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Competitive immunoassay for cyclosporine using capillary electrophoresis with laser induced fluorescence polarization detection

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Abstract

Frequent monitoring of immunosuppressive drug cyclosporine A (CsA) in blood samples of tissue transplant patients is required in clinical practice because of the narrow therapeutic range between the immunosuppressive effect and the toxic effect of this drug. We describe a competitive immunoassay capillary electrophoresis (CE) with laser induced fluorescence polarization detection method, which is rapid and sensitive for the determination of CsA. The method is based on the competitive immunochemical reaction between the analyte and fluorescent hapten (CsA*****) with the antibody, CE separation of the antibody bound and free fluorescent CsA*****, followed by the laser induced fluorescence polarization detection (LIFP) of the fluorescent species. The method detection limit is governed by the stability of the antibody–CsA***** complex rather than by the detector noise. The use of post-column sheath flow cuvette LIFP detection resulted in excellent detection limit, typically 0.9 n*M* (or $9 \cdot 10^{-19}$ mol for 1 nl injection) of CsA. CsA in whole blood samples from organ transplant patients were measured and results agreed well with those obtained by using a standard fluorescence polarization immunoassay. Each determination took less than 3 min. The CsA metabolites AM9 and AM19 were also determined by using this technique, and their cross-reactivities with the antibody were 13% and 2%, respectively.

Keywords: Immunoassay; Fluorescence polarization; Cyclosporine A

1. Introduction

Cyclosporine A (CsA) is now routinely used as an immunosuppressive drug for patients who have received organ transplants. Also, CsA has been proposed as a therapeutic candidate for early treatment of human immunodeficiency virus infection [1,2]. The role of CsA is believed to suppress T cell activation [3]. However, its use is not free of adverse effects, especially nephrotoxicity, hepatoxicity and neurotoxicity [4–6]. Clinical use of CsA needs to be carefully controlled because of the narrow therapeutic range (e.g., 150 to 450 ng/ml in blood [4]). Above this range, the beneficial immunosuppressive effect is diminished and the toxic effect dominates. It is also recognized that clinical response does not correlate well with the administered dose [7]. Therefore, the frequent monitoring of blood CsA levels is

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necessary in clinical practice to determine an optimum dosage for individual patients [7,8].

Commonly used methods for monitoring CsA have been reviewed [7,9–11]. High-performance liquid chromatography (HPLC) with either UV absorbance detection [12–15] or mass spectrometric detection [16,17] have been employed to detect CsA and its metabolites. More recently, an immunoaffinity capillary electrophoresis (CE) method has been reported for the analysis of CsA in tears [18]. In addition, CsA can catalyze the conversion of the aryl oxalate to hydroperoxy oxalate which is the reactive intermediate for chemiluminescence detection. This characteristic has been used for the determination of CsA by using aryl oxalate chemiluminescence in flow injection analysis and HPLC [19]. Time-of-flight secondary-ion mass spectrometry has also been reported for the determination of CsA [20]. However, many of these methods were tedious because they required extensive sample pre-treatment in order to achieve necessary sensitivity and specificity. Various formats of immunoassay have been employed to improve the sensitivity and specificity in the determination of CsA. Although polyclonal antibodies have shown some problems of higher cross-reactivity, applications of monoclonal antibody have significantly improved the specificity for the monitoring of CsA [21–23]. As a consequence, the whole blood enzyme immunoassay [24,25], radioimmunoassay (RIA) [12,26,27], and fluorescence polarization immunoassay (FPIA) [21–23,27–29] using monoclonal antibodies have been developed for the determination of CsA.

The objective of this work is to develop a technique, which combines the advantages of immunoassay, CE separation and LIF detection. CE offers a high separation efficiency and requires only small amount of sample. LIF detection using post-column sheath flow cuvette can provide excellent detection sensitivity [30–33]. Thus, pre-column competitive immunochemical reactions between hapten (i.e., a low-molecular-mass molecule that is recognized by preformed antibody but is not itself immunogenic) and antibody followed by the CE separation of fluorescent-labeled free hapten from the antibody bound hapten can be used to determine unlabeled hapten, antigen or antibody [34–39]. However, this approach has not been applied to the determination of CsA.

