Extending the Usefulness of HPLC with Electrochemical Detection

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Overview

Purpose: To highlight different approaches that can be used to extend the usefulness of HPLC-ECD to a wider variety of analytes.

Methods: Four techniques are examined including pre-column derivatization, immobilized enzymes, photolysis and novel working electrode materials.

Results: All approaches extend the range of analytes that can measured by HPLC-ECD but differ in their specificity and range of molecules that can be analyzed.

Introduction

High performance liquid chromatography with electrochemical detection (HPLC-ECD) is typically chosen for its extreme sensitivity, with low femtogram (pM) limits of detection readily achievable. Unfortunately, when compared to UV detection, ECD is very selective with relatively few organic compounds capable of undergoing redox reactions. A number of approaches can be used to extend the range of compounds detected by ECD while maintaining sensitivity. In this poster we evaluated four such approaches.

1. Pre-column derivatization: application to amino acid analysis
2. Use of immobilized enzymes to indirectly measure electrochemically inert species: the measurement of acetylcholine in microdialysis perfusates will be presented.
3. Use of on-line pre-electrode high-energy photolysis to generate transiently electrochemically active species from inert compounds: a global method for the measurement of explosive residues will be shown.
4. Use of novel working electrodes that can render inert compounds electrochemically active through electrotagging: use of a boron-doped diamond (BDD) working electrode to measure genotoxins, thiols/disulfides, and polyaromatic hydrocarbons will be evaluated.

1. Pre-Column Derivatization

Derivatization is used to render inert species electrochemically active. This can be accomplished in two ways: a) the covalent modification of the inert species by a molecule that is electrochemically active or b) the formation of an electrochemically active product from inert species. Derivatization can be further divided into pre-column approaches where the product is typically formed automatically on the autosampler prior to chromatographic separation of the derivatized species; post-column approaches where analytes are derivatized after chromatographic separation and prior to detection and the use of more complex off-line chemistries that require longer reaction times and possibly cleanup prior to analysis. A wide variety of derivatizing agents have been employed with HPLC-EC detection, typically reacting with amine, carboxylic acid or thiol residues, and these have been reviewed elsewhere.[1] The derivatization of amino acids by o-phthaldialdehyde (OPA) and beta-mercaptoethanol (ß-ME) is a classic example of pre-column derivatization (Figure 1). The reaction kinetics for derivatization are extremely fast, the isoindole product is extremely stable, and the derivatizing agent OPA is not electrochemically active so, even though it is used in excess, it does not interfere with the chromatographic separation or detection of the amino acid derivatives (Figure 2).

FIGURE 1. The pre-column formation of electrochemically active isoindole derivatives from mostly inert alpha amino acids, OPA and ß-ME.

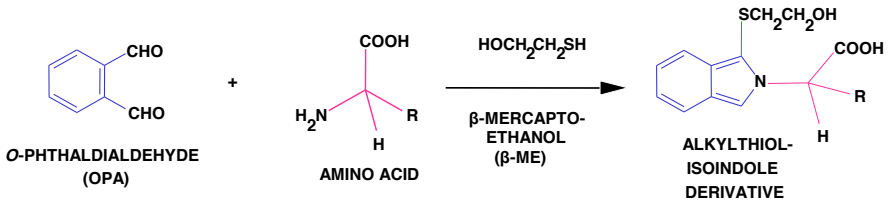
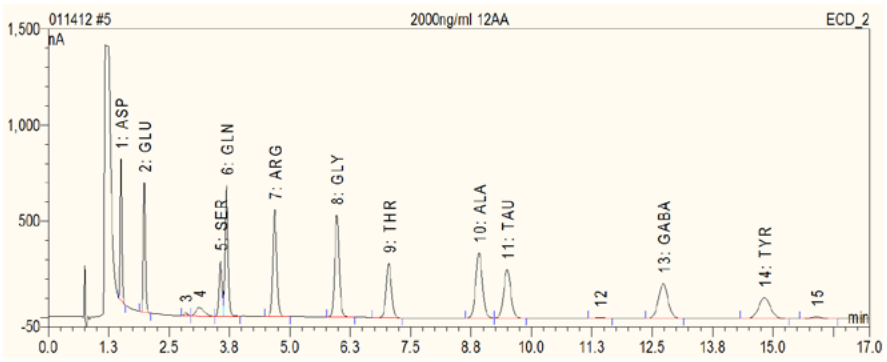


