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Determination of Glucose in Submicroliter Samples by CE−LIF Using Precolumn or On-Column Enzymatic Reactions

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Two enzymatic reactions combined with capillary electrophoresis (CE) are used to determine glucose contained in sample volumes of ≤500 nL. In the first enzymatic reaction, glucose is oxidized in the presence of glucose oxidase producing hydrogen peroxide, which reacts quantitatively with the fluorogenic compound homovanillic acid catalyzed by the enzyme peroxidase. The second reaction generates a fluorescent species that is proportional to the glucose concentration. The reaction product is determined by CE using laser-induced fluorescence (LIF) as the detection mode. The overall reaction scheme is faster than commonly used precolumn derivatization procedures and can be performed using very small sample quantities. Alternatively, the enzymatic reactions can be performed on-column, similar to the electrophoretically mediated microanalysis approach, accommodating sample quantities in the nanoliter range. The on-column reaction is a simple and practical approach for the determination of glucose contained in low-volume samples by CE-LIF, where samples are injected directly into the capillary column without any pretreatment. However, sample handling and detectability of the precolumn approach proved to be superior. Determination of glucose using the precolumn and on-column reactions showed detection limits of 50 and 800 nM, respectively. The methods were shown to be linear in the range tested, 1−100 μM and 100 nM−30 μM, for the on-column and precolumn reactions, respectively. The reproducibility for each scheme was <5% RSD. To determine the possibility of using a noninvasive procedure for glucose monitoring, we used the CE−LIF methods to analyze human tear samples for glucose. The tear fluid samples were contained in a volume of ∼200 nL. The concentration of glucose in the human tear samples analyzed using the precolumn and on-column procedures was ∼138 μM.

Several studies have indicated that changes in glucose concentration in blood and tear fluid are correlated.[1] Consequently, such studies have proposed the analysis of tear fluid as a viable noninvasive alternative to detect diabetes mellitus. The methods used in these studies, however, lack sensitivity and for the most part are only semiquantitative. For example, a color change (of a paper) has been used to determine glucose. The tear sample required for analysis can be a relatively large quantity (e.g., 10 μL),[2] and collecting such a sample can involve extra sampling time. Furthermore, the natural state of the tear fluid can be changed because in most cases tearing is induced, altering the concentration of the fluid's constituents. The wide range of glucose concentration in tears reported by different researchers (micro- to millimolar range) can be a result of these problems. However, the glucose concentration in the tear fluid for normal individuals is generally taken to be about 136−230 μM.[3] If indeed the analysis of tear fluid can be used as a noninvasive approach for glucose monitoring, it is important that the analytical procedure used overcomes the above-mentioned problems.

The development of new approaches to determine glucose in biological fluids continues to receive great attention.[4,5] Most of the current methods are based on the enzymatic oxidation of glucose as in the following reaction:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \quad (1)$$

The glucose concentration is proportional to both the depletion of oxygen and the increase in hydrogen peroxide concentrations. Thus, glucose is determined indirectly by measuring a decrease in oxygen or an increase in hydrogen peroxide concentrations. Electroanalytical means are frequently used to follow these concentrations,[6] even in ultrasmall environments.[7] Although these electrochemical methods are simple and reproducible, most of these sensors are used for glucose determinations in the milli-

(1) (a) Giardini, A.; Roberts, J. R. E. *Br. J. Ophthalmol.* **1950**, *34*, 737−743. (b) Lewis, J. G.; Stephens, P. J. *Br. J. Ophthalmol.* **1958**, *42*, 754−775. (c) Gasset, A. R.; Braverman, L. E.; Fleming, M. C.; Arky, R. A.; Alter, B. R. *Am. J. Ophthalmol.* **1968**, *65*, 414−420. (d) Motoji, K. *Jpn. J. Clin. Ophthalmol.* **1971**, *25*, 1945−1950. (e) Sinha, A.; Prasad, I. M.; Rahman, R. *Ind. J. Ophthalmol.* **1983**, *31*, 473−475. (f) Mediratia, R. K.; Rohatgi, J. N. *Ind. J. Optthalmol.* **1983**, *31*, 635−638. (g) Desai, B. M.; Desai, M. T.; Lavingia, B. C. *J. Assoc. Physicians India* **1987**, *35*, 576−577. (h) Romano, A.; Rolant, F. *Metab. Pedriatr. Syst. Ophthalmol.* **1988**, *11*, 78−80.

(2) van Haeringen, N. J.; Glasius, E. *Graefe's Arch. Clin. Exp. Ophthalmol.* **1977**, *202*, 1−7.

