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An integrated electrochemical capillary liquid chromatography–dual microelectrode system for bromine based reaction detection

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Amperometric reaction detection of model peptides containing either a thiol or disulfide was successfully achieved with a novel coupled capillary liquid chromatography–dual microelectrode system. Detection is facilitated *via* dual platinum wire electrodes fabricated directly on a liquid chromatography capillary column. The detection strategy is based on the electrogeneration of bromine at the upstream working electrode followed by reduction of the bromine to bromide at the downstream working electrode. When bromine reacts with analyte eluting from the capillary column, the amount of bromine that reaches the downstream electrode decreases. As a result, the current at the downstream electrode will decrease in proportion to the amount of analyte eluting from the capillary column. Glutathione, glutathione disulfide, α-TGF and oxytocin were used to characterize the system and to determine system parameters. The feasibility of the determination of disulfide containing peptides is demonstrated with glutathione disulfide, α-TGF and oxytocin.

Introduction

Miniaturized capillary chromatography columns provide improved separation efficiency as a result of the reproducibility of packing, improved permeability, increased column length and the wall effect.[1] Capillary columns require lower mobile phase volumes during the separation (0.01–10 μl min^{-1}), leading to decreased cost of operation. The reduced cost of fabricating capillary columns in-house relative to conventional columns ($30 per capillary column) is a further advantage. Typical injection volumes of 1–2 nl, are the most attractive benefit of capillary liquid chromatography; however, low-volume separation methods inevitably require that new detection strategies be developed and implemented to accommodate such small masses of analyte.

Electrochemical detection (EC) offers unique advantages for capillary column chromatography. In contrast to most optical detection methods, the electrochemical detector can be readily miniaturized without a loss in sensitivity. Concentration detection limits of 10^{-9} to 10^{-10} M have been reported.[2] EC is inherently selective for those compounds that are easily oxidized or reduced at a particular potential. By judicious selection of the applied potential, the selectivity of the detector can be further altered. If the analyte is not natively electroactive within the working range of the electrode, it must be derivatized. However, the incorporation of derivatization increases the labor, cost and total analysis time. Furthermore, the benefit of low sample volume requirements is lost when large sample volumes are required for the derivatization chemistry.

Thiols are difficult to oxidize at conventional electrode materials because the necessary applied potential is too high to make the method feasible. Alternative electrode materials (chemically modified electrodes,[3,4] gold/mercury amalgam electrodes[5]) and waveforms (pulsed amperometric detection[6–8]) have been used with success. Difficulties in implementing alternative electrode materials include lack of ruggedness and poor reproducibility; however, the primary drawback is the necessary disulfide reduction. Chemical reduction of disulfides requires increased sample pre-treatment and multiple analyses in order to differentiate thiols from disulfides. Electrochemical reduction of disulfides is a limited approach as the conversion efficiency is poor. Pulsed amperometric detection relies on software generated waveforms that allow for surface oxide-catalyzed detection and electrode cleaning. The method has been coupled to liquid chromatography for the detection of thiols (cysteine, dithioerythritol, *trans*-1,2-dithiane-4,5-diol, 2-aminoethanethiol, glutathione, reduced glutamylcysteine and reduced cysteinylglycine)[6–8] and also the disulfides cystamine, glutathione disulfide and oxidized cysteinylglycine.[6,7] A detailed description of the optimized waveform parameters for these compounds has been reported.[6]

In order to expand further the use of capillary liquid chromatography–electrochemical detection to a broader range of analytes, post-column amperometric reaction detection has been developed. An elegant example of this approach was first reported by King and Kissinger in 1980 for conventional liquid chromatography.[9] This work incorporated the electrogeneration of a reactive halogen that was subsequently reduced at a detection electrode. In the absence of analyte, the electrogenerated halogen (bromine, chlorine or iodine) produced a baseline current at the detection electrode ($X_2 + 2e^- \rightarrow 2X^-$, X = Cl, Br, I). If an analyte peak is present and this analyte reacts with the halogen, the result is consumption of the halogen. This in turn results in a decreased halogen level reaching the detection electrode and a subsequent decrease in the reductive current measured at the detection electrode. In other words, when a reactive analyte elutes from the chromatographic column, the signal measured at the detection electrode will decrease in proportion to the amount of analyte present.