FIGURE 2. HPLC separation and electrochemical detection of 11 neurochemically relevant alpha amino acids as their isoindole derivatives. Column: Thermo Scientific Accucore PhenylHexyl, 3 mm × 100 mm 2.6 µm, Accucore Ph/Hex 10 × 3.0 mm 2.6 µm Guard column and Uniguard Holder. Mobile Phase: 100 mM disodium hydrogen phosphate anhydrous 22% methanol, 3.5% acetonitrile, pH=6.75 with phosphoric acid. Flow rate = 0.600 mL/min. Column temperature = 45 °C. Injection volume = 10 µL. Thermo Scientific Dionex Coulochem III with Thermo Scientific Dionex model 6011 analytical cell: E1 = +150 mV: E2 = +550 mV. The derivatization protocol is described in an application note from Thermo Fisher Scientific.[2]



2. Immobilized Enzymes

Immobilized enzymes, as part of an immobilized enzyme reactor (IMER) or solid phase reactor (SPR), are typically used on-line with HPLC for the indirect measurement of non-electrochemically active analytes. Here the specificity of the enzyme is used to enhance selectivity of the HPLC-ECD approach. Although many different types of enzymes are used[3], it is the bacterial oxidases that are most common, as they generate hydrogen peroxide (measured electrochemically) in direct proportion to the amount of substrate being converted. Acetylcholine (ACh) is a critical neurotransmitter in the brain. Unfortunately, it occurs at very low levels in the extracellular space and is difficult to detect. Figure 3 shows a schematic of a typical HPLC-ECD system used for the determination of ACh. Here the SPR contains two immobilized enzymes – Acetylcholinesterase (AChe), responsible for the hydrolysis of ACh to choline (Ch), and Ch oxidase, responsible for the production of hydrogen peroxide from Ch. Hydrogen peroxide is determined using a Pt electrode at +275 mV (vs. Pd reference). Therefore, although ACh is not electrochemically active, enzymes can be used to convert it to a product that is. The hydrogen peroxide produced by the SPR provides a suitable EC-active moiety that can be measured at extremely low levels.

FIGURE 3. Schematic for the analytical HPLC-ECD system used for the indirect measurement of ACh.

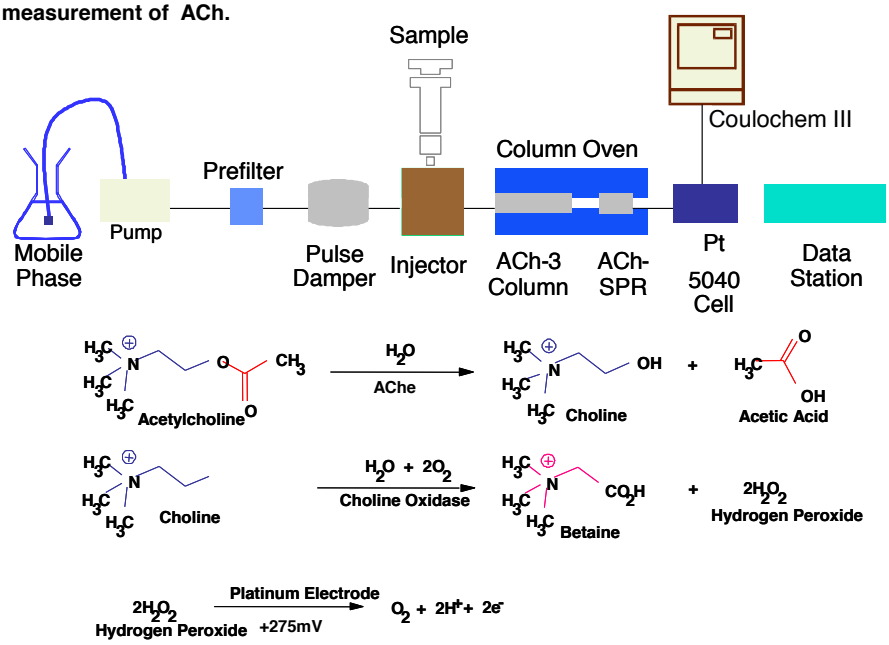
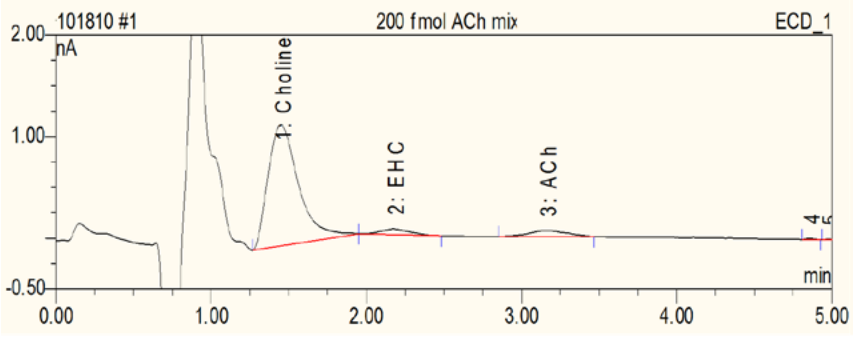