In this paper we describe a competitive immunoassay using CE–LIFP for the determination of CsA. Fluorescence polarization has shown wide applications in immunochemistry [40]. However, its unique features have not been applied to electrophoretic and chromatographic analysis. For the first time, we show a LIFP system for CE detection and competitive immunoassay. We also discuss several important parameters which affect the assay, including the CE separation of unbound CsA* and CsA*–antibody complex, the effect of methanol on the stability of CsA*–antibody complex, and the cross-reactivity of two CsA metabolites (AM9 and AM19) with the antibody. We also demonstrate an application of the method to the determination of CsA in whole blood samples from three organ transplant patients.

2. Experimental

2.1. Instrumentation

Fig. 1 shows a schematic diagram of the CE–LIFP system, which was used throughout this work. The electrophoresis was driven by a high-voltage power supply (CZE1000R, Spellman High Voltage Electronics, Plainview, NY, USA). The separation voltage, injection voltage and injection time were controlled by a Macintosh computer, with program written in LabVIEW (National Instruments, Austin, TX, USA). Separation was carried out typically in a

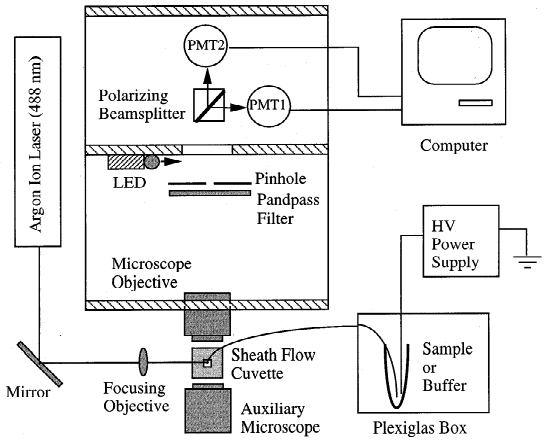

Fig. 1. A schematic diagram showing CE separation with laser induced fluorescence polarization detection.

40 cm×20 μm I.D.×150 μm O.D., fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) at an electric field of 500 V/cm. The high-voltage injection end of the capillary, along with a platinum electrode, was inserted into a sample solution (when injecting sample) or running buffer (when performing separation) and was held in a Plexiglas box equipped with safety interlock. The other end of the capillary was inserted into a sheath flow cuvette, which was grounded. The sheath flow cuvette was used as a post-column fluorescence detector cell. The length of capillary from the injection end to the detector is the same as the total length of the separation capillary (typically 40 cm).

The LIFP system (Fig. 1) was constructed on an optical table. A 65-mW argon ion laser (Model 2214-65ml, Uniphase, San Jose, CA, USA) with a wavelength of 488 nm was used as the excitation source. The laser beam was focused with a 10× microscope objective into a sheath flow cuvette (NSG Precision Cells, Farmingdale, NY, USA). The sheath flow cuvette has been previously used as the fluorescence detection cell, as developed by Dovichi and co-workers [30–33]. Briefly, the cuvette was constructed with high optical quality quartz, and it had 0.9-mm thick walls, a 0.2-mm square inner chamber, and a length of 20 mm. The quartz cuvette was mounted in a locally constructed stainless steel body. The detection end of a capillary was inserted into the cuvette such that the laser beam was illuminated approximately 200 μm below the tip of the capillary. The sheath fluid, identical to the running buffer, was introduced into the cuvette by hydrodynamic pressure at a flow-rate of a few microliters per hour. The sheath flow surrounding the effluent from the reaction capillary produced hydrodynamic focusing of the analyte band, leading to an intensified small fluorescent spot for measurement. Fluorescence was collected at right angle with respect to both the laser beam and the sample stream by using a high-numerical aperture microscope objective (60×, 0.7 NA, Universe Kogaku, Oyster Bay, NY, USA), which has the required working distance of greater than 2 mm. The fluorescence was then spectrally filtered with a bandpass filter (515DF20) to reject scattered laser light. A 200-μm radius pinhole was placed in the reticle position of the microscope objective to restrict the field of view of the photomultiplier tubes (PMTs) to the illuminated sample stream. The fluorescent light was split, by using a polarizing beamsplitter (Melles Griot, Nepean, Canada) to two photomultiplier tubes (R1477, Hamamatsu Photonics, Japan) for measuring horizontally and vertically polarized light, respectively. The output from the two PMTs was digitized by a PCI data acquisition board and a LabVIEW software (National Instruments) in a Power Macintosh computer.

