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Fluorogenic assay for β-glucuronidase using microchip-based capillary electrophoresis

Dustin E. Starkey[a], Arum Han[b], James J. Bao[c], Chong H. Ahn[b], Kenneth R. Wehmeyer[c,*], Marla C. Prenger[c], H. Brian Halsall[a], William R. Heineman[a]

[a]*Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221-0172, USA*
[b]*Department of Electrical and Computer Engineering and Computer Science, University of Cincinnati, Cincinnati, OH 45221-0018, USA*
[c]*Procter and Gamble Pharmaceuticals, Health Care Research Center, 8700 Mason-Montgomery Rd., Mason, OH 45040, USA*

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Abstract

Microchip capillary electrophoresis (CE) was used with a model enzyme assay to demonstrate its potential application to combinatorial drug screening. Hydrolysis with β-glucuronidase of the conjugated glucuronide, fluorescein mono-β-D-glucuronide (FMG), liberated the fluorescent product, fluorescein. FMG and fluorescein were detected by fluorescence, with excitation and emission at 480 and 520 nm, respectively. Microchip CE was used to separate FMG and fluorescein. Fluorescein production was monitored to assess β-glucuronidase activity. Michaelis–Menten enzyme kinetics analysis yielded the K_m value. The results were compared with those from experiments done by conventional CE. The K_m value for β-glucuronidase with FMG is being reported for the first time as 18 μ*M*. The inhibition of β-glucuronidase by the competitive inhibitor D-saccharic acid-1,4-lactone (SL) was also determined using microchip CE. Reactions were done with various concentrations of inhibitor and constant β-glucuronidase and FMG concentrations. A dose–response plot was acquired and the IC_{50} value for SL was determined to be 3 μ*M*.

Keywords: Microchips, CE; β-Glucuronidase; Enzymes

1. Introduction

Microchip capillary electrophoresis (CE) has been the focus of ongoing research because of its potential applications and advantages. Smaller volumes of sample and reagents are required for the micrometer-sized channels, in which the pumping mechanism is electroosmotic. This provides accurate control of the small reagent volumes and eliminates the need for pumps and valves. Electrophoretic separations on microchips are faster than and comparable in efficiency to conventional CE [1–3]. These advantages make microchip CE potentially useful in a number of research areas, such as biochemical and clinical applications [4]. Several applications in these areas have been described including immunoassay [5–8],

*Corresponding author. Tel.: +1-513-622-2149; fax: +1-513-622-0086.
E-mail address: wehmeyer.kr@pg.com (K.R. Wehmeyer).

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measuring antibody binding affinity [9] and diagnosing molecular-based disease [10–12].

The high-throughput screening (HTS) of pharmaceutical drug libraries is another potential application of microchip CE. Microchip CE in a multiple separation-channel format could provide a hundred or more parallel analyses within minutes or even seconds. Multiple-channel microchip CE devices have already been used to analyze DNA restriction fragments [13,14]. Enzymes, which are often targets for drug therapy, can be assayed fluorescently in a high-density microplate format for the HTS of combinatorial libraries that have large numbers of compounds with enzyme inhibition properties [15–17]. However, direct plate-based enzyme inhibition assays can suffer from interferences if library compounds fluoresce or quench the fluorescence of the product. Using a rapid, separation-based enzyme inhibition assay can avoid false positive and negative results. Enzyme assays have been done successfully on single separation-channel, microchip CE devices. Two of these were fluorogenic assays for β-galactosidase and acetylcholinesterase, in which microchips were used more for flow injection analysis rather than for separation purposes [18,19]. Microchip CE was also used to assay protein kinase A [20]. The protein kinase A catalyzed a reaction between ATP and a fluorescently labeled heptapeptide, where a γ-phosphate group was transferred from ATP to the peptide. The phosphorylated and non-phosphorylated peptides were separated electrophoretically to monitor the enzymatic conversion. The inhibition of enzyme activity by known inhibitors was demonstrated in all three of these studies.