Following the initial report of bromine based reaction detection, a number of applications of the approach have been reported.[10–15] These reports included the determination of phenols,[14] thiols,[11,16,17] disulfides,[11,15,16] proteins,[10,11,17] drugs[13,16] and amino acids.[18] In spite of the interest in the use of bromine facilitated amperometric reaction detection, the method has only been coupled to conventional or microbore liquid chromatography. There has been no attempt to couple the detector to capillary liquid chromatography.

The coupling of post-column reaction detection with capillary liquid chromatography is by no means trivial. The

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allowable reaction time, detector cell volume and even the mobile phase itself can interfere with and even prevent electrochemical reaction detection. In this paper, we report the first capillary liquid chromatography–electrochemical system utilizing bromine based post-column reaction detection. Through proper design and construction, we characterized the system with model peptides. Glutathione and glutathione disulfide are ideal compounds to evaluate a capillary column–electrochemical reaction detection system for the determination of thiol and disulfide containing peptides. Determinations of the peptide are well characterized as glutathione is involved in detoxification reactions.[19] The potential of the approach for peptide analysis is demonstrated with glutathione disulfide, α-TGF and oxytocin.

Experimental

Materials and reagents

Glutathione, glutathione disulfide, sodium bromide and sodium phosphate were purchased from Sigma (St. Louis, MO, USA) and α-TGF and oxytocin from American Peptide (Sunnyvale, CA, USA). De-ionized water was obtained from a Barnstead Nanopure Infinity ultrapure water system (Barnstead Thermolyne, Dubuque, IA, USA). Stock standard solutions were prepared with purified water and stored at −20 °C. All standard solutions were prepared in the mobile phase.

Equipment

Capillary liquid chromatography. The capillaries were fritted using an advanced capillary former (InnovaTech, Stevenage, Hertfordshire, UK) according to a previously described procedure.[20] The reversed phase capillary column was a 50 μm id fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) packed with 5 μm spherical octadecylsilyl particles of 300 Å pore size (Jupiter, Phenomenex, Torrence, CA, USA). The liquid chromatography column was packed using a previously described procedure.[1] The mobile phase was a mixture of 50 mM sodium bromide and 50 mM phosphate buffer (pH 6.0) in either water or water–methanol (1 : 1).

Mobile phase was delivered with a conventional pump [Model 600E (Waters, Milford, MA, USA) or Model 140B (Applied Biosystems, Perkin-Elmer Analytical Instruments, Norwalk, CT, USA)]. Injections were facilitated with a four-port C4UW or six-port C6W injection valve (Valco Instruments, Houston, TX, USA).

The implementation of a capillary column-electrochemical system requires appropriate mobile phase delivery. The dimensions of the capillary columns used for this work necessitated the use of an optimum volumetric flow rate near 30 nl min^{-1}. Conventional chromatographic equipment was modified to accommodate the low volumetric flow rate and small working volume of the system by splitting the mobile phase flow. The operation of a liquid chromatography capillary column with conventional HPLC pumps by splitting the pump flow has been described previously.[21] The purpose of the flow splitter is to divert a majority (>99%) of the mobile phase flow delivered from the pump at convenient flow rates (*e.g.*, 1.0 ml min^{-1} for a reciprocating pump or 15 μl min^{-1} for a syringe pump) in order to ensure that the flow rate delivered to the column is appropriate.

Electrochemical detection. The electrochemical cell consisted of four electrodes: two platinum working electrodes, a platinum auxiliary electrode and an Ag/AgCl reference electrode (RE-4, Bioanalytical Systems, West Lafayette, IN, USA). The electrodes were connected to a bipotentiostat (LC-4C, Bioanalytical Systems). Data collection was possible with a multi-function portable data acquisition card (DAQCard-A1-16XE-50, National Instruments, Austin, TX, USA) and commercially available software (Igor NIDAQ Tools, Wavemetrics, Lake Oswego, OR, USA). Data analysis and representation were achieved with the aid of commercially available software (Igor Pro v. 3.1, Wavemetrics).