It is necessary to align a tightly focused laser beam with a small-diameter sample stream so that the fluorescence passes through a high numerical aperture objective and is detected by the PMTs. In the present optical arrangement, we fixed the location of the PMTs, the collection optics and the limiting aperture. All other components were aligned with respect to the collection optics. The sheath flow cuvette and the laser beam focusing objective were each mounted on a set of three-axis translation stages, and thus their positions could be adjusted with ease and precision. An auxiliary microscope was placed opposite the collection optic and used to assist the alignment.

We initially used LIFP to identify the antibody–CsA* complex. We then only needed to use one PMT for detection, to perform the competitive immunoassay. When using only one PMT for detection, the LIFP system is equivalent to the conventional LIF detection. There was no need to change any instrument components.

2.2. Reagents

All buffer solutions were prepared using distilled deionized water purified by Maxima Ultra-Pure Water System (ELGA, Topsfield, MA, USA) and analytical grade reagents were used throughout. Stock solutions of 200 m*M* phosphate (pH 7.2), 200 m*M* borate (pH 9.2), 100 m*M* histidine (pH 9.0) and 100 m*M* aspartate (pH 9.0) were prepared from disodium hydrogen orthophosphate (BDH, Toronto, Canada), boric acid (Anachemia, Mississauga, ON, Canada), L-histidine (Aldrich, Milwaukee, WI, USA) and L-aspartic acid monopotassium salt (Aldrich), respectively, in deionized water. Sodium hydroxide (BDH) was used to adjust the pH of phosphate, borate and histidine solutions and potassium hydroxide (BDH) was used to adjust the pH of aspartate solution. All the electrophoretic buffer

solutions were diluted from these stock buffer solutions. The pH values of all diluted buffer solutions were confirmed prior to use.

HPLC-grade methanol (BDH) was used for the extraction of CsA from blood samples and for the preparation of standard CsA solutions. Stock solution of CsA (1000 mg/l) was prepared by dissolving appropriate amount of CsA (Sigma, St. Louis, MO, USA) in methanol. Solutions containing lower concentrations of CsA were obtained by serial dilution of the stock solution with methanol. CsA labeled with fluorescein (CsA*****) and monoclonal antibody to CsA were obtained from the Cyclosporine Monoclonal Whole Blood TDx test kit (Abbott Labs., Abbott Park, IL, USA). The dilute solutions of fluorescein-labeled CsA and antibody were obtained by diluting these test kit reagents with deionized water.

Whole blood samples were obtained from the University of Alberta Hospital (Edmonton, Alberta, Canada). The samples were collected into tubes containing heparin as anticoagulant and stored in refrigerator at 4°C. Two metabolites of CsA (AM9 and AM19) were purified from urine samples of patients administered CsA. The metabolites were characterized using several chromatography and mass spectrometry (MS) techniques for their identity and purity. The metabolites were dissolved in methanol.

2.3. Procedures

All standard solutions and blood samples were prepared in 2.5 m*M* phosphate (pH 7.2) and methanol–water (1:12, v/v) solutions. Standard solutions of cyclosporine A (CsA) were prepared by mixing different concentrations of CsA with constant amount of fluorophore-labeled cyclosporine A (CsA*****) and limiting monoclonal antibody for CsA (Ab). The concentrations of CsA in standard solutions were varied from 0 to 70 n*M* and the concentrations of CsA***** and Ab were both maintained constant at approximately 80 n*M*. For the studies of cross-reactivity of metabolites with Ab, the solutions were prepared in the same manner as for the preparation of CsA standards except that metabolites were used instead of CsA.

A similar method to that described in literature [41] was used to prepare whole blood samples for CsA assay. Methanol (2 ml) was added to 1 ml whole blood to precipitate blood protein and to release bound drug. The mixture was centrifuged and the clear supernatant was removed for analysis. An aliquot of the supernatant was added to a solution consisting of the same components as in the standard solutions (i.e., 2.5 m*M* phosphate, methanol–water, 1:12, CsA***** and Ab). The mixture was vortexed for approximately 15 s prior to CE–LIFP analysis.

Before use, the capillary was rinsed with 0.1 *M* NaOH solution for 5 min, then with water for another 5 min, and finally with electrophoretic buffer for 3 min. When changing different electrophoretic separation buffers, the same washing sequence was applied. All samples were electrokinetically injected onto the capillary by applying 5 kV for 5 s. The separations were carried out under 20 kV (500 V/cm). Running current was approximately 4–5 μA. Triplicate sample runs were performed.