β-Glucuronidase (EC 3.2.1.31) is an acid hydrolase that also has fluorescent substrates, and therefore could be used in a microchip enzyme assay. In humans, the enzyme degrades conjugated glucuronides in lysosomes. It is believed that it may also hydrolyze the conjugated glucuronides of foreign chemicals in vivo [21,22]. This has been used beneficially in that the increased activity of β-glucuronidase in cancerous tumors has led to the development of anti-cancer prodrugs, where the prodrug is delivered to the tumor as a non-toxic glucuronide, and is then activated by the enzyme [22]. Conversely, β-glucuronidase may promote cancerous formations by cleaving conjugated glucuronides of carcinogenic compounds. Inhibiting β-glucuronidase in this case would promote excretion of the carcinogens [22]. Fluorescence assays of β-glucuronidase activity have used 4-methylumbelliferyl-β-D-glucuronide (MUG) [21,23–25] and HPLC and CE were used in three of these to separate and monitor the hydrolysis product, 4-methylumbelliferone, from the MUG substrate [21,24,25]. Fluorescein mono-β-D-glucuronide (FMG) is another fluorescent substrate of β-glucuronidase that could be used in a separation-based assay. This conjugate is hydrolyzed by β-glucuronidase, liberating fluorescein [26–28]. Fluorescein has a larger extinction coefficient than 4-methylumbelliferone, a larger quantum yield and a lower sensitivity to photochemical decomposition, making FMG an attractive choice [29].

We describe in this paper the application of microchip CE to the model assay of β-glucuronidase with FMG and with the competitive inhibitor D-saccharic acid-1,4-lactone (SL). The results demonstrate the potential use of microchip CE in the rapid screening of combinatorial compounds in enzyme inhibition formats.

2. Experimental

2.1. Chemicals and reagents

Disodium fluorescein was from ICN Pharmaceuticals (Costa Mesa, CA, USA). Type-1 bovine liver β-glucuronidase (1 640 000 modified Fishman units/g, where 1 unit liberates 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at 37°C), FMG, boric acid and SL were from Sigma (St. Louis, MO, USA). Sodium acetate, hydrogen peroxide (30%), ammonium hydroxide (14.8 *M*), hydrochloric acid (12.1 *M*), potassium iodide, iodine, hydrofluoric acid (28.9 *M*) and nitric acid (15.8 *M*) were from Fisher Scientific (Pittsburgh, PA, USA). Sodium hydroxide (1.0 and 0.1 *M*) and glacial acetic acid were from J.T. Baker (Phillipsburg, NJ, USA).

All solutions were prepared with distilled deionized water (Millipore, Bedford, MA, USA). A 50 m*M* borate buffer was prepared by dissolving boric acid in water and adjusting the pH to 10 with 1.0 *M* and 0.1 *M* NaOH. A 100 m*M* sodium acetate buffer

was prepared by dissolving sodium acetate in water and adjusting the pH to 5.0 with dilute acetic acid. A 0.919 mg/ml stock solution of FMG was prepared by dissolving 1 mg FMG in water and adjusting the concentration for water content. The enzyme was prepared daily in sodium acetate buffer as either 50 units/ml or 250 units/ml. A 971 μ*M* SL stock solution was prepared in water and was diluted in sodium acetate buffer before use. All buffers were filtered using a 0.22-μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) before using with the microchips.

2.2. Microchip fabrication

Microchip channels were fabricated using standard lithography followed by wet chemical etching and thermal bonding [30]. Borosilicate glass wafers (250 μm thick) (US Precision Glass, Elgin, IL, USA) were annealed at 560°C for 6 h and cleaned with a 70°C H_2O_2–NH_4OH (4.3%:2.1 *M*) solution before depositing on them 400 Å Cr and 4000 Å Au by electron beam evaporation. The wafers were spin-coated with photoresist (Shipley 1818, Marlborough, MA, USA) at 4000 r.p.m. for 30 s and soft-baked at 90°C for 30 min. A mask aligner was used to expose the photoresist layer through the mask design shown in Fig. 1. The exposed channel pattern was developed with Shipley 1818 developer solution and hard-baked at 120°C for 30 min. Cr and Au were removed from the exposed portions with a 6.1 *M* HCl solution and aqueous KI–I_2 (0.6 *M*:0.2 *M*) solution, respectively. After removing the metal films, the glass was etched with a HF–HNO_3 (5.8 *M*:2.2 *M*) solution for 20 min at room temperature to a depth of 15 μm and width of 70 μm. Channel-access holes were drilled with a 1.6-mm diameter diamond-plated drill bit. The remaining photoresist and metal films were removed with acetone and metal etchant. The processed wafer and a second wafer were thermally bonded at 630°C for 6 h in a furnace to encapsulate the channels. A second bonding cycle was repeated if necessary to remove localized, incomplete bond areas.