Both the generating (WE_1) and detection electrodes (WE_2) were fabricated from platinum wire (Goodfellow, Berwyn, PA, USA) using a previously described procedure.[22,23] Briefly, the platinum wire was pre-treated with a cleaning solution and sodium hydroxide and bent with the aid of forceps under an optical microscope. The generating electrode was 1 mm in length and was inserted into the end of the capillary column as shown in Fig. 1. It is important that this electrode is placed in the end of the capillary column so that it is in contact with the frit to minimize the detector cell volume, otherwise volumetric dilution in the detector cell prior to reaction with the bromine will degrade the limit of detection. The detection electrode was fitted over the end of the capillary column as shown in Fig. 1. Both working electrodes were held in place with epoxy (Epoxy 907, Miller-Stephenson Chemical, Danbury, CT, USA). For all experiments the generating electrode was maintained at +0.85 V or greater and the detection electrode was maintained at a potential of approximately 0.025 V.

Results and discussion

The on-column electrode configuration (Fig. 1) is well suited for the detection of thiols and disulfides. The electrodes are fabricated directly on to the liquid chromatography capillary column and these devices are currently constructed in-house. The on-column design eliminates the need for micromanipulators to position the electrodes as they are permanently attached to the column. The fixed electrode position eradicates variability resulting from slight differences in electrode position upon insertion. The electrode design used here also keeps the insulating epoxy out of the path of the residual bromine, which is important as bromine will attack this epoxy and destroy the electrodes. In addition, the small detector cell volume for the on-column design (1.5 or 2.2 nl) reduces analyte dilution and post-column band broadening, while still allowing an adequate reaction time. Scaling the technique to micrometer dimensions

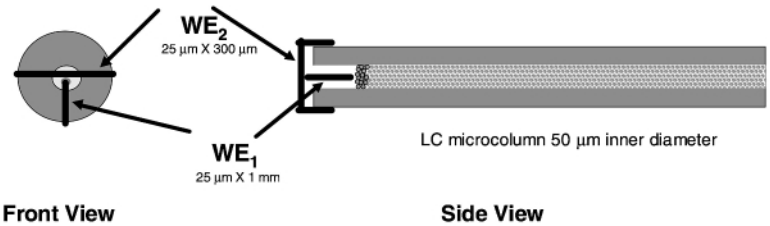

Fig. 1 Configuration of the on-line capillary column electrode. WE_1 is the upstream generating electrode and WE_2 is the downstream detection electrode. Both electrodes are fabricated from 25 μm od platinum wire as described in the text.

and incorporating a reactive species (bromine) in the detection scheme require thoughtful design of an appropriate low volume detection cell.

The applicability of the reaction detection scheme with capillary column chromatography was initially assessed by incorporating a 50 μm id capillary column packed with 5 μm underivatized silica particles. The configuration is the same as that shown in Fig. 1. The use of underivatized silica particles allows the system to be operated at similar mobile phase flow rates due to the flow resistance created by the packing material. This is important as the analyte–bromine reaction time is related to mobile phase flow rate and detector cell volume. The use of underivatized silica eliminates analyte retention on the capillary column (analyte partitions between a polar stationary phase and polar aqueous mobile phase). The data collected for Fig. 2 were obtained with a mobile phase flow rate of 0.118 cm s^{-1}. Based on this linear flow rate, the reaction time in the 1.5 nl detector cell is estimated to be 1.25 s by calculating the volume of the packed capillary. A solution of 10 μM reduced glutathione injected on to this system is shown in Fig. 2. The generating electrode was on when the upper trace (solid line) was obtained at the detection electrode. The run was repeated with the generating electrode off as shown in the lower trace (dashed line). A solution of 100 μM glycine was also injected in to the liquid chromatography–electrochemical system and no discernible signal was observed. All runs were obtained using identical conditions (50 mM bromide, 50 mM phosphate, pH 6.0). The purpose of this experiment was to confirm that the signal obtained at the detection electrode is due solely to the depletion of bromine generated by the first electrode. The fact that no signal was obtained from the injection of glycine, an analyte that does not contain a thiol or disulfide group, confirms that the signal obtained from glutathione is a result of bromine depletion rather than a simple displacement mechanism.