CsA concentrations in whole blood samples were also determined by using fluorescent polarization immunoassay performed on a commercial TDx instrument [22]. The results were compared with those obtained by using the competitive immunoassay CE–LIFP method.

3. Results and discussion

Native CsA does not possess a fluorophore and cannot be directly detected with high sensitivity by using laser induced fluorescence. However, LIF detection is very sensitive for the determination of fluorescein-labeled CsA (CsA*****). We have achieved a detection limit of $1 \cdot 10^{-11}$ *M* for CsA*****, estimated by using Knoll's method [42]. Therefore, if CsA in the sample can be readily labeled with good efficiency, the CE–LIFP technique would provide a sensitive and easy approach to the assay of CsA. However, fluorescent labeling of trace levels of analyte in the presence of sample matrix is very difficult because of non-quantitative labeling and poor specificity. In a competitive immunoassay approach, there is no need to label the analyte in the sample. Both the fluorescently labeled hapten (CsA*****) and the analyte (CsA) compete for the limiting amount of antibody (Ab) according to the following equilibrium:

$$CsA^{*} + CsA + Ab \rightleftharpoons CsA{-}Ab + CsA^{*}{-}Ab \quad (1)$$

The fluorescent species, the free CsA* and the antibody bound CsA* (CsA*–Ab) can be detected by the LIFP detector. Although the analyte (CsA) is not directly detected, its concentration is related to the amount of CsA* and CsA*–Ab in the system. Therefore, the response of CsA* and CsA*–Ab can be used to quantify the concentration of non-fluorescent CsA.

3.1. Separation of free CsA from CsA*–antibody complex*

To perform the competitive immunoassay, it is essential to separate the free (unbound) fluorophore-labeled cyclosporine A (CsA*) from the antibody bound CsA* (CsA*–Ab) in the reaction mixture. To achieve a baseline resolution of these two fluorescent components, we have optimized CE parameters. We found that the composition and pH of electrophoretic running buffer system dramatically affect the CE separation and the formation of antibody complex. At a pH lower than 6, we did not observed the CsA*–Ab peak. This is probably due to the low stability of the CsA*–Ab complex at the extreme pH. Therefore, we chose to use separation buffers with pH between 7 and 9.5.

Fig. 2 illustrates the effect of four buffers on the separation of the fluorescent species in the immunoreaction mixture. The baseline resolution of free CsA* and CsA*–Ab complex is achieved by using phosphate buffer at pH 7.2. The separation is complete within 3 min. Other three buffer systems studied do not provide adequate separation, although a partial separation is achieved using the aspartate buffer. Therefore, 10 m*M* phosphate buffer (pH 7.2) was used for the rest of this study.

The third peak (peak 3) in Fig. 2 is due to free fluorophore, fluorescein, which is also present in the CsA* reagent. Because this peak is close to the migration of free CsA* and also because its concentration is constant in all the immunoreaction mixtures, it can be used as an ideal internal standard for the quantitative determination of CsA*. Therefore, the addition of another internal standard to the samples is not necessary.

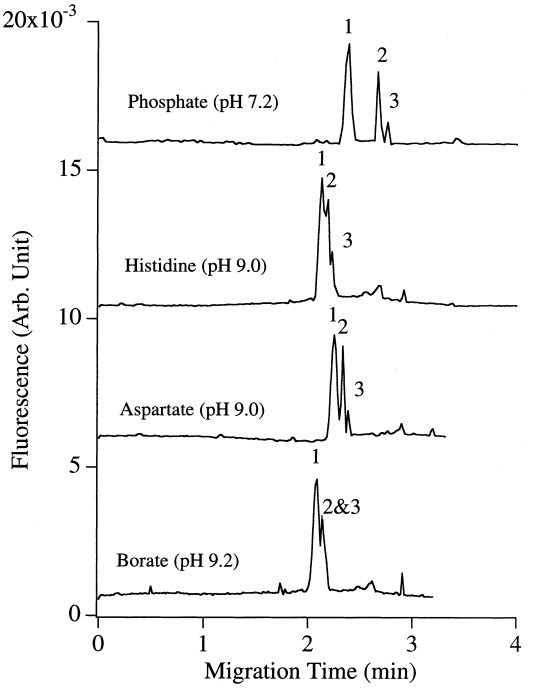

Fig. 2. Electropherograms showing the separation of antibody bound cyclosporine tracer (CsA*–Ab) from the unbound CsA* by using different electrophoretic buffers. (from top to bottom) Ten m*M* phosphate, pH 7.2; 10 m*M* histidine, pH 9.0; 10 m*M* aspartate, pH 9.0; 20 m*M* borate, pH 9.2. Peak 1: CsA*–Ab complex; peak 2: unbound CsA*; peak 3: free fluorescein dye.