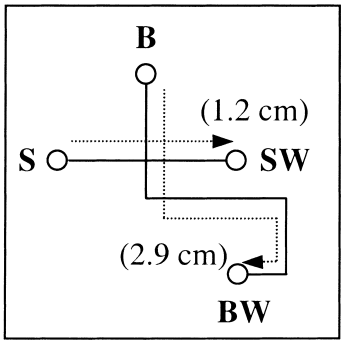

Fig. 1. Microchip channel design and lengths used in all experiments. The channel-access hole/reservoir abbreviations are sample (S), buffer (B), sample waste (SW) and buffer waste (BW).

2.3. Instrumental

Microchip experiments were observed with a Nikon Eclipse TE300 inverted epifluorescence microscope (Melville, NY, USA). A 150-W xenon lamp was the fluorescence excitation source. A filter block in the microscope consisted of a 480±20 nm excitation filter, a 505 nm dichroic mirror and a 535±25 nm emission filter. A 10× microscope objective coupled the 480 nm excitation light to the chip and collected the 520 nm emission signal. The emission signal was spatially filtered with a 1-mm pinhole before being detected with a photomultiplier tube (Hamamatsu R3896, Bridgewater, NJ, USA). The PMT was powered at −900 V with a Stanford Research Systems, Model PS325 high voltage power supply (Sunnyvale, CA, USA) and a Stanford Research Systems, Model SR570 low-noise current preamplifier converted the PMT output to a voltage signal. Data were acquired with a PE Nelson 900 Series interface and TURBOCHROM software (San Jose, CA, USA) controlled by a PC. Voltages were applied to the microchip through platinum electrodes using a μTK Microfluidic Tool Kit (Alberta Microelectronics, Alberta, Canada), which was controlled by a LABVIEW (National Instruments, Austin, TX, USA) program written by the Alberta Microelectronics Corporation.

Conventional CE experiments used a Bio-Rad BioFocus 3000 capillary electrophoretic system (Hercules, CA, USA) interfaced to a PC. Detection was by a Bio-Rad BioFocus LIF^2 laser induced fluorescence system. Excitation and emission wavelengths were 488 and 520 nm, respectively.

2.4. CE conditions

Microchip separations were done in a channel with total length of 2.9 cm and effective length of 2.3 cm (Fig. 1). Channels were conditioned before each experiment by slowly pulling reagents through with a vacuum hose. Conditioning consisted of distilled water for 5 min, 0.1 *M* NaOH for 20 min, distilled water for 5 min and running buffer for 10 min. The channels and access holes (B, SW and BW) were filled with running buffer after conditioning. Micropipette tips were inserted into the access holes (B, SW and BW) to serve as buffer reservoirs and to house the Pt electrodes. Sample was added to and a Pt electrode was inserted into the channel-access hole (S) rather than a pipette tip in order to make sample exchange easier. The separation voltage was 2.7 kV (field strength = 931 V/cm) under normal polarity. Electrokinetic injection was for 15 s by applying 1.5 kV across the injection channel. Pinched injection was achieved by applying 1.0 and 1.3 kV to the buffer and buffer waste reservoirs, respectively, during the 1.5-kV application to the injection channel. Single point fluorescence detection was attained by aligning the excitation light at the end of the separation channel, just before the buffer waste reservoir.

Conventional CE separations were done in an uncoated, fused-silica capillary (Bio-Rad) with a total length of 26.3 cm and effective length of 22.5 cm (50 μm I.D.). The capillary was conditioned before each experiment with 1.0 *M* NaOH for 5–8 min, distilled water for 3 min and running buffer for 5 min. The applied voltage was 20 kV under normal polarity (field strength = 760 V/cm). The capillary temperature was maintained at 25°C. Samples were pressure injected at 2 p.s.i.·s (1 p.s.i. = 6894.76 Pa).