After initial assessment, a reversed phase capillary column with on-column electrodes was used to assess the overall feasibility of the technique for the determination of thiols and disulfides. The reversed phase capillary column consisted of a 50 μm id column packed with 5 μm diameter packing material as described in the experimental section. Again, the mobile phase was an aqueous solution of 50 mM phosphate–50 mM bromide (pH 6.0). The isocratic separation of glutathione and glutathione disulfide is shown in Fig. 3.

Statistical data (reproducibility, linear range and limit of detection) were determined for both glutathione and glutathione disulfide using the conditions described for Fig. 3. For glutathione, the linear dynamic range of the system for detection is 1.33–100 μM (n = 6) with a correlation coefficient of 0.996 and a slope of 0.2 nA μM^{-1}. The within-day relative standard deviation (RSD) of the retention time was 0.2% (n = 4 runs) and the day-to-day RSD was 1% (n = 5 days). The within-day RSD of the peak height was 6% (n = 4 runs) and the day-to-day RSD was 8% (n = 5 days). The limit of detection for reduced glutathione at a signal-to-noise ratio of 3 was 64 nM.

For glutathione disulfide, the within-day peak height and retention RSDs were 7 and 0.1%, respectively (n = 4 runs). The dynamic linear range was 2.0–25 μM (n = 4) with a correlation coefficient of 0.994 and a slope of 0.6 nA μM^{-1}. The smaller linear range and larger slope for glutathione disulfide (relative to glutathione) is a result of increased stoichiometric consumption of bromine by disulfides as compared with thiols, as follows:[11]

$$R–SH + 3Br_2 + 3H_2O \leftrightharpoons RSO_3H + 6HBr$$

$$R–SS–R + 5Br_2 + 6H_2O \leftrightharpoons 2RSO_3H + 10HBr$$

Reaction detection involves a compromise between linearity and limit of detection as quantification of analyte is dependent on the amount of electrogenerated reagent present. In cases where a good limit of detection is required, generation of a small concentration of reagent is necessary, but will lead to a reduced linear range as a result of depletion of the electrogenerated reagent. Conversely, the production of a high concentration of reagent will lead to a larger range for linear response, but the limit of detection will of course be higher. Others have approached this problem by determining the optimum concentration of bromine that should be generated[14] or by using a feedback method to adjust continuously the amount of bromine generated according to the signal response.[24] Determination of optimum generation levels is a straightforward approach; however, a feedback method allows for the determination of samples consisting of a wide concentration range. This can be particularly useful for the determination of glutathione disulfide in the presence of glutathione as the disulfide can be at levels 100 times lower than the thiol in biological samples.

It has been noted that the presence of methanol does not interfere with bromine generation,[9] but does affect analyte reaction time.[14] The effects of methanol on the detection of thiols and disulfides can be seen in the chromatograms shown in Fig. 4 containing glutathione disulfide and/or glutathione. These reversed phase separations were obtained using a mobile phase modified with methanol [50 mM phosphate–50 mM bromide (pH 6.0) in water–methanol (1 : 1)]. The data in Fig. 4 contain three overlaid chromatograms. The lower trace (labeled GSH) was obtained after injecting 100 μM of glutathione, the middle trace (GSSG) after injecting 100 μM of glutathione disulfide and the upper trace (GSSG + GSH) after injecting a sample containing both 100 μM glutathione and 100 μM

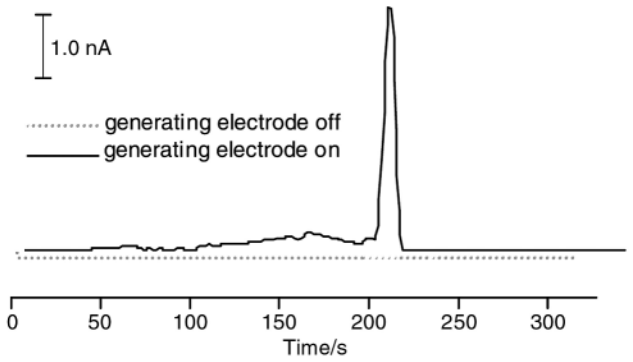

Fig. 2 Detection of 10 μM reduced glutathione. The upper trace was obtained with the generating electrode on and the lower trace with it off. The column was 25.0 cm × 50 μm id, packed with 5 μm diameter underivatized silica particles. Run conditions were as follows: mobile phase, 50 mM phosphate–50 mM bromide (pH 6.0); flow rate, 0.118 cm s^{-1}; E_{gen} = 0.850 V; E_{det} = 0.025 V, detector cell volume, 1.5 nl; and estimated reaction time, 1.25 s. The signal has been inverted in order to display the chromatogram in conventional format.

