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Amino acid and peptide analysis using derivatization with *p*-nitrophenol-2,5-dihydroxyphenylacetate bis-tetrahydropyranyl ether and capillary electrophoresis with electrochemical detection

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

The amine derivatization reagent *p*-nitrophenol-2,5-dihydroxyphenylacetate bis-tetrahydropyranyl ether (NDTE) was used in conjunction with capillary electrophoresis (CE) and electrochemical detection (EC) for the pre-separation derivatization of primary amine analytes present in aqueous solution. Glycine, several dipeptides and angiotensin II were used as model analytes. A miniaturized EC detection cell was designed and fabricated, which featured a fractured-joint field decoupler with a fixed end-column carbon fiber electrode. When a series of glycine and angiotensin II calibration solutions were derivatized with NDTE followed by CE–EC determination, linear calibration plots resulted with pre-derivatization concentration limits of detection of 500 nM (106 attomoles on-column) and 6 μM (1.275 femtomoles on-column), respectively.

Keywords: *p*-Nitrophenol-2,5-dihydroxyphenylacetate; bis-Tetrahydropyranyl ether; NDTE; Electrochemical detection; Capillary electrophoresis; Electrochemical derivatization; Amine derivatization

1. Introduction

The use of electrochemical detection (EC) in capillary electrophoresis (CE), first demonstrated in 1987 by Wallingford and Ewing [1], has proven to be an interesting option for the analysis of trace quantities of electroactive compounds. In general,

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EC is ideally suited for use within the microdetection environment of a capillary as compared with the use of optical detection methods. Signal to noise ratios often become more favorable with miniaturization in EC [2], whereas these ratios become less favorable when using optical detection methods.

Despite past successes, and the fundamental advantages of using EC within microseparation environments, routine use of this detection format for CE remains isolated to only a few laboratories due to some interrelated limiting factors. These include the inherent operator-intensive nature of this technique, the lack of commercial instrumentation, and the limited number of analytes that possess adequate electrochemical detectability. Despite these limitations, incremental advances have been made in CE–EC over the past decade and these have been reviewed [3–5]. In addition to advances in conventional CE–EC, several reports have been made detailing the use of CE chips with integrated EC [6,7]. It is perhaps within the confines of the microchip where the future utility of CE–EC is most strongly implicated, again due to favorable response to miniaturization. In comparison with EC, the shear size of the other microcompatible detection technologies, including both laser-induced fluorescence (LIF) and mass spectrometry, place a substantial burden on the otherwise compact and portable microchip separation format.

Past applications of CE–EC have mainly been to the determination of natively electrochemically active analytes including neurotransmitters [8], thiols [9], certain amino acids [10,11], and sugars [12]. Unfortunately, many biological compounds (e.g. most amino acids and peptides) lack the physical–chemical properties required for trace determination by native electrochemical or optical detectability. One option for the detection of non-electrochemically active molecules when using CE–EC is the use of derivatization chemistry to transform these analytes to electrochemically detectable moieties. Past reports of the derivatization/transformation of bioanalytes for subsequent analysis using CE–EC have been limited. Nonetheless, there have been some successful applications of this methodology including the analysis of peptides after complexation with Cu(II) [13,14] and the use of naphthalene-2,3-dicarboxaldehyde (NDA) derivatization [15,16] for the determination of cellular amino acids.

In the present research, a limited feasibility study was undertaken for the determination of poorly detectable amino acids and peptides using CE–EC after derivatization with a novel electrochemical derivatization reagent, *p*-nitrophenol-2,5-dihydroxyphenylacetate bis-tetrahydropyranyl ether (NDTE) [17]. In a two-step process (Fig. 1), NDTE derivatization provides a hydroquinone moiety to analytes possessing a primary or secondary amine functional group, making them amenable to EC at mild potentials. As part of this investigation, novel construction methods of the CE–EC system for testing the compatibility of NDTE derivatization to CE–EC were investigated with a special emphasis placed on simplicity, ruggedness and reproducibility. Construction of this device is described herein.