(3) Milder, B. In *Adler's Physiology of the Eye: Clinical Applications*; Mosses, R. A., Hart, W. M., Jr., Eds.; C. V. Mobsby: St. Louis, MO, 1987; pp 15−35.

(4) Wang, J. *Anal. Chem.* **1993**, *65*, 450R−453R.

(5) Wang, J. *Anal. Chem.* **1995**, *67*, 487R−492R.

(6) (a) Lucisano, J. Y.; Armor, J. C.; Gough, D. A. *Anal. Chem.* **1987**, *59*, 736. (b) Atanasov, P.; Wilkins, E. *Biotechnol. Bioeng.* **1994**, *43*, 262. (c) Carr, P. W.; Bowers, D. L. *Immobilized Enzymes in Analytical and Clinical Chemistry*; Wiley: New York, 1980; p 460. (d) Pickup, J. C.; Shaw, G. W.; Claremont, D. *J. Diabetologia* **1989**, *32*, 213. (e) Bindra, D. S.; Zhang, Y.; Wilson, G. S.; Sternberg, R.; Thevenot, D. R.; Moatti, D.; Reach, G. *Anal. Chem.* **1991**, *63*, 1692. (f) Moussy, F.; Jakeway, S.; Harrison, D. J.; Rajotte, R. V. *Anal. Chem.* **1994**, *66*, 3882. (g) Wang, J.; Liu, J.; Chen, L.; Lu, F. *Anal. Chem.* **1994**, *66*, 3600. (h) Csoregi, E.; Quinn, C. P.; Schmidtke, D. W.; Lindquist, S. E.; Pishko, M. V.; Ye, L.; Katakis, I.; Hubbell, J. A.; Heller, A. *Anal. Chem.* **1994**, *66*, 3131.

(7) Abe, T.; Lau, Y. Y.; Ewing, A. G. *Anal. Chem.* **1992**, *64*, 2160−2163.

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molar range and are prone to interferences. Furthermore, a relatively large amount of sample volume is often required. Other approaches have used separation techniques in combination with amperometric schemes to determine glucose and other carbohydrates.[8,9] These methods incorporate the selectivity of a separation technique to eliminate possible interferences. However, the detection is limited to the micromolar range, in the best case.

Capillary electrophoresis (CE) in combination with photometric schemes has been used to improve the detectibility of carbohydrates, including glucose (see review by El Rasi and Mechref).[10] These schemes take advantage of the separation power offered by CE to selectively determine carbohydrates. These CE procedures also offer the possibility of analyzing very minute sample quantities in a routine fashion, which is important when studying biological fluids contained in very low volumes (e.g., tear fluid). Photometric schemes developed for CE are, in principle, applicable for the determination of glucose in small quantities of biological fluids. In these schemes, glucose is derivatized with a suitable reagent to facilitate detectibility, either by UV absorbance or laser-induced fluorescence (LIF). Although these derivatization procedures provide detectabilities below the micromolar range, it is at the expense of relatively long reaction times. In some instances, the signal intensity of the final product can be very sensitive to the glucose/reagent ratio.[11] Furthermore, most derivatization schemes have been performed at a relatively high concentration of saccharide; once derivatized, the samples are diluted to the desired concentrations. Recently, the derivatization of picomole levels of carbohydrates followed by CE analysis has been reported.[12,13] However, the derivatization procedures were either relatively long[12] or the reaction byproducts complicated the electropherograms.[13] In addition, the possibility of labeling other compounds with similar functionalities still exists, which can create some complications. It is indeed very difficult to determine low-micromolar levels of glucose contained in sample volumes of ≤ 1 μL; therefore, there still is the need to develop procedures for the rapid, sensitive, and specific determination of glucose in low sample volumes. Such a procedure, for instance, is required to monitor glucose in the tear fluid.

Herein, we describe a sensitive method for the determination of glucose using an enzymatic reaction scheme combined with CE−LIF. The scheme involves the enzymatic reaction in eq 1 coupled to a reaction with a fluorogenic compound, first proposed by Guilbault and co-workers.[14,15] The hydrogen peroxide produced from the oxidation of glucose oxidizes the nonfluorescent homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid, HVA) in the presence of peroxidase, giving the fluorescent species 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (HVA_{ox}). This oxidation product is a fluorescent compound with maximum excitation and emission wavelengths centered at 315 and 425 nm, respectively.[14] The reaction can be performed with submicroliter sample volumes and is then injected into the CE−LIF system, which is operated with a helium−cadmium laser (325 nm) that closely matches the excitation wavelength of HVA_{ox}. Alternatively, the enzymatic reactions can be performed on-column as in an electrophoretically mediated microanalysis (EMMA).[16] The procedure is very useful in the determination of glucose in small amounts of biological fluids, especially if large quantities of samples are not readily available, which is the case of the tear fluid. We demonstrate the utility of the method for the specific case of determining glucose in human tear samples. The method has been shown to be sensitive, simple, and relatively easy to implement.