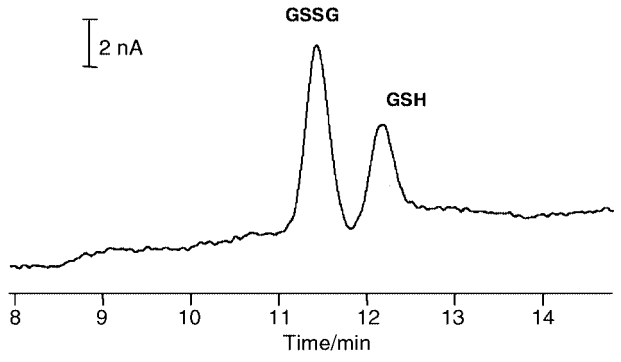

Fig. 3 Separation of 100 μM glutathione disulfide and 100 μM glutathione by reversed phase capillary column liquid chromatography. The column was 23.5 cm × 50 μm id, and the packing material was 5 μm diameter. Run conditions were as follows: mobile phase, 50 mM phosphate–50 mM bromide (pH 6.0); flow rate, 0.0332 cm s^{-1}, E_{gen} = 0.874 V; E_{det} = 0.025 V; detector cell volume, 1.5 nl; and estimated reaction time, 4.6 s. The signal has been inverted in order to display the chromatogram in conventional format.

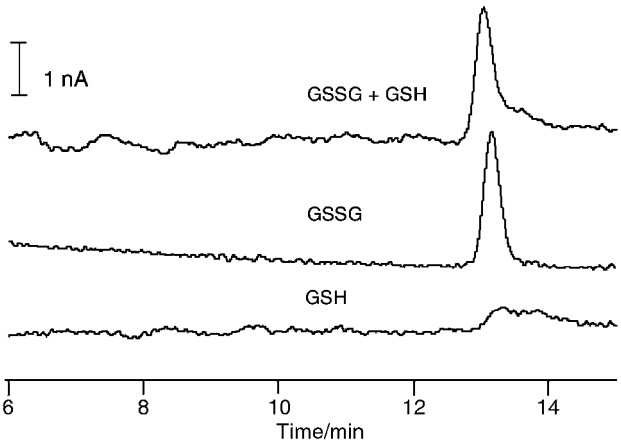

Fig. 4 Detection of 100 μM glutathione and/or 100 μM glutathione disulfide, by reversed phase capillary column liquid chromatography. The upper trace (labeled GSSG + GSH) represents the separation of a mixture of 100 μM glutathione and 100 μM glutathione disulfide, the middle trace (GSSG) the detection of 100 μM glutathione disulfide and the lower trace (GSH) the detection of 100 μM glutathione. The column was 20.5 cm × 50 μm id, and the packing material was 5 μm diameter. Run conditions were as follows: mobile phase, 50 mM phosphate–50 mM bromide (pH 6.0) in water–methanol (1 : 1); flow rate, 0.0260 cm s^{-1}; E_{gen} = 0.851 V; E_{det} = 0.020 V; detector cell volume, 2.2 nl; and estimated reaction time, 4.3 s. The electrodes were fabricated from 10 μm platinum wire. The signal was inverted in order to display the chromatogram in conventional format.