2. Experimental

2.1. Materials

p-Nitrophenyl-2,5-dihyroxyphenylacetate bis-tetrahydropyranyl ether (NDTE) was prepared in our laboratories using a previously described method [17]. Angiotensin II was obtained from Bachem Bioscience, Inc. (Bubendorf, Switzerland). Glycine–proline (Gly–Pro), glycine–glutamine (Gly–Gln), glycine–alanine (Gly–Ala), glycine–glycine (Gly–Gly) and glycine–glutamic acid (Gly–Glu) were obtained from Sigma Chemical Company (St. Louis, MO). Glycine, homogentisic acid, 4-dimethylaminopyridine and phosphoric acid (99.99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile (HPLC grade) was obtained from Fischer Scientific, Inc. (Pittsburgh, PA). Chemicals and solvents were used as received. Water was purified with a bulk carbon and mixed bed deionization cartridge and then ultra-purified with a Millipore (Bedford, MA) Milli-Q Water System to a resistance of 18 MΩ/cm.

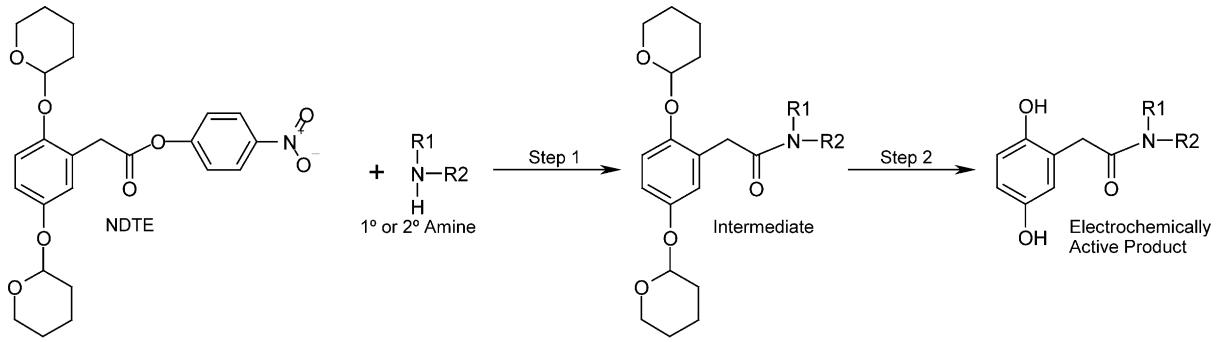

Fig. 1. Two-step reaction of *p*-nitrophenol-2,5-dihydroxyphenylacetate bis-tetrahydropyranyl ether (NDTE) to label a primary or secondary amine with hydroquinone.

2.2. Derivatization reagent stock solutions

Derivatization stock solutions were prepared by analytical balance measurement of the required mass of NDTE and the catalyst, 4-dimethylaminopyridine (DMAP), and then the addition of dry acetonitrile to the solids by volumetric pipette. Reagent stock solutions were prepared at twice the reaction concentration during the analysis of authentic standards to allow for dilution by the standard-containing solution. Pre-mixing concentrations were 20 and 40 mM for NDTE and DMAP, respectively. These stock solutions were stable for approximately 1 week after which time they were discarded.

2.3. Preparation of standard solutions

Angiotensin II, glycine and dipeptide standard solutions for calibration and detection limit experiments were prepared by measurement on a Cahn microbalance (Ventron Corp., Cerritos, CA) followed by dilution with 50:50 acetonitrile:water. Working solutions for standard curve construction were prepared by dilution of an initial stock solution with 50:50 acetonitrile:water using volumetric pipettes. The reported concentrations for derivatized standards represent the concentration of analyte in solution during derivatization step 1.

