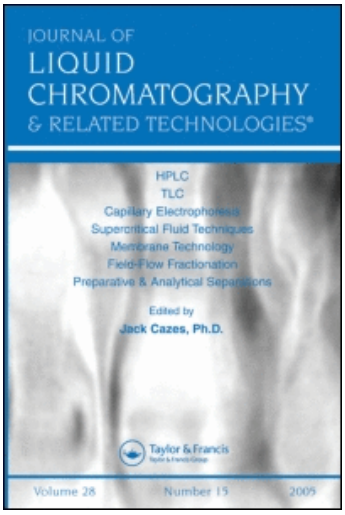

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Reductive Mode Thin-Layer Amperometric Detector for Liquid Chromatography

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REDUCTIVE MODE THIN-LAYER AMPEROMETRIC DETECTOR FOR LIQUID CHROMATOGRAPHY

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ABSTRACT

An electrochemical detector for liquid chromatography is described based on the use of a mercury film electrode in a thin-layer cell with the auxiliary electrode placed across the channel from the working electrode. Considerations relevant to optimizing performance of the detector are described including removal of dissolved oxygen from the sample and mobile phase. Detection limits are reported for a number of reducible organic compounds of interest in environmental and biomedical research. Detection limits at the picomole level are readily achieved for easily reduced nitro compounds. Typical applications are illustrated for the determination of parathion in field runoff water and chloramphenicol in human plasma.

INTRODUCTION

Amperometric detectors for liquid chromatography are in widespread use for the trace determination of easily oxidized

organic compounds of environmental, clinical, and pharmaceutical interest. Recent technological advances and applications in this area have been reviewed (1-5). Relatively little attention has been paid to electrochemical detection of easily reducible organic compounds because, until recently, it has been difficult to prepare a reliable mercury electrode, and carbon electrodes have not been seriously considered for this application due to their relatively low hydrogen overvoltage. Progress in this area has been slow due to problems associated with dissolved oxygen and metal ion impurities, but perhaps a more important reason is that many substances which are readily reduced on a mercury-film electrode are also good candidates for detection by optical absorption methods.

Since the introduction of the dropping mercury electrode (DME) for LCEC detection of easily reducible compounds in 1952 (6), several investigators have worked to improve the design of the DME based detector (7-10). These devices have shown only a limited use in a few academic laboratories due to the time dependent surface area, the expense and toxicity of mercury, the poor tolerance to a rapidly moving solution, and awkward cell construction. Improvements in tolerance to a moving solution have been accomplished by reducing the flow rate and by decreasing the drop time of the DME (7,10). This was accomplished at the expense of higher detection limits. Nevertheless, even though the problems are serious, several promising applications of DME-based detectors have recently been reported (11-14). Carbon-based electrode materials including glassy carbon (15), a basal plane pyrolytic graphite (16), and carbon impregnated silicone rubber (17) have been evaluated for reductive LCEC. These materials hold promise for many applications especially because preparation of the electrode surface is notably simplified. Nonetheless, mercury continues to be the material of choice for most electrochemical reductions.

Our laboratory (5,18) and others (19,20) have successfully used amalgamated gold, silver, and platinum electrodes. This approach results in easily prepared and mechanically rigid electrodes which provide adequate performance for monitoring trace amounts of many easily reducible compounds. Nevertheless, in certain specilized applications, DME-based transducers will remain useful because they possess two distinct advantages over other electrodes: a more negative potential range and a constantly renewable surface.

In the present paper we discuss various factors influencing the successful utilization of reductive mode LCEC for determination of organic compounds.

MATERIALS AND METHODS

Apparatus

A model LC-304 liquid chromatograph (Bioanalytical Systems) equipped with a PM-20 solvent delivery system, 70-10 fixed volume (20 or 100 μL) rotary sample injection valve, LC-4 electronic controller and LC-19 transducer package was used for all experiments. The columns were 15 cm x 3 mm i.d. packed with 10 μm LiChrosorb RP-18, 15 cm x 2 mm i.d. packed with 10 μm LiChrosorb RP-2, or 25 cm x 4.6 mm i.d. 5 μm Biophase C_{18} (Bioanalytical Systems Inc.).