3.2. Fluorescence polarization

Fig. 3 illustrates fluorescence polarization phenomenon observed using the CE–LIFP system. Fluorescence polarization is based on the measurement of the intensity of polarized light emitted by fluorescent compounds. Polarization of fluorescence depends on the fluorescence lifetime (time between excitation and emission) and the rotational relaxation time of the molecule (the time required for an oriented molecule to return to a random orientation in solution). Small molecules, such as fluorescently labeled CsA, rotate rapidly in solution, have short relaxation times, and do not exhibit significant fluorescence polarization. But when the small molecule is bound by a large antibody molecule, fluorescence polarization is considerable because the large molecule rotates slowly in solution and the relaxation time is prolonged.

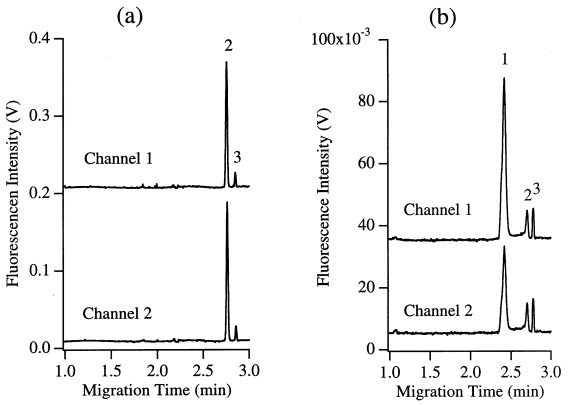

Fig. 3. Comparison of the electropherograms monitored with two channels of the laser induced fluorescence polarization detection system. (a) Fluorescent-labeled cyclosporine (CsA*) reagent; and (b) a mixture containing monoclonal anti-CsA antibody and CsA*. A 40 cm×20 μm I.D.×150 μm O.D. capillary was used for separation with a voltage of 20 kV. Phosphate (10 m*M*, pH 7.2) was used as running buffer. Peak identities were the same as shown in Fig. 2.

Fig. 3a shows electropherograms from the CE analysis of CsA*, monitored with both PMTs, which detect horizontally and vertically polarized fluorescence, respectively. Because CsA* (peak 2) and fluorescein (peak 3) are small molecules, they do not have significant fluorescence polarization. Therefore, signal intensities detected by the two channels are essentially the same.

After mixing anti-CsA antibody with CsA*, antibody–CsA* complex forms. The CE separation of the reaction mixture shows three peaks detected by both PMTs (Fig. 3b). Similarly, CsA* (peak 2) and fluorescein (peak 3) show identical signal intensity detected by both PMTs, because these small molecules do not exhibit significant fluorescence polarization. However, the antibody bound CsA* (peak 1) shows dramatically different intensities detected by the two channels. This is because the antibody–CsA* complex is a large molecule and exhibits significant fluorescence polarization. Using this characteristic of fluorescence polarization, we are able to identify the antibody bound analyte easily.

After the antibody bound and free analytes are identified using the LIFP approach, the results obtained from using any one of the two channels can be used for competitive immunoassay. In the next few sections, we show electropherograms monitored with only one channel.

3.3. Effect of organic solvent on the stability of CsA–antibody complex*

Methanol was used in the preparation of standard CsA solution and of the whole blood samples for CsA assay. Thus we decided to study the effect of methanol in the reaction mixture on the stability of CsA*–Ab complex. Fig. 4 shows electropherograms comparing the presence (top) and absence (bottom) of methanol in the reaction mixture containing Ab and CsA*. Without methanol (Fig. 4, bottom), the CsA*–Ab peak (peak 1) is dominant, indicating the formation of stable CsA*–Ab complex. When 33% (v/v) methanol is present in the reaction mixture containing the same amounts of Ab and CsA* as in (b), the intensity of CsA*–Ab complex peak is dramatically reduced and the intensity of unbound CsA* is increased (Fig. 4, top). These results suggest that the presence of methanol weakens the stability of CsA*–Ab complex.