2.5. Microchip characterization conditions

A 1.6 m*M* stock solution of disodium fluorescein was prepared in water. The fluorescein solution was diluted in 50 m*M* borate, pH 10, to various concentrations for monitoring electroosmotic flow and testing injection in the microchip. Various injection schemes were tested by applying different voltages to the microchip reservoirs and access holes, and monitoring the quality of the injected sample plug and chromatographic peak shape. A fluorescein calibration was also done to test the quantitative abilities of our chip and detection system. Fluorescein was diluted serially in 50 m*M* borate, pH 10, to make standards between 1 n*M* and 1.5 m*M*.

2.6. Assay conditions

A β-glucuronidase working stock solution of 250 units/ml was used in the microchip assays. FMG working stock solutions of 91.9 μ*M* and 23.0 μ*M* were prepared in sodium acetate buffer. Appropriate volumes of FMG and sodium acetate buffer were added to a microcentrifuge tube followed by 20 μl of β-glucuronidase stock solution (5 units), which made the final assay volume 150 μl. All assays were carried out at room temperature. The β-glucuronidase working stock solution had been aliquotted into separate vials and kept in ice, and was brought to ambient temperature before use. The assay components were mixed immediately with a disposable transfer pipette and were transferred to the microchip channel-access hole (S) within a minute of initiation of the reaction. Injections and separations of the substrate and hydrolysis products were done repeatedly. The reaction time was defined as from when β-glucuronidase was added until the sample was injected. After data were acquired for each reaction, the access hole was emptied and flushed with buffer to prepare for the next reaction.

A β-glucuronidase working stock solution of 50 units/ml was used in the conventional CE assays. The FMG working stock concentrations were as used in the microchip assay and reactions were done at ambient temperature. Appropriate volumes of FMG stock solution and sodium acetate buffer were added to a microcentrifuge tube followed by 20 μl of β-glucuronidase stock solution (1 unit), which made the final assay volume 150 μl. The reaction components were mixed immediately with a transfer pipette and the tube was placed in the autosampler. The reaction time was defined as from when β-glucuronidase was added until the sample was injected.

2.7. Inhibition assay conditions

The competitive inhibitor SL was diluted serially in sodium acetate buffer, from an initial stock solution, to make working stock solutions of 388,

77.7, 7.8 and 0.78 μ*M*. The FMG concentration (27.6 μ*M*) and β-glucuronidase concentration (5 units) were the same for all experiments. FMG was added to a microcentrifuge tube, followed by the appropriate volume of inhibitor and lastly the β-glucuronidase, giving a final assay volume of 150 μl. The remaining procedure was the same as the previous microchip assay. Reactions were repeated three times for each inhibitor concentration, except for 0.78 μ*M* inhibitor, which was repeated four times. The data were analyzed with the graphing program GRAFIT 3.0 (Erithacus Software, Horley, Surrey, UK).

3. Results and discussion

3.1. Microchip characterization

The microchip system was characterized initially with solutions of disodium fluorescein. This permitted the electroosmotic flow and injection process in the microchip channels to be seen. Initial efforts to introduce the sample using a simple cross-channel injection format resulted in band broadening and reduced peak resolution due to sample diffusing into the separation channel (data not shown). Similar results have been reported by other workers [31–33]. Sample leaking from the injection channel into the separation channel during the microchip separation also contributed to band broadening. Hydrodynamic flow, caused by a discrepancy in buffer heights between the S access hole and the others (SW, B and BW), may have contributed to the problems as well. Sample injection using a pinching scheme [34] was evaluated as an improvement to the injection process. Voltages were also applied to the injection side channels during separation to move sample away from the separation channel. After these steps were taken, the problems were eliminated and a reasonable peak shape was obtained. A quantitative experiment was done using replicate ($n=3$) injections of a series of standards to evaluate the precision of injection and microchip quantitation under the pinched voltage conditions. The migration times were precise with RSD values of 3% or less while the peak area and height reproducibility varied from 0.83 to 8.8% across the concentration range (Table 1). The system gave a linear response to concentration from 25 nM to 250 μ*M*. The $1/x^2$ weighted linear regression gave a slope of 0.43 ± 0.01 and intercept of -2.3 ± 0.5, with a correlation coefficient of 0.998. The presence of an internal standard would improve the reproducibility of both the migration time and peak integration.