2.4. Derivatization of amines using NDTE

Reagent stock solutions were added to equivalent volumes of a known concentration of amine in 1:1 acetonitrile:water. Step 1 was allowed to proceed for a minimum of 30 min at 25 °C. The reaction mixture was then diluted with an equal volume of pH 1.2 phosphoric acid (step 2) and allowed to remain at room temperature for a minimum of 2 h. To reduce ionic strength, dilution with distilled water (four parts of water:one part of sample) was performed prior to injection.

2.5. Manufacture of the detector assembly (Fig. 2)

Carbon fiber detector electrodes were constructed by making electrical connection between a 10 μM carbon fiber from Dupont, Inc. (Chicago, IL) or a 33 μM carbon fiber from Avco Specialty Materials (Lowell, MA) and a copper wire using colloidal silver paint from Ted Pella, Inc. (Redding, CA). This assembly was then immobilized in a pulled glass capillary ($(1.5–1.8) \times 100$ mm^2) using quick setting epoxy from Devcon Corp. (Danvers, MA) with the carbon fiber extending through the tapered end. The field decoupler joint [18] was manufactured as follows. A 1 cm length of 0.01 in. PEEK HPLC tubing from P.J. Cobert Associates, Inc. (St. Louis, MO) was sealed onto the detection end of a polyimide-coated quartz capillary (25 or 75 μM) from Polymicro Technologies, Inc. (Phoenix, AZ) using quick setting epoxy. After allowing the epoxy to cure, a notch was carved in the PEEK tubing using a Dremel® multipurpose tool (Racine, WI) to expose the polyimide-coated capillary. The exposed capillary was scored with a ceramic capillary cutter and fractured by slight bending of the PEEK tube

housing. The detection outlet of the capillary was trimmed to approximately 2 cm from this fracture. The detection reservoir and the detector electrode were fixed in an immobilized flow-cell fashioned from a 5 ml polypropylene syringe body (Becton-Dickenson and Co., Franklin Lakes, NJ) modified to contain two liquid and two epoxy reservoirs using excess silicon plunger tips from the same syringes. The capillary was fixed first and allowed to dry within a reservoir containing C7 epoxy resin from Protective Coating Company (Allentown, PA). After the capillary was firmly supported, the carbon fiber detection electrode was placed in position in close proximity to the end of the quartz capillary, the electrode reservoir was filled with C7 epoxy resin and the carbon fiber was manually aligned under a microscope into the end of the quartz capillary. After electrode placement, the assembly was protected from sharp vibration until the epoxy hardened (30 min). The completed assembly was allowed to sit overnight before use. Two combinations of capillary i.d. and carbon fiber o.d. were used for these experiments (25 and 10 μm, and 75 and 33 μm, respectively).

2.6. Capillary electrophoresis

Assays were conducted using a modular CE system consisting of a Glassman High Voltage Power Supply (Whitehouse Station, NJ), a lab built pressure/time injector, a LC4C-EC amperometric detector and a RE-4 Ag/AgCl reference electrode, both from Bioanalytical Systems, Inc. (West Lafayette, IN). Data collection was accomplished using either a Fisher (Pittsburgh, PA), Recordall Series 5000 Chart Recorder or a PE Nelson (Cupertino, CA), Turbochrom data system with a 900 series A/D interface. Injection was controlled by modification of time and pressure and the exact volumes were determined using the measured flow rate and capillary internal diameter. The carbon fiber detection electrodes were pretreated before each injection using a potential step of $\pm$1800 mV (10 Hz, 20 s). Parameters specific to the individual CE assays are noted in the figure legends.

3. Results and discussion

3.1. NDTE derivatization

The structure of NDTE and the reaction pathway with a primary or secondary amine is shown in Fig. 1. The general development, characterization and application of this reagent in the trace analysis of amine bearing analytes has been reported previously [17]. Overall, amine derivatization using NDTE is a two-step procedure performed at room temperature. Most amines react with greater than 90% yield in less than 3 h to form highly electrochemically detectable products that are stable at room temperature in the final derivatization solution. Amines thus derivatized are compatible for determination by CE–EC. The reagent appears to be similar in ease of use as compared with other reagents available for derivatization followed by EC; however, the low potential required for detection is an advantage not shared by most other reagents described at this time (Fig. 5).