Chemicals

Reagent grade acetonitrile, n-propanol, and triple distilled mercury from Fisher Scientific were used as purchased. Technical grade methanol and deionized water were distilled in an all glass apparatus prior to solution preparation. Citric acid, sodium phosphate (dibasic), monochloroacetic acid, sodium acetate, and glacial acetic acid purchased from Fisher Scientific

were used without further purification. All mobile phases were filtered through 0.22 μm cellulose acetate Millipore filters (Millipore Corp., Bedford Mass). Parathion and methyl parathion were purchased from Analabs Inc. (North Haven, CT). RDX, TNT, and PETN were gifts from Dr. Dietz (Bureau of Alcohol, Tobacco, and Firearms, Department of the Treasury, Cincinnati, OH). Diazepam, chlordiazepoxide and nitrazepam were received as gifts from Dr. R. P. W. Scott, Hoffman La-Roche Co., Nutley, NJ, XAD-2 resin, 120 mesh (Rohm and Hass) was conditioned according Paschal et al. (21).

Preparation of Mercury-Film Electrode

Preparation of Gold Substrate

It is very important to remove any residue present on the gold surface, in order to obtain an electrode with low background and noise characteristics. The old amalgam surface must be removed before polishing the gold substrate. This can be easily achieved by placing a few drops of 6 M nitric acid on the amalgam surface. After 2-3 minutes the electrode surface was washed with distilled water. Surface residues were removed by rubbing the electrode cube over a 600 grit silicone carbide abrasive paper. A mirror-like finish was obtained by rubbing the electrode cube over a microcloth (Buehler) polishing pad which was covered with Gamma Alumina (Fisher Scientific). Water was used as a lubricant. Alumina and gold residues were removed by sonicating the electrode cube in water/ethanol solution.

Preparation of Mercury-Film Electrode Surface

The mercury-film surface was prepared by two methods.

Method I. A small amount of mercury is placed on the polished gold surface with a mirror-like finish (making sure that the entire gold surface is covered by mercury).

After 2-3 minutes the excess mercury is removed into a beaker using a computer card. The mercury layer is then "smoothed" out using a dry tissue. The electrode is now ready to be used.

Method II. Electrolytic amalgam preparation is carried out in two steps. In the first step a solution containing 0.01 M $Hg(NO_3)_2$, 0.5 M KCl, and 0.1 M HCl is passed slowly through the cell using a 10 mL syringe. The syringe is connected directly to the flow cell. The potential of the working electrode is set at -0.7 V vs. Ag/AgCl for a period of 10-15 minutes. In the second step a solution containing 0.5 M KNO_3 and 0.1 M HNO_3 is pumped through the cell and the potential of the working electrode is set at -1.5 V for a period of three minutes. After rinsing the electrode surface with water the electrode is ready to be used.

Oxygen Removal

Two modifications of the LC system were made for reductive LCEC. The entire LC system was constructed with 316 stainless steel tubing and an oxygen removal apparatus (schematically shown in Figure 1) was placed in line with the pump and sampling valve.

The dissolved oxygen in the mobile phase was removed by modifying a procedure of Michael and Zatka (7). The mobile phase was heated to 60-70°C and nitrogen gas was vigorously bubbled through the mobile phase for approximately 20-30 minutes before initiating flow through the LC system (helium or argon have also been used). After starting the LC pump, a gentle flow of nitrogen gas was maintained over the surface of the mobile phase in order to prevent oxygen from diffusing back into the mobile phase, and the temperature of the mobile phase