Table 1
Reproducibility ($n=3$) of migration time, peak area and height

Conc. (n*M*)	RSD (%)		
	Time	Peak area	Peak height
25	1.6	6.1	5.8
50	0.54	4.3	2.4
100	0.82	2.5	3.5
250	0.62	5.0	3.8
500	2.3	4.4	5.4
1000	1.9	4.4	4.1
5000	3.1	4.7	4.4
10 000	3.0	7.6	8.8
50 000	1.2	4.3	3.9
100 000	0.82	2.8	3.4
250 000	2.0	1.3	0.83

3.2. Assay development

The reaction between β-glucuronidase and FMG is shown in Fig. 2 [28]. We chose a 100 m*M* sodium acetate, pH 5.0, buffer for the enzyme assay based on previously reported work with other substrates [35]. A separation of FMG and fluorescein using microchip CE is shown in Fig. 3a. The running buffer was 50 m*M* borate, pH 10, because this provided optimal conditions for the fluorescence detection of fluorescein and gave a reasonable electroosmotic flow for rapid separation. These conditions gave a separation of FMG and fluorescein with good resolution in under 30 s. The running buffer used in conventional CE assays of β-glucuronidase was also 50 m*M* borate, pH 10.0. The separation of FMG and fluorescein with the Bio-Rad system also gave good resolution, but required 1.5 min to resolve the peaks (data not shown).

3.3. Enzyme assay and kinetics

An electropherogram obtained by repeatedly injecting a reaction mixture of 5 units of β-glucuronidase and 24.1 μ*M* FMG is shown in Fig. 3b. Each set of peaks represents a new injection of the reaction

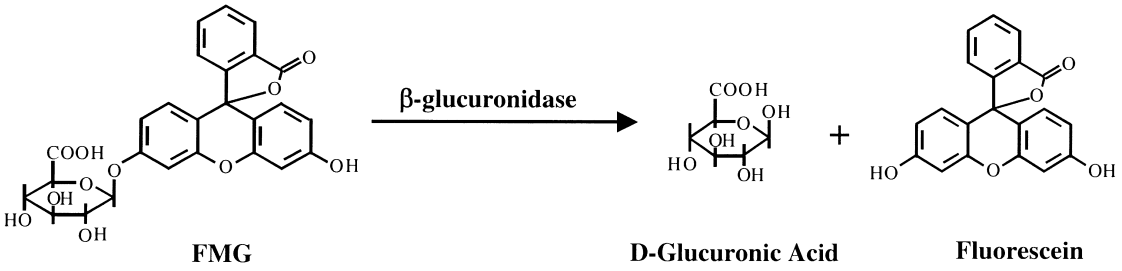

Fig. 2. Hydrolysis of FMG by β-glucuronidase yielding fluorescein and D-glucuronic acid.

mixture and separation of FMG and fluorescein (Fig. 3a). Fluorescein production was linear during the 30-min reaction.

The FMG concentration was varied and the β-glucuronidase concentration was held constant in a series of reactions. Reactions were done with FMG concentrations between 0.92 and 55.1 μ*M* and were monitored for 5–6 min using microchip CE. The peak areas for fluorescein were integrated, plotted versus reaction time, and the slope was taken as initial velocity. These were plotted versus [S] and a nonweighted, nonlinear least-squares best-fit to the Michaelis–Menten equation was done with SIGMA PLOT 4.0 (SPSS Science, Chicago, IL, USA). Fig. 4a shows the combined results of two microchip assays for β-glucuronidase. Data points are the average of multiple reactions for each substrate concentration. The K_m value was determined by the SIGMA PLOT 4.0 curve fitting routine as 19±4 μ*M*, where the error is the standard error of the best-fit curve.

A K_m value for β-glucuronidase with FMG has never been reported to our knowledge, therefore a comparison experiment was done on a conventional CE system under similar conditions. Reactions were monitored for about 17 min and the averaged results of two conventional β-glucuronidase assays are shown in Fig. 4b. The line drawn through the data points represents the nonweighted, nonlinear least-squares best-fit to the Michaelis–Menten equation. The K_m value was determined by the SIGMA PLOT 4.0 curve fitting routine as 17±3 μ*M*, where the error is the standard error of the best-fit curve.