FIGURE 4. HPLC separation and electrochemical detection of acetylcholine (ACh), choline (Ch) and the internal standard ethylhomocholine (EHC). Column: Capcell Pak® MGII C18, 75 × 1.5mm, 3 µm. SPR: Thermo Scientific Dionex ACH-SPR. Mobile Phase: 100 mM disodium hydrogen phosphate, 0.8 mM 1-octanesulfonic acid sodium salt, 0.005% Reagent MB (microbicide), pH 7.0 ± 0.2 with phosphoric acid. Flow rate = 0.30 mL/min. Column temperature = 38 °C. Inj. volume = 10 µL. Coulochem™ III with Thermo Scientific Dionex model 5040 cell and Pt target. E1 = +275 mV.

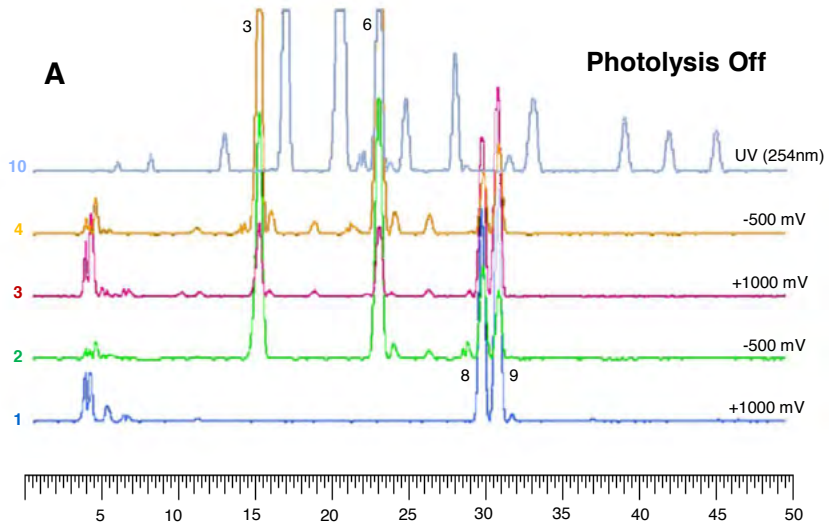


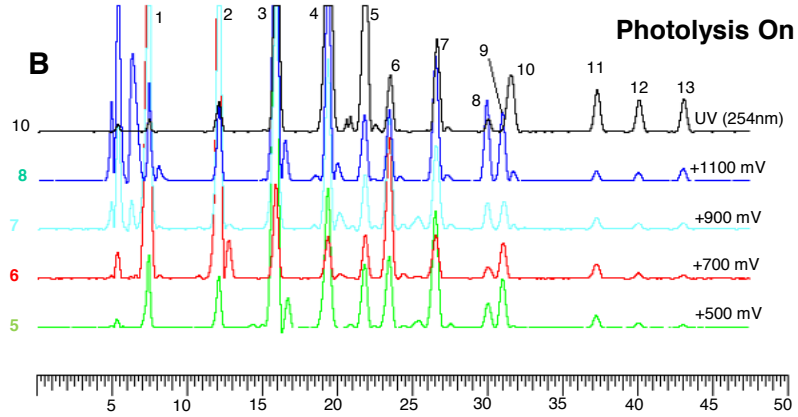
3. Photolysis

On-line post-column photolysis is now routinely used to extend the range of analytes measured by EC detection while still taking advantage of its sensitivity and selectivity. Extensively reviewed by Fedorowski and LaCourse[4], photolysis can be regarded as a form of derivatization using photons instead of chemical reagents. Photolytic EC has been successfully applied to the measurement of numerous drugs, pesticides, insecticides and, as presented below, to explosives.