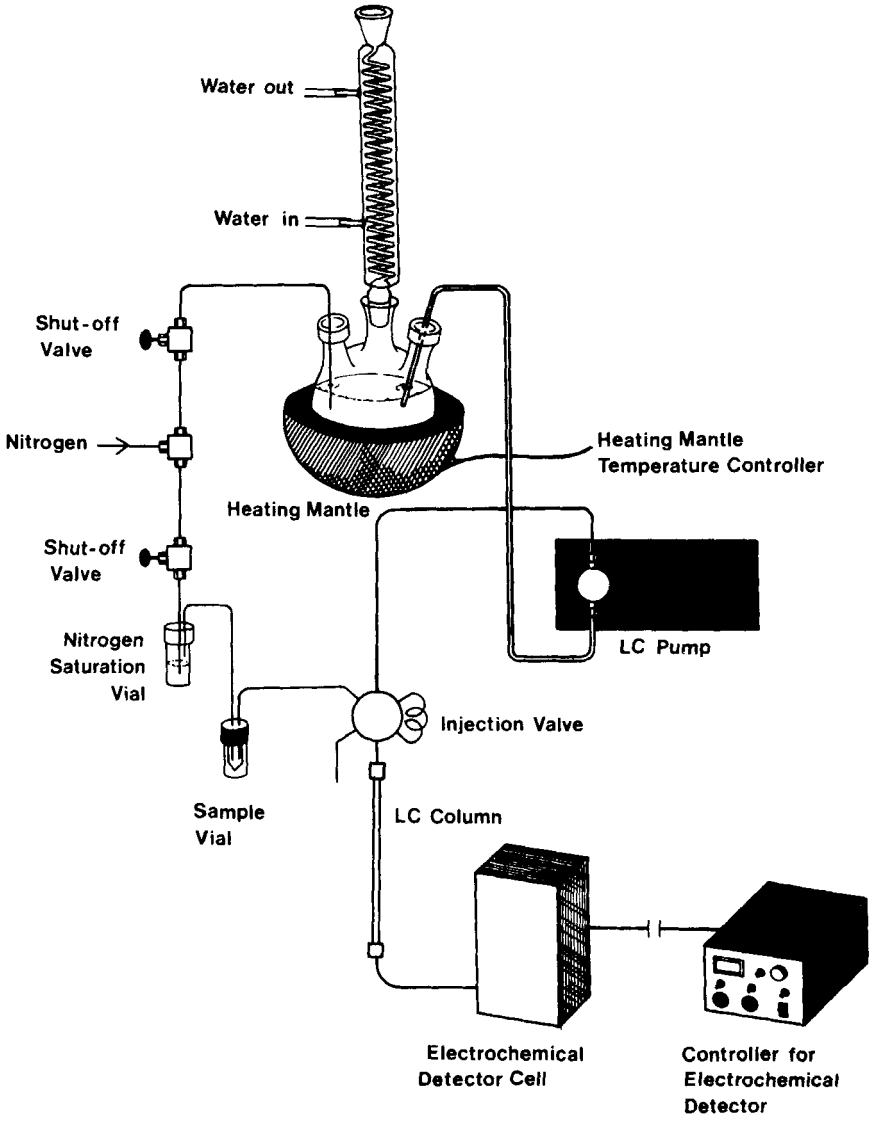

FIGURE 1. Schematic diagram of the reductive mode LCEC system.

was lowered to 35-40°C. The mobile phase was heated and a flow of nitrogen was maintained throughout the course of all LC experiments. Valuable time was saved when deoxygenation of the mobile phase was performed overnight at minimum flow (0.2 to 0.3 mL/min) through the LC system.

Sample deoxygenation was achieved by bubbling nitrogen gas through a sample solution. In order to prevent evaporation of solvent from a sample solution, dry nitrogen gas was passed through a saturation chamber which contained the same solvent as the sample solution. The deoxygenated sample solution was pulled into a sample loop without exposure to the ambient atmosphere. This sampling procedure was more convenient and effective than injecting deoxygenated solution into a sample loop with a syringe.

RESULTS AND DISCUSSION

Performance of the Detector

The utility of reductive mode LCEC detection is greatly limited if the dissolved oxygen is not removed from the mobile phase and sample solution prior to injection. Even at relatively low potentials, very large background currents are observed when oxygen is not removed from the mobile phase as illustrated in Figure 2. As mentioned previously, the LC system was modified to prevent diffusion of oxygen through the teflon tubing which is widely used in making low pressure connections in LC systems. It is not necessary to complicate the design of the reductive mode LCEC system by enclosing it in a box purged with nitrogen, if all teflon connections are replaced with a stainless steel tubing.