The K_m values obtained from the microchip and CE assays, 19 and 17 μ*M* respectively, are in close

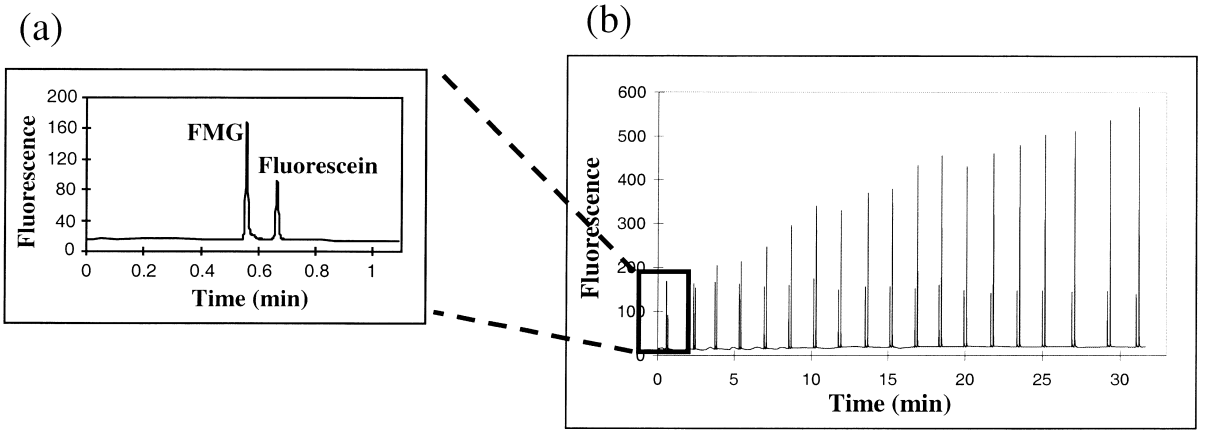

Fig. 3. (a) Each set of peaks was the separation of FMG and fluorescein using a field strength of 931 V/cm. (b) Electropherogram obtained by microchip CE, which shows a 30-min on-chip reaction between 5 units of β-glucuronidase and 26.2 μ*M* FMG. The reaction was done at room temperature in 100 m*M* sodium acetate, pH 5.0.

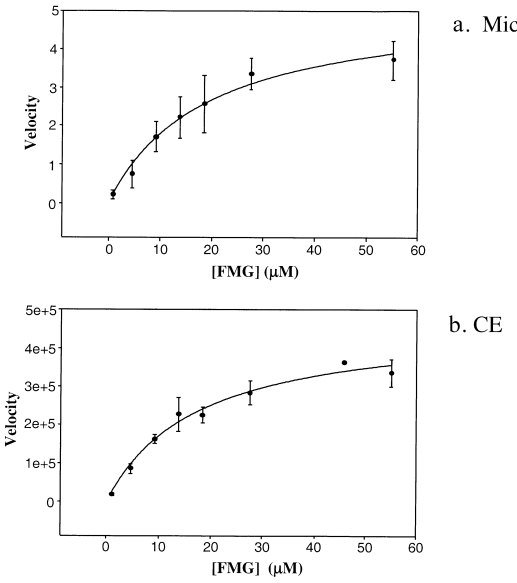

Fig. 4. Kinetic plots of initial velocity versus substrate concentration for the (a) microchip and (b) CE assay of β-glucuronidase with FMG. The data in (a) are the average of two assays, where $n=4$ for 0.92 μ*M*, 9.2 μ*M*, $n=6$ for 4.6 μ*M* and $n=5$ for the remaining points. The error bars in each plot are standard deviations of the average of signal measurements for each substrate concentration. The data in (b) are the average of two CE assays, where $n=2$ for 45.9 μ*M*, $n=3$ for 55.1 μ*M* and $n=4$ for the remaining concentrations. The FMG concentration 45.9 μ*M* was used in only one assay, therefore, it was the average of only two points and did not receive an error bar.

agreement. Therefore, the K_m value for the enzyme is being reported as 18 μ*M*, which is the average of the two methods.