3.2. CE–EC instrumentation

A schematic diagram of the CE–EC detection cell designed for these studies is shown in Fig. 2. It is similar to systems described previously [1,15] consisting of a high-field grounding reservoir followed by a second low-field detection reservoir. The design of this cell was motivated by the desire to test the CE–EC compatibility of NDTE-derivatized analytes with a high degree of reproducibility at minimal expense. The various components in contact with the reservoirs, including the capillary, the field–decoupler joint, and the carbon fiber detection electrode were held in a fixed position by a viscous, slowly curing, epoxy resin. The novel aspects of this CE–EC detection cell were its static design and its ease of construction and application, which foregoes the need for micromanipulators or specialized equipment for electrode placement. Electrode alignment into the end of the capillary, often a difficult task without a micromanipulator, was assisted in this case by the damping qualities of a slowly curing viscous epoxy resin. The detector assembly, once completed, was

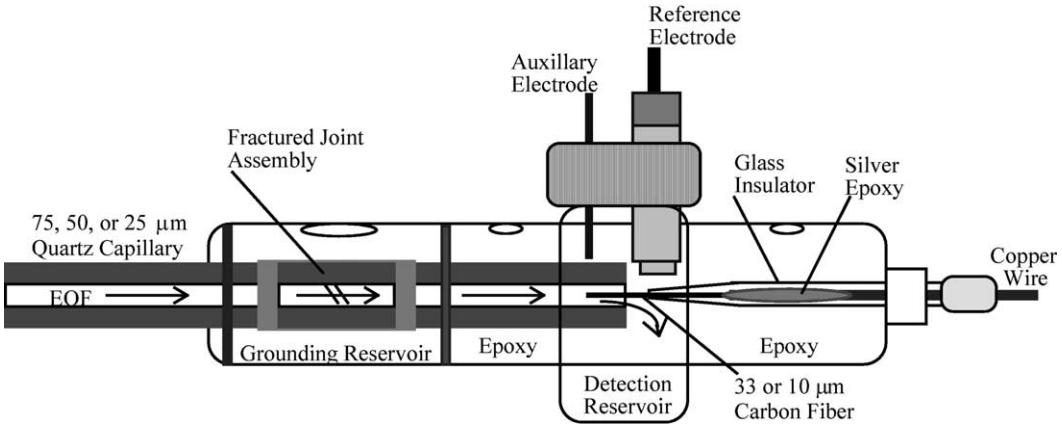

Fig. 2. Diagram of the CE–EC detection cell used for the analysis of amines derivatized using NDTE reagent.

immune to vibrations and frequent handling and required no isolating precautions for electrochemical analysis. The fixed nature of the detection assembly eliminated the need to align the end-column detector electrodes on the fly before CE–EC analysis. The inability to remove the microelectrode from the end of the capillary did not prove to be detrimental and added an aspect of reproducibility to the system, enabling extended experiments to be performed while using the same electrode/capillary combination. Electrode/capillary assemblies of this type were constructed at a rate of 10 per 6 h with approximately one-third of those constructed delivering satisfactory sensitivity. The cause of rejection for the final assemblies was in most cases related to defects in the field decoupler at the first reservoir. Rejections of final assemblies would have been reduced by pretesting this component, but due to the relatively low cost incurred to reaching the final detector assembly pretesting was unwarranted. Once deemed acceptable, while there may be variation between the various detection cells constructed, any given cell was quite reproducible on a day to day basis.