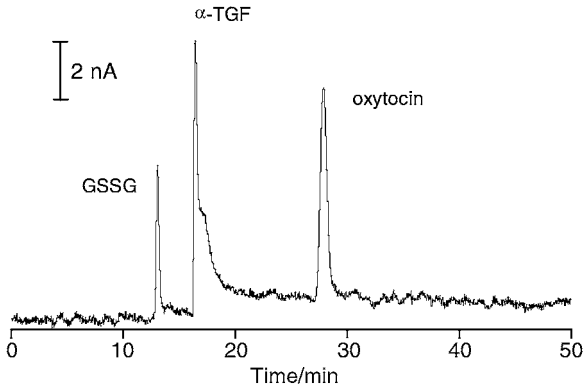

Fig. 5 Separation of 100 μM glutathione disulfide, 350 μM α-TGF and 350 μM oxytocin by reversed phase capillary column liquid chromatography. The column was 20.5 cm × 50 μm id and the packing material was 5 μm diameter. Run conditions were as follows: mobile phase, 50 mM phosphate–50 mM bromide (pH 6.0) in water–methanol (1 : 1); flow rate, 0.0260 cm s^{-1}; E_{gen} = 0.851 V; E_{det} = 0.020 V; detector cell volume, 2.2 nl; and estimated reaction time, 4.3 s. The electrodes were fabricated from 10 μm platinum wire. The signal has been inverted in order to display the chromatogram in conventional format.

glutathione disulfide. The response to the thiol is significantly lower than the response to the disulfide.

The separation of three peptides containing disulfide linkages (glutathione disulfide, α-TGF and oxytocin) is shown in Fig. 5. $\alpha\text{-TGF}_{(34\text{-}43)}$ is a 10 residue growth factor and oxytocin is a nine residue hormone. This separation was obtained under isocratic conditions; however, the mobile phase was 50 mM phosphate–50 mM bromide (pH 6.0) in water–methanol (1 : 1). Addition of methanol to the mobile phase allowed faster elution times for the α-TGF and oxytocin than would be obtained using a 100% aqueous mobile phase. This separation demonstrates the applicability of this approach to biologically relevant peptides containing disulfide linkages that are larger than glutathione disulfide.

Statistical data were determined for glutathione disulfide, α-TGF and oxytocin in the presence of 50% methanol (conditions as described for the chromatograms in Fig. 4 and 5). For glutathione disulfide, analyte was detected in the range 50–200 μM (n = 3) with a correlation coefficient of 0.999 and a slope of 0.06 nA μM^{-1}. For α-TGF, analyte was detected in the range 100–350 μM (n = 3) with a correlation coefficient of 0.943 and a slope of 0.04 nA μM^{-1}. For oxytocin, analyte was detected in the range 175–500 μM (n = 3) with a correlation coefficient of 0.986 and a slope of 0.02 nA μM^{-1}. The within-day RSD of the retention time for glutathione disulfide, α-TGF and oxytocin was 0.4, 0.2 and 0.5%, respectively (n = 3 runs). The within-day RSD of the peak height for glutathione disulfide, α-TGF and oxytocin was 11, 13 and 15.9%, respectively (n = 3 runs). The limit of detection for glutathione disulfide, α-TGF and oxytocin at a signal-to-noise ratio of 3 was 37.5, 162 and 120 μM, respectively.

Although the separations reported here are isocratic, reversed phase chromatographic separations often incorporate organic gradients. The effect of methanol on the reaction between the disulfide-containing peptides and bromine does not pose a problem for isocratic elution, but it is of concern for gradient elution. It has been demonstrated previously that a shallow, single step organic gradient is feasible with bromine based amperometric reaction detection.[16] In cases where more rigorous gradient elution conditions are necessary, the use of other chromatographic separation modes, such as ion exchange, is possible. For example, ion exchange takes advantage of charge differences of peptides and gradient elution is possible by varying the salt concentration of the mobile phase. In contrast to organic gradients, salt gradients will not affect bromine facilitated reaction detection and are therefore better suited for quantitative determinations.

Conclusions

The results presented here employing capillary column liquid chromatography and bromine based electrochemical reaction detection clearly indicate that the application of the method to the determination of glutathione is feasible. The technique has the potential to be used for the determination of a number of individual thiol or disulfide containing peptides and related peptide fragments within the same analysis. Furthermore, because the technique provides a means of separation and detection of the thiols and disulfides it can indicate whether a peptide is in the reduced or oxidized state. Future work is directed towards the optimization of chromatographic separations based on ion exchange, the use of direct injection techniques and the application of the system to other clinically relevant peptides.

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