EXPERIMENTAL SECTION

Reagents. HVA was obtained from Aldrich (Milwaukee, WI). D-(+)-Glucose, glucose oxidase (GOx) type II-S (EC 1.1.3.4, 18 000 units/g), and horseradish peroxidase (POx) type VI (EC 1.11.1.7, 180 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide, sodium phosphate, sodium borate, and methanol were purchased from Fisher Scientific (Springfield, NJ). All reagents were used as received without further purification. Aqueous solutions were prepared using purified water from a Milli-Q UV Plus water purification system, fed from a Milli-RO 10 Plus reversed osmosis system (Millipore, Bedford, MA). When not in use, enzymes and HVA solutions were stored at 4 °C.

Apparatus. Capillary electrophoresis and on-column fluorescence detection were performed using a P/ACE 2200 unit (Beckman Instruments, Fullerton, CA). The CE system and data acquisition were controlled by means of the System Gold software version 7.12, provided by the same company. A helium−cadmium laser, Series 56 (Omnichrome, Chino, CA), operating at 325 nm was employed as the excitation source. The analyte fluorescence emission was collected through a 380 nm cut-on filter.

The fused-silica capillaries used in the experiments were 27 cm in length and 50 μm i.d. The length of the capillary from the inlet to the detection window was 20 cm. A new capillary was conditioned by rinsing with 0.1 M NaOH for 15 min and with water for another 15 min, before rinsing with the separation electrolyte for 5 min. The capillary was usually rinsed with 0.1 M NaOH for 1 min and then with running buffer for 1 min before each injection. The capillary was thermostated at 23 °C throughout the experiments, unless otherwise specified.

Reaction Procedure. For the reaction of glucose standard solutions, the following reagents were mixed: to 100 μL of a glucose solution, 1 μL of 0.5 M phosphate buffer (pH 7.0), 1 μL of HVA solution (1−2 mg/mL, 50:50 methanol/water), 1 μL of POx solution (0.1−1 mg/mL), and 1 μL of GOx solution (1−4 mg/mL). After mixing the solution, the reaction was allowed to proceed for 30 min at room temperature. Sample solutions were then introduced into the capillary by hydrodynamic injection (0.5 psi), for 10 s. In the on-column reaction experiments, the required reagents for the enzymatic reactions were added to the running electrolyte (HVA ~0.5 mg/mL, GOx ~10 mg/mL, POx ~0.1 mg/mL). The samples containing glucose were injected directly into the capillary, where the reactions were allowed to proceed prior to detection.

(8) Johsnon, D. C.; LaCourse, W. R. *Anal. Chem.* **1990**, *62*, 589A−597A.
(9) Colón, L. A.; Dadoo, R.; Zare, R. N. *Anal. Chem.* **1993**, *65*, 476−481.
(10) El Rasi, Z.; Mechref, Y. *Electrophoresis* **1996**, *17*, 275−301.
(11) Oefner, P.; Chiesa, C.; Bonn, G.; Horváth, C. *J. Capillary Electrophor.* **1994**, *1*, 5−26.
(12) Evangelista, R. A.; Liu, M.; Chen, F. A. *Anal. Chem.* **1995**, *67*, 2239−2245.
(13) Perez, S. A.; Colón, L. A. *Electrophoresis* **1996**, *17*, 352−358.
(14) Guilbault, G. G.; Brignac, P., Jr.; Zimmer, M. *Anal. Chem.* **1968**, *40*, 190−196.
(15) Kiang, S. W.; Kuan, J. W.; Kuan, S. S.; Guilbault, G. G. *Clin. Chem.* **1976**, *22*, 1378−1382.
(16) (a) Harmon, B. J.; Patterson, D. H.; Regnier, F. E. *J. Chromatogr., A* **1993**, *657*, 429−434. (b) Bao, J.; Regnier, F. E. *J. Chromatogr.* **1993**, *608*, 217−224. (c) Wu, D.; Regnier, F. E. *Anal. Chem.* **1993**, *65*, 2029−2035. (d) Patterson, D. H.; Harmon, B. J.; Regnier, F. E. *J. Chromatogr., A* **1994**, *662*, 389−395. (e) Avila, Luis Z.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 5508−5512.