The majority of the CE–EC data shown in this report was collected with detector assemblies having 75 μm i.d. capillaries and 33 μm o.d. carbon fiber electrodes due to the ease of construction and also the more rugged nature of these larger dimension CE–EC components. Though more complicated to construct because of electrode placement, the combination of 25 μm i.d. capillaries and 10 μm o.d. carbon fiber electrodes was also investigated in these experiments. The use of small diameter (≤ 25 μM) capillaries has previously been proven to offer advantages in CE due their ability to limit the effects of joule heating through reduced current flow and superior heat dispersion characteristics [19]. Despite this advantage, 25 μm i.d. capillaries are not used routinely in CE due to their limited optical pathlength, which negatively affects detection. When determining NDTE-derivatized analytes using CE–EC, better signal-to-noise ratios were observed using 25 μm i.d. capillaries than those obtained using 75 μm i.d. capillaries, probably due to the decreased separation current observed in the smaller capillary.

3.3. Multicomponent amino acid and peptide determination

Fig. 3 shows an electropherogram for the CE–EC analysis of five dipeptides derivatized using NDTE. The analytes, shown as their final hydroquinone-labeled products in Fig. 4, were derivatized as a mixture at a pre-derivatization concentration of 50 μM before injection onto the CE–EC system. Four of the five derivatized dipeptides were resolved by the CE system. The large peak eluting at about 42 min is due to excess NDTE derivatization reagent converted in derivatization step 2 to homogentisic acid (Fig. 4f).

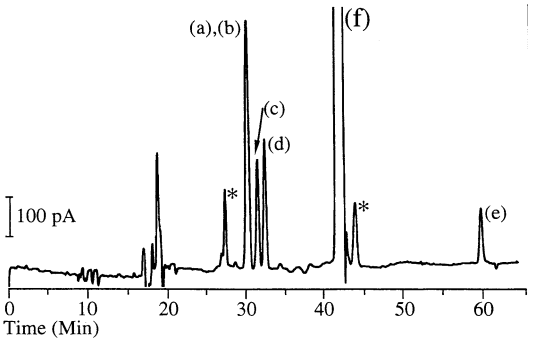

Fig. 3. Dipeptides derivatized using NDTE and analyzed using CE–EC. (a) Gly–Pro, (b) Gly–Gln, (c) Gly–Ala, (d) Gly–Gly, (e) Gly–Glu, (f) excess reagent (homogentisic acid), and (*) peaks present in blank derivatization. CE conditions: capillary i.d.: 75 μm, capillary length: 70 cm, separation potential: 25 kV, separation current: 30 μA, injection volume: 77 nl, running buffer: 100 mM morpholine-ethanesulphonic acid (pH 6.1 w/ NaOH), 40 mM tetrabutylammonium dihydrogen phosphate, 10% acetonitrile, detection: amperometric +450 mV versus Ag/AgCl (3 M)/fixed end-column 33 μm o.d. carbon fiber microelectrode with a fractured-joint field decoupler.

Table 1 summarizes the molecular weight and mass to charge ratio of the five dipeptides in their derivatized form and their respective elution orders when determined using CE at pH 7. Derivatization of the N-terminal amine in this case is beneficial as four of the five dipeptides have a net neutral charge over most of the working pH range when in their native form, but a net negative charge after derivatization. As per Table 1, the derivatized dipeptides eluted according to their charge to mass ratio, but excess NDTE reagent, converted to homogentisic acid, was observed to elute before derivatized Gly–Glu in an elution order counter to predictions. This elution anomaly for homogentisic acid has not been fully explained, but seems to be related to the dynamic structure of homogentisic acid. The open chain form of this compound has been proven to undergo equilibrium lactone ring closure under some conditions [20] indicating that the 2-hydroxy and the carboxylic acid functionality are in close proximity, which may result in a hydrogen bonding interaction that results in the observed decreased electrophoretic mobility.