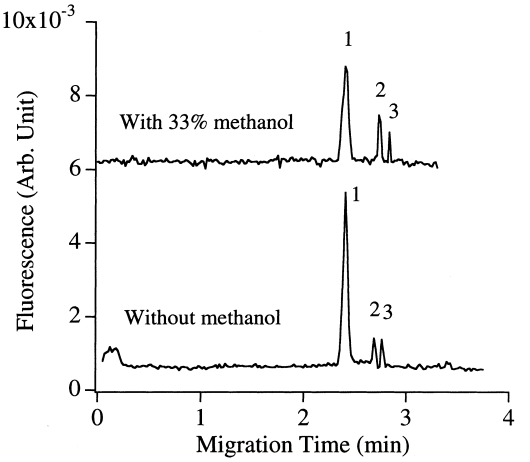

Fig. 4. Electropherograms showing the effect of methanol on the relative intensity of CsA* and CsA*–Ab. The composition of both samples was identical except that the sample in the upper electropherogram was dissolved in 33% methanol and that no methanol was added to the sample shown in the bottom electropherogram. Peak identities were the same as shown in Fig. 3.

It is necessary to use methanol in the treatment of the whole blood sample and in the preparation of standard solutions. In order to eliminate any quantitation error that may be caused by the effect of methanol, equal amount of methanol (8.3%) was added to all sample and standard solutions, and all the immunoreaction mixtures were prepared in the same manner.

3.4. Quantitative determination of CsA

Using the competitive immunoassay approach as shown in Eq. (1), we have explored the possibility of using the intensity of free CsA* peak in electropherograms to determine the amount of CsA in the solution. Fig. 5 shows a series of electropherograms obtained from CE–LIFP analyses of competitive immunoreaction mixtures, containing the same concentration of Ab and CsA* reagents but varying concentrations of the analyte CsA. The corresponding increase of unbound CsA* (peak 2) and the decrease of CsA*–Ab complex (peak 1) are associated with the increase of CsA.

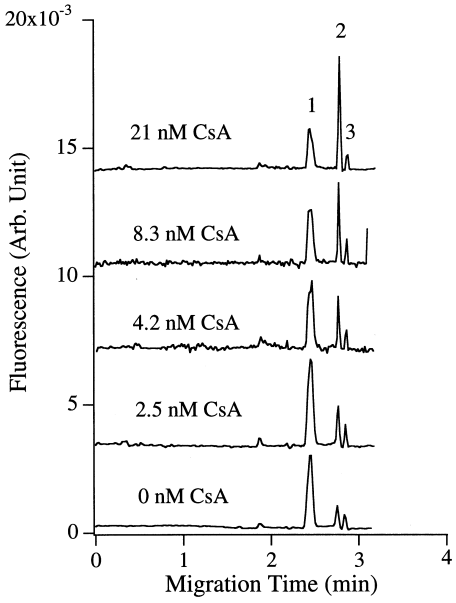

Fig. 5. Electropherograms showing the competition of analyte (CsA) with the fluorescent tracer (CsA*) for the antibody (Ab), with increasing amounts of CsA. Same separation conditions as shown in Fig. 3 were used. Peak identities and the separation conditions were the same as shown in Fig. 3.

A regression calibration curve was plotted based on the relative intensity of peak 2 to peak 3 (i.e., the ratio of two peak areas) versus concentration of CsA. The linear range was observed from 2.5 n*M* to 25 n*M* CsA. The slope of the regression curve was 0.216 ± 0.006, *y*-intercept was 1.742 ± 0.063, and the regression correlation coefficient (r^2) was 0.998.

The detector routinely provides detection limit for the free fluorescent dye on the order of $6 \cdot 10^{-21}$ mol, based on three-times baseline noise [42], consistent with previously reported [30–33]. However, the detection limit of CsA using CE–LIFP competitive immunoassay is not governed by the detector noise. The limiting factor is the blank CsA* signal due to the dissociation of the CsA*–Ab complex. Thus the detection limit is estimated as the concentration equivalent to the blank signal plus three-times the standard deviation from the replicate blank runs [34]. Using this method we have achieved a detection limit of 0.9 n*M* for the determination of CsA. For a typical sample injection volume of approximately 1 nl, the mass detection limit is 0.9 amol. Both concentration detection limit and the mass detection limit achieved using this method are better than those reported by using other methods for CsA assay, such as fluorescence polarization immunoassay [22,23] (detection limit 21 n*M*), HPLC with UV absorbance detection (detection limit 8.3 n*M*) [15], and chemiluminescence (detection limit 5 n*M*) [19].