Tear Sample. Tear samples were collected from a healthy male volunteer using a piece of glass capillary. The capillary tube was placed near the lower outer corner of the eye, and the tear fluid entered the tube by capillary action. The total volume of tear sample was calculated from the inner diameter and the length of the capillary occupied by the sample (200−500 nL). It is very difficult to accurately add individual reagents in nanoliter quantities to the small sample volume. Therefore, a reagent mixture was first prepared by adding 1 μL of 0.5 M phosphate buffer (pH 7.0), 1 μL of HVA (1.7 mg/mL), 1 μL of GOx (4 mg/mL), and 1 μL of POx (0.1 mg/mL) to 100 μL of water. The tear sample was transferred to a 0.5 mL plastic centrifuge vial containing 5 or 10 μL of this reagent mixture. The capillary tube used for sampling was rinsed with the solution. The vial was capped to prevent evaporation, and the reaction was allowed to proceed for 30 min at room temperature before analysis by CE−LIF. A sample blank was prepared in a similar fashion but without the addition of glucose oxidase. For the on-column reactions, the tear sample was transferred from the collecting capillary to a microvial provided for the P/ACE system, from which injections were performed directly.

RESULTS AND DISCUSSION

Enzymatic Reactions. The oxidation of a variety of fluorogenic compounds by H_2O_2 can be catalyzed effectively using the enzyme horseradish peroxidase or hematin.[17,18] The oxidation products are highly fluorescent species that under a given set of experimental conditions have a fluorescence intensity proportional to the concentration of H_2O_2. This establishes the basis for the determination of glucose by means of eq 1. We investigated the fluorogenic reagents dihydrofluorescein diacetate (DHFD) and HVA. The solutions of DHFD showed a high background signal, limiting detectability; this compound was prone to rapid degradation and had to be prepared within 4 h of the analysis. Furthermore, it has been reported that several species can interfere with the reaction when DHFD is used (e.g., uric acid, ascorbate, and vitamin E).[17c] The solutions of HVA, on the other hand, were stable for months, and the reaction has been shown to be relatively free of interferences for several important metabolites found in body fluids (e.g., ascorbate, bilirubin, uric acid, and others).[19] Therefore, we used HVA as the substrate for the peroxidase enzyme.

The generated species HVA_{ox} has maximum excitation and emission wavelengths centered at 315 and 425 nm, respectively. Guiltbault and co-workers[17a,19] have shown that the initial rate of reaction for the formation of HVA_{ox} is proportional to the concentration of glucose when introduced by means of eq 1. In their experiments, they found that the change in fluorescence per unit time was best at pH 8.5. Because the reaction and detection were performed at this pH, it was a compromise between the optimum pH for the enzyme-catalyzed reactions (pH ∼7) and the optimum pH for the maximum fluorescence intensity of HVA_{ox} (pH >9).

To investigate how the fluorescence intensity is affected by the pH of the electrolyte used for detection, we performed the enzymatic reactions using a phosphate buffer system with a pH of 7 (a final concentration of 5 mM) and a 3 μM glucose solution. The pH of the detection electrolyte was adjusted and the fluorescence intensity monitored by CE−LIF. Figure 1 illustrates how the relative fluorescence intensity depends on the pH of the electrolyte used for detection. The relative fluorescence intensity reaches its maximum at a pH close to 9.3. We also performed the enzymatic reactions at different pHs (6−9) and monitored the reaction product at pH 9.3. Maximum signal was observed when the reactions were performed at pH 7. Thus, we performed the enzymatic reactions at a more favorable pH condition (pH 7) and then analyzed the reaction products by CE−LIF using a detection electrolyte with a pH of 9.3.