FIGURE 5. Isocratic separation of 13 explosives and related compounds and measurement by coulometric electrode array detection and UV detection with A) post-column pre-electrode photolytic unit (Phred™ consisting of a 254 nm lamp and 15 m × 0.25 mm PTFE knitted reactor coil) off and B) on. Column: Thermo Scientific BetaSil C18, 4.6 × 250 mm; 5 µm. Mobile phase: 50 mM sodium phosphate pH 3.5, 50% methanol. Flow rate = 0.7 mL/min. Column temperature = 35 ° C. Injection volume = 20 µL. Thermo Scientific Dionex CoulArray detector with applied potentials (mV vs. Pd reference) as shown in the Figures 5A and 5B.

1 – HMX; 2 – RDX; 3 – TNB; 4 – DNB; 5 – NB; 6 – Tetryl; 7 – TNT; 8 – 4-Amino-2,6, DNT; 9 - 2-Amino-4,6-DNT; 10 - 2,4-DNT; 11 – 2NT; 12 – 4NT; 13 – 3NT; (200 ng on column).



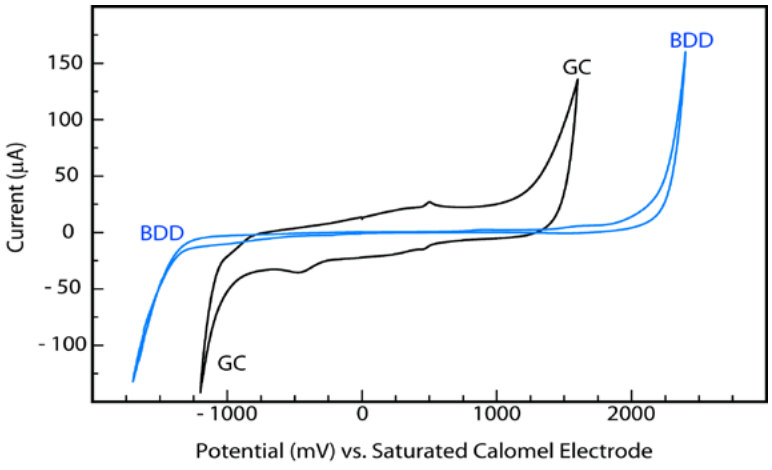


As shown in Figure 5A, although most of the 13 explosives showed adequate UV signal, only two compounds, the amines 4-amino-2,6 DNT and 2-amino-4,6 DNT, showed a significant oxidation signal when the photolytic unit was off. Once turned on (Figure 5B), all 13 explosives showed an increased EC response, with an improvement in limit of detection (LOD) over UV of approx. 5-100 fold. The limits of detection were found to be 20-100 pg (on column) for HMX, RDX and TETRYL, 0.5-2ng (on column) for the nitrotoluenes, and 200-500 pg (on column) for the other explosives and residues.[1]