Three whole blood samples from patients who were administrated CsA were analyzed for CsA by using the CE–LIFP method. Typical electropherograms from the analyses of these samples are shown in Fig. 6. Replicate analyses of these samples showed reproducible electropherograms, indicating that there was no interference from the sample matrix. The concentrations of CsA in these three whole blood samples, obtained from the three replicate assays are summarized in Table 1. For comparison, the samples were also submitted to a clinical laboratory for CsA assay using a standard fluorescence polarization immunoassay (FPIA) method [22,23]. The inter-laboratory comparison results are also listed in Table 1. The results obtained by using the two methods are in good agreement, with a deviation of less than 10%.

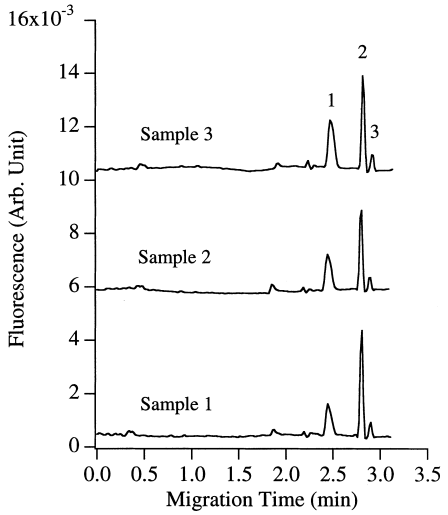

Fig. 6. Typical electropherograms obtained from the determination of CsA in three whole blood samples. Peak identities and the separation conditions were the same as shown in Fig. 3.

3.5. Cross-reactivity

The potential interference for the determination of CsA by using competitive immunoassay is from the cross-reaction of the monoclonal antibody with metabolites of parent drug CsA. The cross-reaction of the metabolites with the antibody can result in an over estimate of CsA concentration. It is important to obtain information on the extent of such cross-reaction.

More than 14 CsA metabolites resulting from the biotransformation of CsA in human body have been chemically characterized [7,43–45]. Cross-reactivities for seven of these metabolites have been reported. The highest cross-reactivity was found from AM9 [43]. We have selected two common CsA metabolites, AM9 and AM19, and have studied their cross-reactivity with the antibody to CsA. The cross-reactivity was determined by comparing the amount of metabolite measured against the CsA standard calibration with the actual amount of metabolite added. Using the present method, we observed cross-reactivity of (13.3±0.1)% for AM9 and (2.0±0.1)% for AM19. These results agree well with those reported in the literature [43].

4. Conclusions

The competitive immunoassay combined with CE–LIFP was demonstrated to be a rapid, sensitive and selective technique for the detection of cyclosporine A. Both concentration detection limit (0.9 n*M*) and mass detection limit (0.9 amol) are the best among all methods available for CsA assay. The CE–LIFP analysis of each sample was complete within 3 min. Only small volume of sample was needed and the sample treatment process was minimum. The use of only small volume of sample is particular advantageous when frequent monitoring of CsA and frequent sampling of patients' blood are required. Similar competitive immunoassay approach using CE–LIFP can be extended to assay of other clinical drugs where the antibody and fluorescent tracer are available.

Most current approaches of competitive immunoassay rely on pre-column reaction followed by a separation of antibody bound and free antigen (or hapten). When the antibody cross-reacts with several haptens (e.g., parent drug and its metabolites) of similar structure, the CE separation is unable to resolve various antibody bound haptens. The cross-reactivity is a problem encountered in almost all competitive immunoassays. This problem may be circumvented by using CE separation of individual drug and metabolites before post-column competitive immunoreaction and laser induced fluorescence po-

Table 1
Comparison of concentrations of CsA (n*M*) in whole blood samples measured by competitive immunoassay CE–LIFP and FPIA methods

Sample	CE–LIFP method[a]	FPIA method	Relative deviation (%)
1	323.5±21.3	346.4	−6.6
2	277.3±10.4	250.6	10.6
3	245.0±2.5	244.8	0.1

[a] Mean±one standard deviation from triplicate sample analyses.

larization detection. This is a very attractive approach and is under further development.

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