As discussed previously, 25 μm i.d. capillaries were also applied to the determination of amines derivatized using NDTE. Fig. 5 shows a hydrodynamic voltammogram for hydroquinone-labeled glycine using a 25 μm i.d. capillary with a 10 μm o.d. carbon fiber detection electrode. This plot indicates an optimum detection potential of 400–500 mV versus Ag/AgCl ($[Cl^-] = 3$ M). Glycine working solutions were derivatized with NDTE and found to result in a linear concentration versus detector response over the pre-derivatization concentration range 500 nM–10 μM. The standard curve was adequately represented by the equation $y = 2.8E-05x + 0.015$, $r^2 = 0.998$, where y = peak area (nA × s) and x = glycine concentration (nM). An electropherogram at the concentration limit of detection (CLOD; 500 nM) is shown in Fig. 6. Sample requirements for this analytical system were extremely small and required only 1.7 nl injections for these experiments. Considering the total 8:1 dilution of the original glycine concentration (500 nM) during derivatization (see Section 2 for details), this represents an absolute detectability for the hydroquinone-labeled glycine of 106 attomoles. Though mass detectability has minimal bearing on limits of detection for real samples, it does demonstrate the limits of detection that would be possible for a hydroquinone-labeled analyte using a simple CE–EC instrument if the derivatization was optimized to minimize dilution.

As part of this initial evaluation process, NDTE derivatization with CE–EC was used for the analysis of angiotensin II (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe). The quantitation of angiotensin II is of interest due to the propensity of this molecule to transverse the blood brain barrier (BBB). Many of the specifics of the transport of angiotensin II across the BBB have been elucidated using bovine brain microvessel endothelial cell monolayers [21] as an in vitro model to characterize the movement of this peptide into the central nervous system. These studies represented an analytical challenge due the low angiotensin II concentrations (low nM) and the small sample volumes (≤ 200 μl) available from transport experiments. Since the determination of angiotensin II was previously reported in such an application by these laboratories using gradient microbore CE–EC [22], it was of interest to determine if the presently described NDTE/CE–

