terms of sensitive of our proposed method compared with the classic spectrophotometric method. The precision of the IA-CE method was checked with control serum at 20, 50 and 100 U/mL *H. pylori*-specific antibody concentrations. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results obtained are summarized in Table 1. The *H. pylori* assay showed good precision; the CVs for within-assay values were below 2.5% and for between-assay values were below 4.7%. The accuracy was tested with

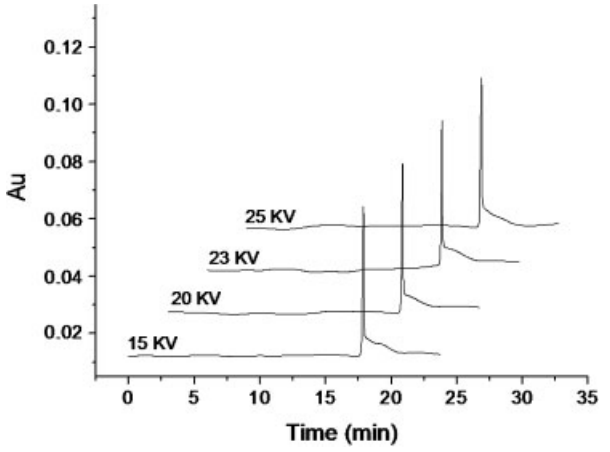


Figure 2. Stability of retained MNBs applying different voltages (15, 20, 23 and 25 kV) for 15 min. After this, the particles were removed by high pressure (1 psi) followed by a UV detector at 210 nm in 20 mM PBS (pH 7.2).

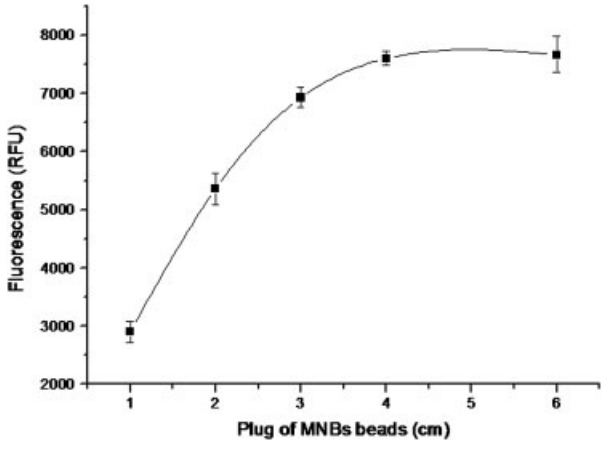


Figure 3. Effect of different lengths of the plug of retained modified MNBs using a 100 U/mL *H. pylori*-specific antibodies standard. Capillary: total/effective length, 30/20 cm; excitation, 488 nm; emission intensity, 520 nm in 0.1 M glycine–HCl, pH 2 ($n = 5$).

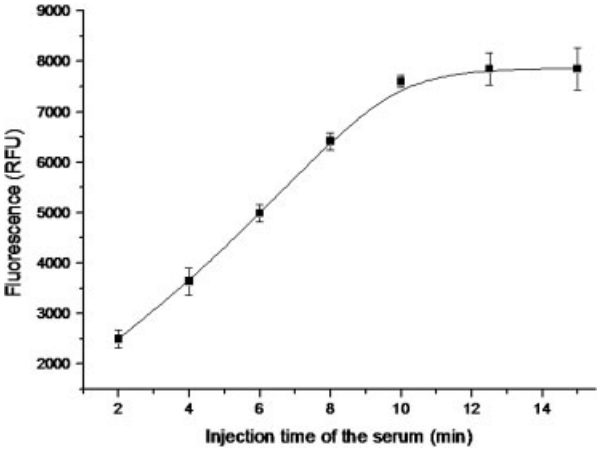


Figure 4. Effect of the sample injection time for a 100 U/mL *H. pylori*-specific antibodies standard. Capillary: total/effective length, 30/20 cm; excitation, 488 nm; emission intensity, 520 nm in 0.1 M glycine–HCl, pH 2 ($n = 5$).