4. Novel Working Electrodes

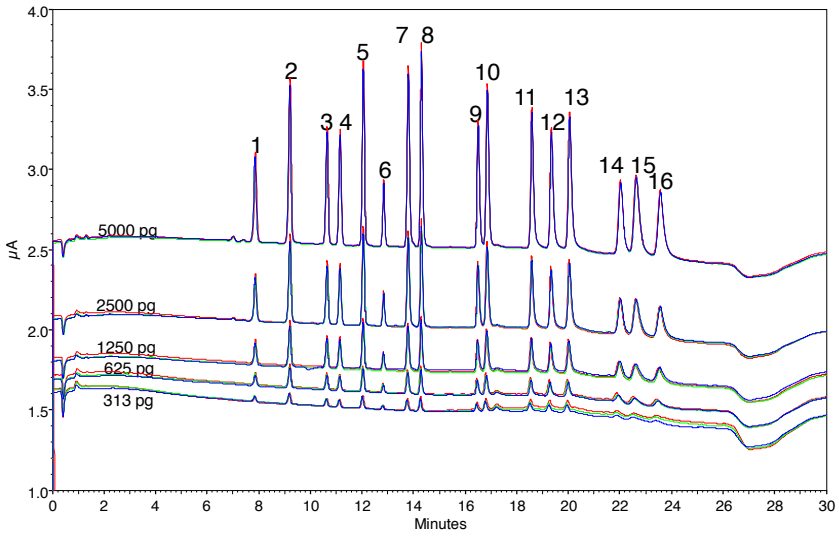
The boron-doped diamond (BBD) electrode is a novel carbon-based working electrode material that overcomes many of the issues of traditional carbon working electrodes. It is inert and resists fouling typically seen when measuring thiols and disulfides. It offers high sensitivity with minimal noise leading to improved LODs. It is both stable and stabilizes rapidly. Most importantly, the BDD working electrode extends the usable potential window for EC analysis, as electrolysis of water in the mobile phase only occurs at extreme potentials (Figure 6). Normal carbon working electrodes can only be used over a limited potential range due to electrolysis of water interfering with analyte measurement. Additionally, at extremely positive applied potentials the BDD acts as both a reactor and detector. Hydroxyl free radicals can be generated in-situ which can then react with electrochemically inert species, forming products that are now electrochemically reactive and measurable by HPLC-ECD – a process termed electrotagging. Figure 7 below is an example of the electrotagging process measuring the electrochemically inert polyaromatic hydrocarbons (PAHs).

FIGURE 6. Cyclic voltammograms obtained on either a traditional glassy carbon (GC) or BDD working electrode. Note that the BDD waveform extends to 2500mV vs. 1500mV for the GC electrode.[5]



The BDD approach is easy to implement and extends the range of analytes that are difficult to measure (e.g., thiols and disulfides) or not normally measured (e.g., PAHs, genotoxins (e.g., tosylates and besylates), and chelating agents) by HPLC-ECD.

FIGURE 7. Analysis of 16 different PAHs using gradient HPLC separation and detection by a BDD electrotagging technique. Restek Pinnacle® C18 (2.1 x 100 mm; 4 µm). Mobile phase A: water/ acetonitrile (90:10), 50 mM sodium perchlorate, 25 mM perchloric acid, 980 µM hydrogen peroxide. Mobile phase B: acetonitrile, 40 mM sodium perchlorate, 20 mM perchloric acid, 980 µM hydrogen peroxide. Gradient (min, %B): 7,58; 16,100; 25,100; 25.1,30; 30,30. Flow rate = 0.7 mL/min; Column temperature = 35 °C. Coulochem III applied potential: 1750 mV (vs. Pd reference) Mixed standards were analyzed from 313 to 5000 pg (on column) each in triplicate. 1. naphthalene; 2. acenaphthylene; 3. acenaphthene; 4. fluorene; 5. phenanthrene; 6. anthracene; 7. fluoranthene; 8. pyrene; 9. benzo(a)anthracene; 10. chrysene; 11. benzo(b)fluoranthene; 12. benzo(k)fluoranthene; 13. benzo(a)pyrene; 14. dibenzo(a,h)anthracene; 15. benzo(ghi)perylene; 16. indeno(1,2,3-cd)pyrene



Conclusion

Extended EC techniques are providing scientists with novel ways of using the superior sensitivity of ECD to analyze classes of compounds that are often difficult to measure using other approaches. A good example of this is the analysis of genotoxic analytes that exist in pharmaceuticals at ppb levels.[6]

Extended EC techniques tend to fall into two classes:

- Derivatization and immobilized enzyme approaches are more specific and offer increased selectivity.
- Photolysis and the BDD electrode are much less specific and offer the analyst a greater range of compounds that can currently be measured using HPLC-ECD.

References

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