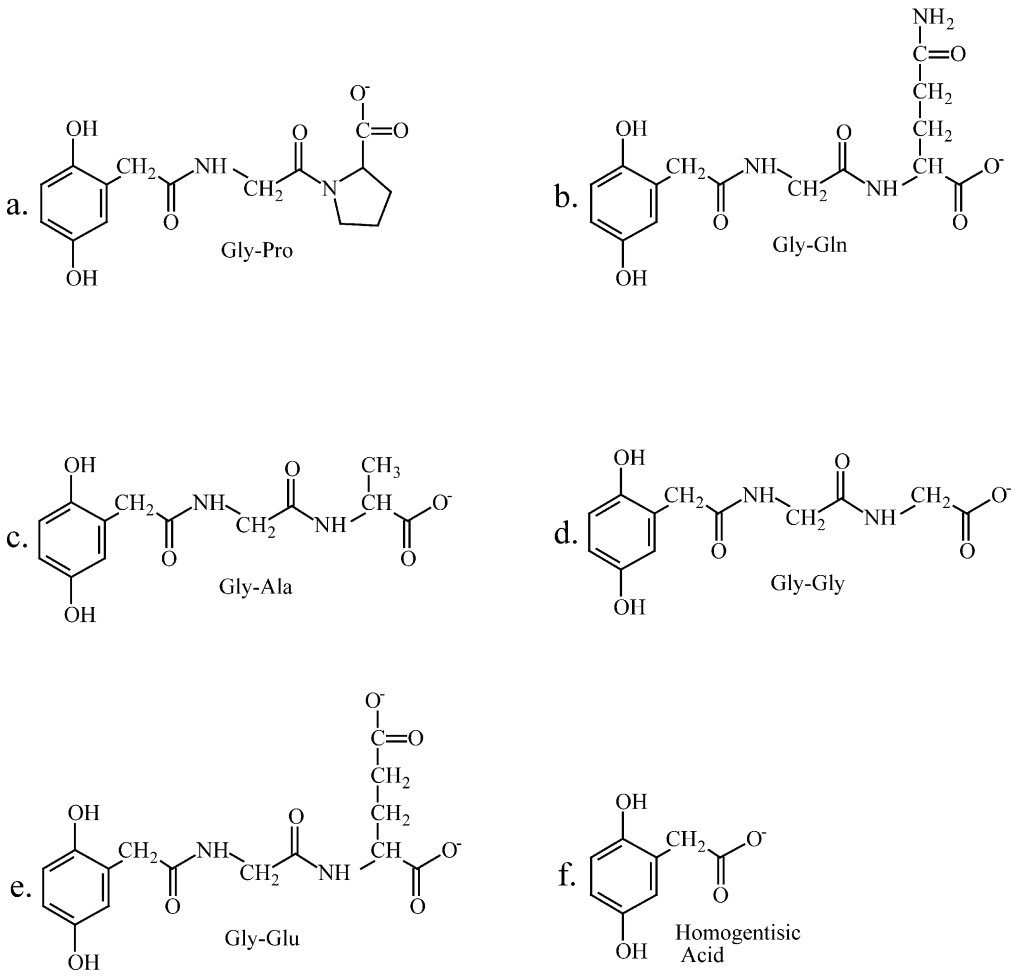

Fig. 4. Products resulting from the NDTE derivatization of (a) Gly–Pro, (b) Gly–Gln, (c) Gly–Ala, (d) Gly–Gly, (e) Gly–Glu and (f) homogentisic acid (conversion product of unreacted NDTE reagent).

Table 1
Charge to mass (q/MW, pH 6.1) dependence of migration time on NDTE-derivatized dipeptides

Compound	Native MW	Derivatized MW	Derivatized $q \times 10^3$/MW	Migration time (min)	Mobility (cm^2/(V s)) $\times 10^4$
Gly–Pro	172.2	322.2	−3.10	30.5	1.0715
Gly–Gln	203.2	353.2	−2.83	30.5	1.0715
Gly–Ala	146.2	296.2	−3.38	31.4	1.0408
Gly–Gly	132.1	282.1	−3.54	32.6	1.0024
Homogentisic acid	168.2	n/a	−5.95[a]	42.0	0.7781
Gly–Glu	204.1	354.1	−5.65	59.8	0.5465

[a] Not derivatized.

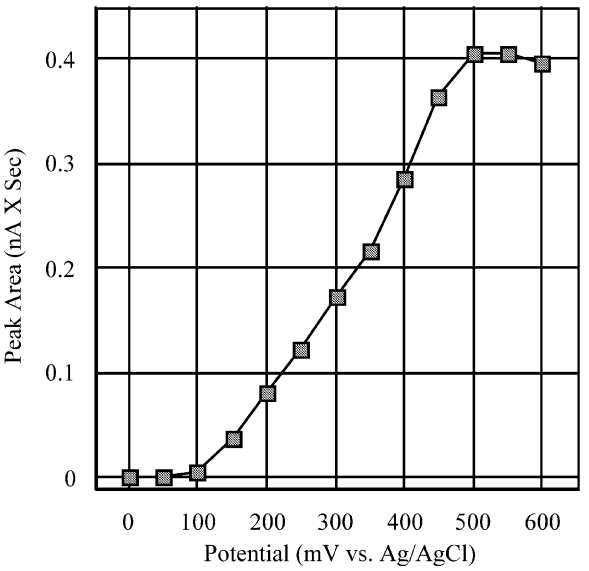

Fig. 5. Hydrodynamic voltammogram for glycine (100 μM) derivatized with NDTE and analyzed using CE–EC. CE conditions: capillary i.d.: 25 μm, capillary length: 60 cm, separation potential: 30 kV, separation current: 1.5 μA, injection volume: 1.7 nl, running buffer: 100 mM morpholine-ethanesulphonic acid (pH 5.6 w/NaOH), 10% acetonitrile, detection: amperometric 0 mV to +600 mV using a fixed end-column 10 μm carbon fiber microelectrode with a fractured-joint field decoupler.