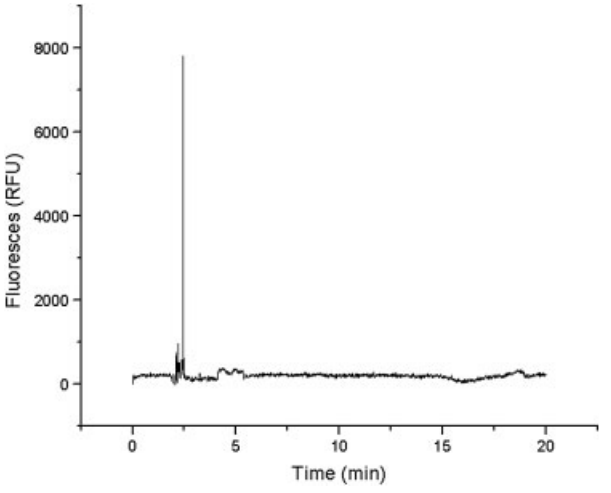


Figure 5. Electropherogram of the labeled *H. pylori*-specific antibodies in a real serum samples. Capillary: total/effective length, 30/20 cm; excitation, 488 nm; emission intensity, 520 nm in 0.1 M glycine–HCl, pH 2 ($n = 5$).

dilution and recovery tests. The total assay time, including all injections and detection steps for the *H. pylori*-specific IgG antibodies measurements, was less than 35 min (much less than the two and half hours normally used with conventional batchwell ELISA), which is more than five times faster than the plate method.

The proposed system was compared with a commercial spectrophotometric system for the quantification of *H. pylori*-specific IgG antibody in serum samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between both methods (Fig. 6). Compared with the commercial ELISA test kit, our method shows large enhancement in sensitivity. These results suggest that the detectable concentration of *H. pylori*-specific IgG antibodies in this system is at the levels of clinical analysis, and the sensitivity has reached to the levels to meet the determination *H. pylori*-specific IgG antibodies in serum even in patients with very low levels.

The results obtained in this study clearly demonstrate that this MNBs-based IA-CE methodology is effective and reliable for serum sample analyses. Compared with ELISA, the main advantage of the use of MNBs in IA-CE is the possibility to reuse the particles for several analyses. In fact, as it has been demonstrated, the MNBs can be easily washed in the capillary without the risk of loss an important amount of them. Under these conditions, if the nonspecific adsorption to the capillary walls is significantly avoided, different patients' serum samples can be analyzed successively in the same capillary without any memory effects. The high surface provided for the MNBs allows bonding a great number of antigens. Thus, the length of the capillary filled with them was only 4 cm. The integral process of modification was developed inside the capillary; it is a great advantage in terms of a less sample consumed than conventional immunoassay techniques. Moreover, the automatization of the CE allowed all the process without any incubation step. In addition, an increased reactive surface area and reduced diffusion distances in this system permitted a faster time of analysis (34 min).The possibility to couple CE with LIF increased the capability to determine low levels of IgG antibodies specific to *H. pylori* with high sensitivity. The disadvantages of this technique, compared with ELISA which is the reference methodology, are the portability and the price of the equipment. Although, we can suggest that nowadays, with the new progress applied to the CE on chip, it is fair to conclude that this technique could be miniaturized.

Table 1. Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated for three consecutive days)

Control sera (U/mL)[a)]	Within-assay		Between-assay	
	Mean	CV %	Mean	CV %
20	19.87	1.26	20.63	3.51
50	50.12	2.38	49.86	2.73
100	100.54	2.01	101.34	4.61

a) U/mL *H. pylori*-specific antibodies.

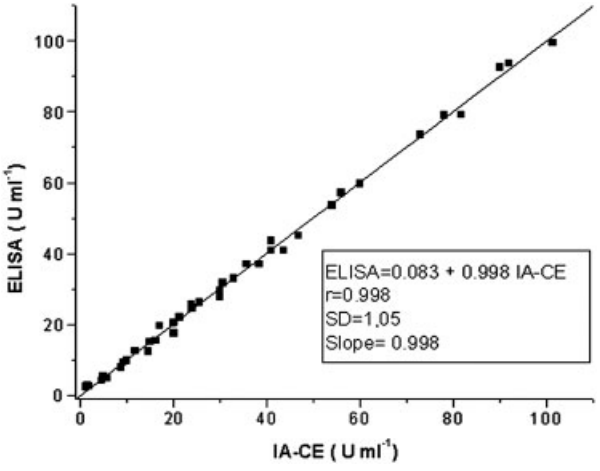


Figure 6. Correlation between proposed method and classic photometric assays.

4 Concluding remarks

In conclusion, we have developed a simple assay like ELISA in the capillary which was capable to determine the lower levels of specific antibodies against *H. pylori* in human serum sample. Otherwise, this system uses small amount of samples which is a critical parameter when young children have to be tested. This method increased the capability to determine the low levels of IgG antibodies specific to *H. pylori* with high sensitivity. The high-reactive surface area and the reduced diffusion distances in our IA-CE permitted a faster time of analysis (34 min) and a less sample consumed than conventional immunoassay techniques. The analytical results for the clinical samples show that the development of an immunoassay has a promising alternative approach for detecting specific antibodies against *H. pylori* in human serum sample in the clinical field. This method can be applied to several kinds of complex samples from different biochemical and environmental origins.

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The authors have declared no conflict of interest.

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