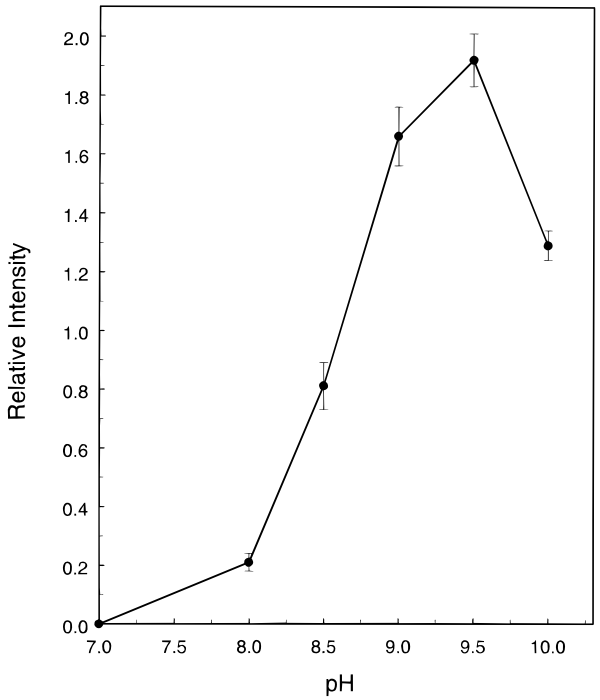

Figure 1. Dependence of the signal intensity on the pH of the CE running electrolyte.

Although in principle either the end point assay or the rate (kinetic) assay method can be used to determine glucose, we selected the end point approach because of its higher precision and lower susceptibility to small changes in temperature, enzyme activity, time of measurements, and incubation.[20] Figure 2 depicts the dependence of the fluorescence intensity on the reaction time at four different temperatures. As the incubation temperature is increased, less time is required to obtain the maximum signal intensity. For example, when the reaction mixture is incubated at 40 °C, the maximum signal is obtained in less than 20 min. When the reaction is performed at room temperature, the maximum signal intensity is achieved ∼35 min after initiating the reaction. All subsequent experiments involving the enzymatic reactions prior to injection were performed in a phosphate buffer (5 mM final concentration) at pH ∼7 at room temperature. The reaction products were analyzed by CE−LIF using 10 mM of the running electrolyte (pH ∼9.3).

Two schemes are possible for the detection of glucose using the above enzymatic reactions. In the first one, the enzymatic

(17) (a) Guilbault, G. G.; Brignac, P. J., Jr.; Juneau, M. *Anal. Chem.* **1968**, *40*, 1256−1263. (b) Zaitsu, K.; Ohkura, Y. *Anal. Biochem.* **1980**, *109*, 109−113. (c) Cathcart, R.; Schwiers, E.; Ames, B. N. *Anal. Biochem.* **1983**, *134*, 11−116.

(18) Genfa, Z.; Dasgupta, P. *Anal. Chem.* **1992**, *64*, 517−522.

(19) Kiang, S. W.; Kuan, J. W.; Kuan, S. S.; Guilbault, G. G. *Clin. Chem.* **1976**, *22*, 1378−1382.

(20) Harris, D. A.; Bashford, C. L. *Spectrophotometry & Spectrofluorimetry: a practical approach*; IRL Press: Washington, DC, 1987, pp 54−55.

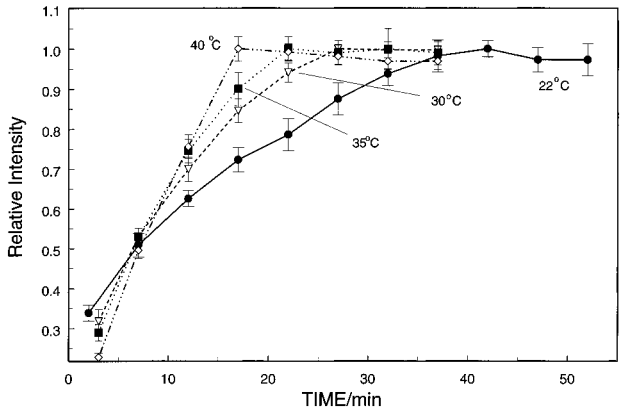

Figure 2. Effect of reaction time and temperature on the signal response.

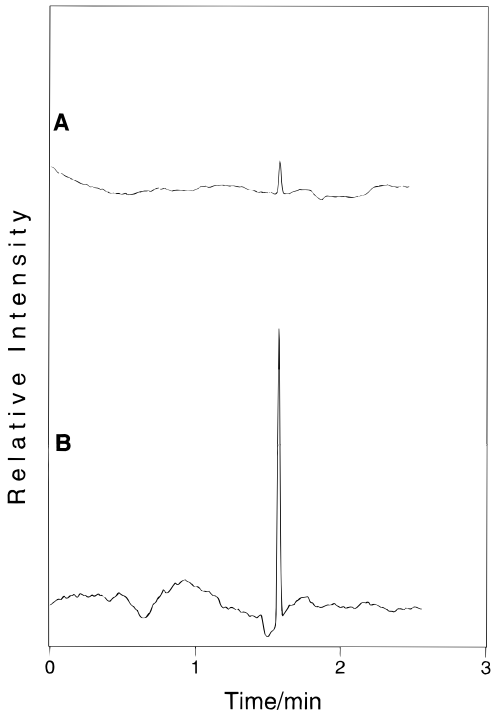

Figure 3. Electropherograms of the precolumn reactions corresponding to a blank (A) and 1 μM glucose (B). CE conditions were 10 s pressure injection (0.5 psi), separation voltage of 20 kV, 10 mM borate (pH 9.2) running buffer, fused-silica capillary (50 μm i.d., 27 cm length, injector to detector 20 cm), and LIF detection (325 nm exitation and emission collected through 380 nm filter).

