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Continuous-flow/stopped-flow system for enzyme immunoassay using a rotating bioreactor: determination of Chagas disease

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Abstract

The high sensitivity that can be attained using an immunoassays coupled to a rotating bioreactor with electrochemical detection mediated by [Os(bpy)2Cl(pyCOOH)]Cl, has been verified for the detection of *Trypanozoma cruzi* (*T. cruzi*), This protozoan parasite causes Chagas disease, affecting more than 18 million people in central and south America. Antibodies in the serum sample are allowed to react immunologically with whole homogenates of the parasite as antigen that are immobilized on a rotating disk. The bound antibodies are quantified by a horseradish peroxidase (HRP) enzyme labeled second antibodies specific to human IgG in presence of hydrogen peroxide using an osmium complex [Os(bpy)2Cl(pyCOOH)]Cl as enzymatic mediators. The amperometric measurement performed at 0.00 V versus Ag/AgCl can be done within 2 min and the analysis time does not exceed 23 min. The calculated detection limits was 0.01 mIU ml^{-1}. Reproducibility assays were made using repetitive serum of 0.182 mIU ml^{-1} *T. cruzi* specific antibody (measured as the activity of the correspondent anti-serum's enzyme conjugated); the percentage standard error was less than 5%. The amperometric immunoreactors showed significantly higher sensitivity and lower time consumed than the standard spectrophotometric detection ELISA method.

Keywords: *Trypanosoma cruzi*; Horseradish peroxidase; Osmium; Flow system; Immunoassays; Biosensors

1. Introduction

Chagas disease (American trypanosomiasis), caused by the hemoflagelle protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), affects nearly 18 million people in endemic regions of central and south America (Carlier et al., 2002) and is transmitted by haematophagous insects of the Reduviidae family. The complex life cycle of *T. cruzi* includes different polymorphic forms in the insect vector and in the vertebrate host. There are two parasite stages in the vector: epimastigote and metacyclic trypomastigote, whereas in the mammalian host, the parasite can adopt an intracellular reproductive form (amastigote) or an infective flagellate form (trypomastigote).

Since there is not efficient chemotherapy for infected patients, the actual control of Chagas disease is restricted to vector control with insecticides and serological screening of blood donors (Moncayo, 1993). Etiological diagnosis of American trypanosomiasis is based on the presence of antibodies against the protozoan parasite *T. cruzi* in the serum of infected individuals. An approach to achieve this objective is based on detection methods using immunological principles. Immunochemical assay are envisaged for application as rapid and simple analytical tools that can be used on site for screening of samples prior to further laboratory analysis; furthermore, in many cases simple procedures can be developed that can be performed by nonspecialists (Van Emon and Lopez Avila, 1992), within this class of techniques, enzyme linked immunosorbent assay (ELISA), is a well established

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analytical method which combines the extreme specificity of antigen/antibody recognition with the sensitive response produced by the enzyme/substrate reaction (Engvall, 1980).

Immunoassay with electrochemical detection appears to have some inherent advantages over the more widely used spectrophotometric techniques. Lower detection limits and less time consumed can be achieved with modern electrochemical techniques. One approach used in such techniques is to employ an enzyme label that generates an electrochemically active product (Babkina et al., 1996; Wehmeyer et al., 1986; Parellada et al., 1998; Lim et al., 1999; Duane and Meyerhoff, 1994; Yao et al., 1993; Manning et al., 1994).

In this work, we established an amperometric immunoreactor with rotation incorporated into a FIA system for rapid and sensitive quantification of human serum IgG antibodies to *T. cruz*i. Antibodies in the serum sample are allowed to react immunologically with the antigens immobilized on a rotating disk and the bound antibodies are quantified by a HRP enzyme labeled second antibody specific to human IgG, using an osmium complex [Os(bpy)2Cl(pyCOOH)]Cl as enzymatic mediators (Danilowicz et al., 1998). This immunoreactor has been applied to the analysis of human serum samples and their results have been compared to those obtained by ELISA.