The variability in the replicate measurements for each substrate concentration by microchip CE was greater than conventional CE, although the calculated K_m values did not differ significantly. The higher variability with the microchip approach is probably due to the fact that the conventional CE system uses automated injection procedures whereas the microchip injections were manual. Further automation of the microchip injection process would be expected to improve the precision for replicate injections. Although the data acquisition by microchip CE was somewhat less precise, it was more time efficient than conventional CE. The conventional CE automation sequence and longer separation time required about 17 min per reaction in order to acquire enough data points for an initial velocity determination. Reactions were monitored for only 6 min by microchip CE. The short microchip separation channel had a shorter separation distance and therefore shorter separation times. Microchip injection was also more convenient since it only required switching voltages to change from separation to injection. A disadvantage of the microchip CE procedure was the manual exchange of sample for each concentration, which made the procedure less convenient and increased the time required to perform the assay. The cross channel design allowed only sample injection and separation. A more complex channel design would permit dilution and reaction mixing on the chip, which would avoid the need to change samples for each concentration [18–20].

3.4. Enzyme inhibition assay

An assay of β-glucuronidase with the competitive inhibitor SL [22] was done by microchip CE, and the results are presented in Fig. 5 as a dose–response plot. Reactions were done with varying inhibitor concentrations while substrate and enzyme concentrations were held constant at 27.6 μ*M* and 5 units, respectively. The signal was measured as the fluores-

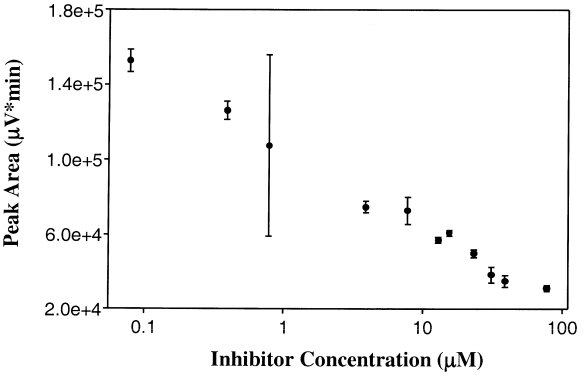

Fig. 5. Dose–response curve of fluorescein peak area versus inhibitor concentration for D-saccharic acid-1,4-lactone with β-glucuronidase, as analyzed by microchip CE. The peak area of fluorescein was measured at 220 s in each reaction. The data points are averages of $n=4$ replicates for 0.78 μ*M* and $n=3$ replicates for the remaining concentrations. Error bars are standard deviations of the average peak areas at each inhibitor concentration.

cein peak area at 220 s reaction time. The data points in Fig. 5 are the averages of the replicate signal measurements for each inhibitor concentration. The IC_{50} value was 3 ± 2 μM, where the error is the standard error of the determination, using the GRAFIT 3.0 graphing program.

It is unknown why the standard deviation for the 0.78 μM SL data point is so large. Since the point occurs during the rapidly changing part of the curve it is possible that small errors in the reaction timing could have contributed to the high variability. The speed advantage of microchip CE was shown once again, since the acquisition time of each reaction was under 5 min. However, the analysis time for the entire experiment was long due to the number of inhibitor concentrations and the number of replicates. The use of multilane chips, as reported previously for a centrifugal microfluidic system [36], would greatly increase the speed and convenience of the approach.

4. Conclusions

We have demonstrated that microchip CE can be applied to a model enzyme assay. A simple cross-channel microchip design was used to inject the reaction components and separate them quickly and efficiently. A comparison of microchip assay results with those of conventional CE showed that similar K_m values were obtained for the enzyme by the two techniques. However, the microchip approach allowed the separations to be completed roughly three times faster than conventional CE.

Monitoring β-glucuronidase inhibition by microchip CE gave an associated dose–response plot that showed the potential use of the technique in drug discovery. The individual reactions in our experiments were performed rapidly, but the total analysis time was quite long due to using only a single-lane chip and the need to change samples during the course of the analysis. Not only could our experiment be finished in less than 5 min if done on a multiple-channel microchip, but using more complex chip designs that provided on-line dilution and addition of reagents could also reduce analysis time further.

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