reactions are performed prior to injection into the CE system (precolumn reactions). This procedure provides the opportunity to perform the enzymatic reactions and detection at optimized pH conditions. In the second scheme, the reactions are performed on-column, which allows the use of very small sample quantities without the need for any precolumn procedure. In this approach, however, the reaction and the detection are performed under the same pH conditions, which compromises enzyme activity and maximum fluorescence intensity of HVA_{ox}. Nevertheless, both schemes were explored.

CE−LIF Precolumn Reactions. A typical electropherogram corresponding to a precolumn reaction of 1 μM glucose at room temperature is shown in Figure 3. A control run (blank) gave a small fluorescence signal, which was easily subtracted from the sample signal. This blank signal arises from mixing HVA and peroxidase and can be attributed to impurities in the commercial peroxidase reagent which oxidizes HVA.[17a] The product of the reaction is analyzed by CE−LIF. The reproducibility in the migration time for HVA_{ox} was ~0.2% RSD (n = 18). The peak height reproducibility associated with the reaction of 1 μM glucose for five different reactions was <5% RSD; injections from the same sample reaction also resulted in <5% RSD (n = 5). Under our experimental conditions, the fluorescence intensity showed a linear response for glucose (r = 0.999) from 0.1 to 30 μM. Increasing the concentrations of HVA and peroxidase resulted in an increase of the background signal, thereby limiting detectability. However, the concentrations indicated in the Experimental Section were satisfactory for the range investigated. The limit of detection of the method was calculated using 3σ of the blank. A signal equal to 3σ of the blank response (n = 18) corresponded to a glucose concentration of 55 nM, based on the response signal of 200 nM glucose. This particular procedure compared favorably with an established method used for the noninvasive analysis of tear fluid in six human subjects.[21]

CE−LIF/On-Column Reactions. In the on-column reaction scheme, all reagents required for the two enzymatic reactions were introduced into the CE running buffer (pH ~8.5). This approach is similar to EMMA (the pertinent considerations regarding EMMA have been properly discussed in the literature).[16] The sample solution was injected directly into the capillary (10 s at 0.5 psi), and the enzymatic reactions were allowed to proceed inside the column. Mixing of the glucose sample with the reagents contained in the running electrolyte can be achieved with electrophoretic mixing or without. Figure 4 depicts electropherograms of the reaction product corresponding to 100 μM glucose injected into the capillary with and without electrophoretic mixing. The electropherogram in panel A was obtained when a potential of 20 kV was applied immediately after injection, while the one in panel B was obtained when the separation potential was initiated 3 min after injection. The incubation time of 3 min resulted in a 6-fold increase in peak height. Mixing of the reagents proceeded simply by diffusion across the sample−running buffer interface. Further incubation times did not provide much improvement in peak height; peak broadening also increased with time. In panels C and D, the analyte and the reagents were mixed electrophoretically. Mixing is achieved because of the mobility differences of the compounds under the electric field. In panel C, 1 kV was applied for 1 min immediately after injection; then, the potential was turned off for an incubation period of 2 min. The potential was reinitiated at 20 kV to bring the reaction product to the detector and the electropherogram recorded. In panel D, 10 kV was used for electrokinetic mixing instead of 1 kV. The increase in mixing potential affected the migration time of the product of interest. This is because product formation takes place closer to the detector. The peak height using 10 kV for mixing instead of 1 kV resulted in a 63% increase. The height was also higher with electrophoretic mixing with an incubation period of 3 min (Figure 4, panel B). A mixing potential of 10 kV for 1 min was found to be optimun for our measurements. Under our experimental conditions, the net migration velocity of glucose (neutral species) is faster than the reagents present in the separation electrolyte solution. The fast-moving zone containing glucose penetrates into the reagent zone, thus resulting in a higher mixing efficiency compared to the mixing by diffusion only.

(21) Chen, R.; Jin, Z.; Colón, L. A. *J. Capillary Electrophor.*, in press.