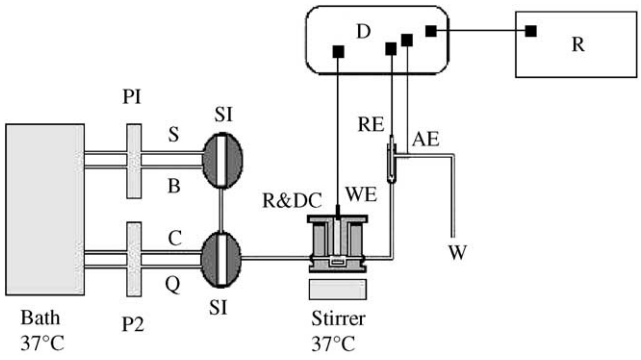

Fig. 1. Block diagram of the continuous-flow system and detection arrangement. P1 and P2: pumps (Gilson Minipuls 3 peristaltic pumps, Gilson Electronics, Inc., Middleton, WI); S: sample (human serum); B: carrier buffer line; C: conjugate, goat anti-human IgG-hoseradish peroxidase; Q: mezcle, [Os(bpy)2Cl(pyCOOH)]Cl 20 μM, H_2O_2 0.10 mM; SI: sample injection; W: waste line; R&DC: reactor and detector cell; WE: working electrode (GCE); RE: reference electrode (Ag/AgCl, 3.0 M NaCl); AE: auxiliary electrode (stainless steel tubing); D: potentiostat/detection unit (LC-4C, Bioanalytical Systems, West Lafayette, IN); R: recorder (Varian, Model 9176, Varian Techtron, Springuale, Australia).

2. Experimental

2.1. Reagents and solutions

All reagents used, except as noted, were of analytical reagent grade. Horseradish peroxidase, HRP [EC 1.11.1.7] Grade II was purchased from Sigma Chemical Co., St. Louis. The concentration of HRP was determined spectrophotometrically using the Soret extinction coefficient of 102 mM^{-1} cm^{-1} at 403 nm (10 000 IU = 55 mg). Goat anti-human IgG-horseradish peroxidase (conjugate) was purchased from Sigma (St. Louis, MO). Glutaraldehyde was purchased from Merk, Darmstadt. 3-Aminopropyl-modified controlled-pore glass, 1400 Å mean pore diameter and 24 m^2 mg^{-1} surface area, was from Electro-Nucleonics (Fairfield, NJ) and contained 48.2 μmol g^{-1} of amino groups. [Os(bpy)2Cl(pyCOOH)]Cl was synthesized as previously described (Danilowicz et al., 1998). Aqueous solutions were prepared using purified water from a Milli-Q-system and the samples were diluted to the desired concentrations using a 10 mL Metrohm E 485 burette.

Epimastigotes of *T. cruzi* were cultivated in a monophasic medium as previously described (Basso et al., 1980, 1991). The ELISA test kit for determination of *T. cruzi* specific IgG class antibodies was purchased from Wiener Lab., Argentina, and was used accordance with manufacture instructions.

2.2. Flow-through reactor/detector unit

A schematic representation of the dual-line continuous-flow setup is shown in Fig. 1. The instrument is composed of three major sets of components (i) a flow injection system. The instrumental setup is based on a pair of three channel peristaltic pumps (Gilson Minipuls 3 peristaltic pumps, Gilson Electronics, Inc., Middleton, WI) connected to multiport injection valves, specifically a two valves switching system common to FIA. The combined flow from the two pumps will be introduced into the flow cell containing the immunoaffinity unit; besides pumping, are used for sample introduction, and flow stopping. (ii) The immunoreactor cell and (iii) a detector unit. This immunoreactor allows a full utilization of immobilized active sites, because rotation facilities the arrival of substrate and cosubstrate. The main body of the cell was made of Plexiglas. Fig. 2 illustrates the design of the flow-through chamber containing the rotating immunoreactor and the detector system. Glassy carbon electrode (GCE) is on the top of the rotating reactor. The rotating reactor is a disk of Teflon in which a miniature magnetic stirring bar (Teflon-coated Micro-Stir bar from Markson Science, Inc., Phoenix, AZ) has been embedded. Typically, a reactor disk carried 1.4 mg of controlled-pore glass on its surface. Rotation of the lower reactor was effected with a laboratory magnetic stirrer (Metrohm E649 from Metrohm AG Herisau, Switzerland). Amperometric detection was performed using a BAS LC-4C potentiostat (Bioanalytical System, West Lafayette, IN). The potential applied to the working electrode was 0.00 V versus Ag/AgCl, 3.0 M NaCl reference electrode BAS MF-2052, and a Pt wire was used as counter electrode. At this potential, a catalytic current was well established. The cell volume will be minimized in order to maximize the amount of sample passing next to the immobilized antigen.

The pump tubing was Tygon (Fisher AccuRated, 1.0 mm i.d., Fisher Scientific Co., Pittsburgh, PA) and the remain-