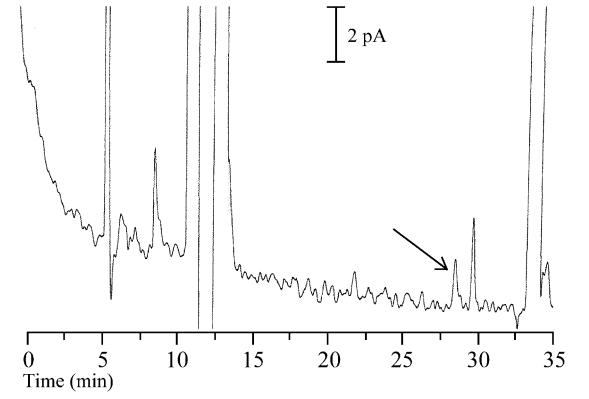

Fig. 6. Sample electropherogram (pre-derivatization concentration of 0.5 μM) from the glycine calibration curve (conditions as in Fig. 5).

EC methodology would provide for similar detection limits.

The NDTE derivatization and CE–EC analysis of serial diluted standards of angiotensin II resulted in a linear calibration curve from 200 μM to the CLOD of 5 μM ($y = 1.4x + 2.5$, $r^2 = 1.000$, where y = peak height (pA) and x = angiotensin II concentration (μM)). Fig. 7 shows an electropherogram from this calibration curve, representing a pre-derivatization concentration of 10 μM angiotensin II. Unfortunately, since the transport experiments require angiotensin II to be determined at low nanomolar concentrations, the NDTE/CE–EC methodology as currently implemented is not a viable alternative for the BBB transport experiments.

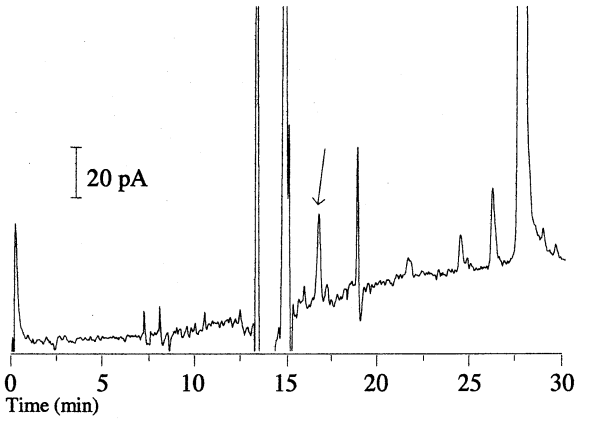

Fig. 7. Sample electropherogram (pre-derivatization concentration of 10.3 μM) from the angiotensin II calibration curve. CE conditions: capillary i.d.: 75 μm, capillary length: 70 cm, separation potential: 25 kV, separation current: 25 μA, injection volume: 88 nl, running buffer: 100 mM morpholine-propanesulphonic acid (pH 6.5 w/NaOH), 10% acetonitrile, detection: amperometric +450 mV with a fixed end-column 33 μm carbon fiber microelectrode with a fractured-joint field decoupler.

As presently described, an identifiable limitation of the NDTE/CE–EC methodology used in establishing the calibration plots of this work is the dilution incurred in conduct of the derivatization reaction and again prior to injection into the CE system. This stems from the requirement for different pH conditions for each step of the reaction and the need to lower the solution ionic strength prior to electrophoresis. In the future, this limitation could be addressed by an appropriate desalting/concentration step; however, further methodology refinement was not pursued in these preliminary evaluations.

4. Conclusions

NDTE is an easily applied reagent for the transformation of poorly detectable amino acids and peptides to electrochemically detectable analytes. Derivatization of glycine for CE–EC using the reported NDTE procedure resulted in a pre-derivatization CLOD in the high nanomolar range, which is similar to or better than CLODs recently reported for the fluorescence derivatization of amines quantified using CE and LIF detection [23–27]. The high ionic strength and low pH of the final derivatization matrix required that the sample be diluted before injection into the CE system, which negatively affected limits of detection. Improvement of the CE–EC CLODs to the middle to low nanomolar range requires either optimization of the derivatization procedure to generate undiluted, low ionic strength samples more compatible to free zone CE, and/or zone concentration of the hydroquinone-labeled sample on the capillary. This would be the subject of future research.

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