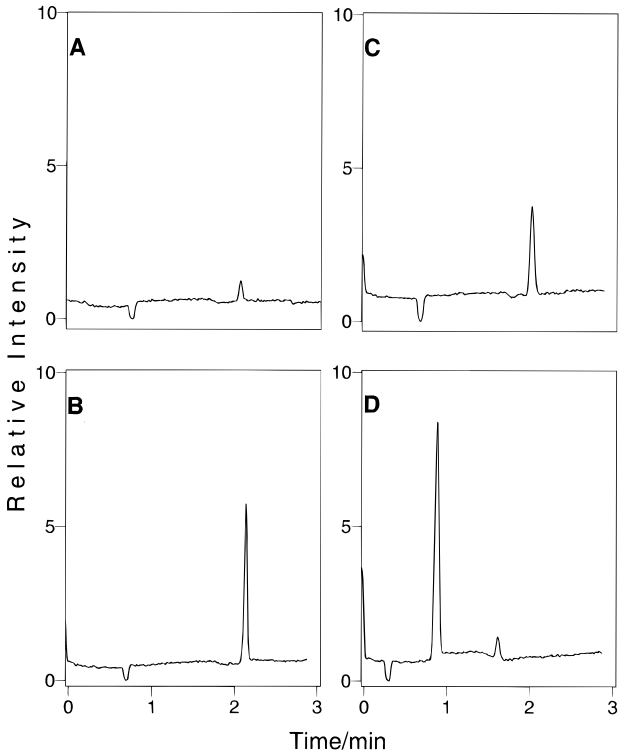

Figure 4. Electropherograms for the on-column reactions of 100 μM glucose. (A) Injection of glucose without mixing and without incubation time. (B) Incubation for 3 min after injection (mixing by diffusion). (C) Injection of glucose, mixing at 1 kV for 1 min, and incubation for 3 min. (D) Mixing at 10 kV for 1 min and incubation for 3 min. The CE conditions were as Figure 3 except that the buffer was composed of 20 mM phosphate (pH 9), 1 mg/mL glucose oxidase, 0.4 mg/mL HVA, and 0.1 mg/mL peroxidase.

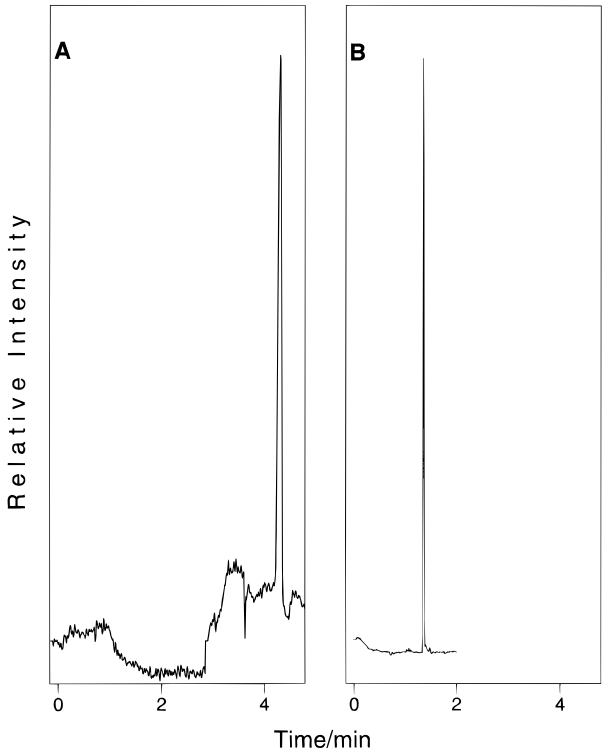

Figure 5. Electropherograms corresponding to glucose in tear samples. (A) On-column reaction, 6 s injection mixing at 10 kV for 1 min, incubation for 2 min; other conditions as in Figure 4. (B) Precolumn reaction; conditions as in Figure 3.

The small peak observed at ~1.8 min in Figure 4D corresponds to reaction product formed immediately after injection, at the interface between the sample and electrolyte zones. After applying 10 kV for mixing, glucose migrates with the electroosmotic flow (EOF) toward the detection window. The negatively charged product initially generated at the interface of the sample zone, and the running electrolyte moves away from the unreacted glucose. During the incubation period, more fluorescent product accumulates in another zone (i.e., glucose zone). Two distinct zones containing the fluorescent product are formed and migrate as such through the column after the electric field is re-initiated. The major peak corresponds to the product generated during the incubation period. The peak height of the small peak is similar to that of the peak shown in Figure 4A, indicating its association with the fluorescent product initially formed at the interface of the sample and the running electrolyte. Product formation also occurs during the 1 min mixing period. This is reflected by a small increase in the baseline between the major and the minor peaks. We performed quantitation using the major peak of the electropherogram.

An increase in the incubation time and the temperature of reaction can render more product formation.[13] We raised the temperature to 35 °C and increased the incubation time to 20 min for a 10 μM glucose solution injection. From the electropherogram obtained, the LOD (S/N = 3) was estimated to be 0.8 μM, based on the major peak. This corresponds to 18 fmol of glucose injected (~20 nL). Longer incubation times at 35 °C can improve detectability; however, the room temperature (~23 °C) and 1 min incubation period were sufficient to analyze our tear samples (vide infra).

Reproducibility was tested using a 100 μM glucose solution, injecting 10 s at 0.5 psi. The migration time of the reaction product had a reproducibility of 0.7% RSD ($n = 6$). Peak height reproducibility was 3.7% RSD ($n = 6$). The peak height of the main peak was shown to be linear ($r = 0.9997$) with respect to glucose concentration up to 0.5 mM. Above this glucose concentration, deviation from linearity was observed. This is due to the presence of unreacted glucose in the reaction plug.

Glucose in Tear Fluid. The analysis of tear fluid has the potential of becoming a noninvasive approach for clinical diagnosis.[22] Changes in the concentration of glucose in tears have shown correlation with changes in blood.[23] However, one of the major problems in analyzing tear samples is the extremely low quantities available for analysis. CE is an excellent analytical technique for the analysis of tears because of its capability to handle the low sample quantities. We used the above two procedures to determine glucose in human tear fluid. Figure 5 shows electropherograms for tear samples using both approaches, precolumn (panel A) and on-column (panel B) reactions. In the on-column reaction scheme, the sample was introduced into the capillary directly, without any sample pretreatment. The separation electrolyte (20 mM phosphate, pH ~8.5) contained all the reagents required for the enzymatic reactions (see Experimental Section).

(22) (a) Mediratia, R. K.; Rohatgi, J. N. *Ind. J. Opthalmol.* **1983**, *31*, 635−638. (b) Romano, A.; Rolant, F. *Metab., Pedriatr. Syst. Ophthalmol.* **1988**, *11*, 78−80. (c) Haeckel, R.; Hanecke, P. *Ann. Biol. Clin.* **1993**, *51*, 903−910 (ATB Conference '92).

(23) (a) Milder, B. In *Adler's Physiology of the Eye: Clinical Applications*, 8th ed.; Moses, R. A., Hart, W. M., Eds.; C. V. Mosby: St. Louis, MO, 1987; Chapter 2. (b) Romano, A.; Rolant, F. *Metab., Pediatr. Syst. Ophthalmol.* **1988**, *11*, 78−80.

The injections were performed by pressure (6 s at 0.5 psi), electrophoretic mixing at 10 kV for 1 min was used, and product was allowed to accumulate for 2 min. The voltage was then established at 20 kV. A calibration curve was constructed between 20 and 500 μM glucose in 20 mM phosphate solution. A tear sample was collected as previously described (~200 nL) and transferred to a microvial. At least three injections were performed. The amount of glucose found was 137 ± 13 μM.

In the precolumn procedure, another tear sample was collected from a healthy volunteer and reacted with the appropriate reagents as indicated in the Experimental Section. A calibration plot was also constructed, accordingly. The concentration was found to be 139 ± 3 μM. This result is not significantly different (95% confidence) than that obtained by the on-column reaction approach. However, a higher variability is observed with the on-column procedure. This can be attributed to the fact that the extremely low sample volume is prone to evaporation; the last of the three injections showed a higher signal response than the first one. In the precolumn method, on the other hand, the sample is diluted in a large volume, relative to the sample (>10-fold). Evaporation of a small fraction will not affect the sample concentration to a great extent.

CONCLUSION

The combination of enzymatic reactions and CE−LIF is a viable scheme to determine glucose present in submicroliter volumes. The method presented here is relatively rapid and simple, offering detection limits below 100 nM (for the precolumn reactions). The procedure has the potential for the noninvasive determination of glucose for diagnostic purposes. The glucose concentration in the tear samples analyzed was within the concentration range found by more tedious and time-consuming procedures.[19a,24] We analyzed human tears to show the applicability of our procedure; however, the method can also be applied to determine glucose in other small volume samples.

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(24) Sen, D. K.; Sarin, G. S. *Br. J. Ophthalmol.* **1980**, *64*, 693−695.

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