The solution resistance (R) can also be a concern when large background currents (> 50 nA) are encountered especially when the transducer schematically shown in Figure 3a was used

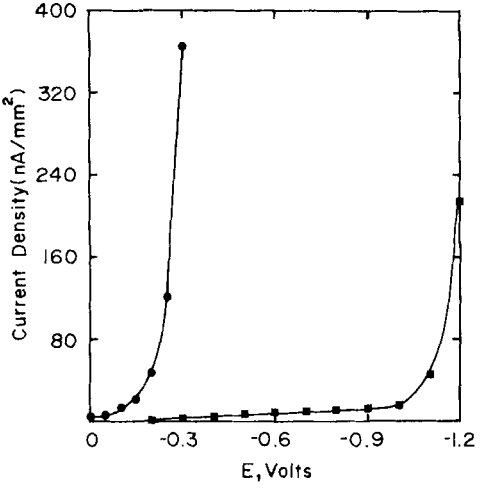

FIGURE 2. The observed background current on a Au/Hg electrode with (■) and without (●) deoxygenation of the mobile phase.

in preliminary experiments. This transducer performs adequately under low background current conditions (low negative potentials and also in oxidative LCEC; background current plus response due to analyte $\lesssim$ 150 nA) otherwise the product of current and resistance (referred to as IR drop) significantly diminishes the potential across the working electrode. The direct result of this IR drop is a decrease in the dynamic range of the detector as discussed in detail elsewhere (18). The solution resistance in the thin-layer channel can be decreased by increasing the ionic strength of the buffer, increasing the thickness of the channel, and decreasing the concentration of non-aqueous solvents. Increasing the ionic strength can also cause an increase in background current because salts often contain easily reducible impurities such as transition metal ions. Metal deposition on the mercury-surface decrease the negative potential range of the detector by decreasing the hydrogen overvoltage which in turn increases the background current.

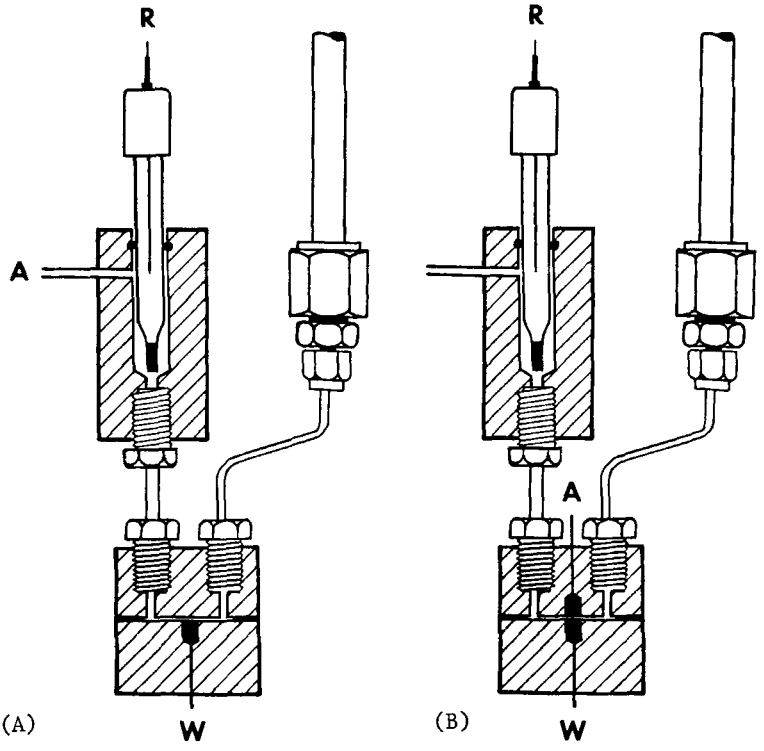

FIGURE 3. Common designs of thin-layer amperometric transducer.

The modified transducer illustrated in Figure 3b offers several important advantages over the more widely used design. The auxiliary electrode is placed in the top block directly across the channel from the working electrode. The path of current (from working electrode to auxiliary) is across the thin channel. The solution resistance even in low ionic strength solvents is extremely small and no longer influences the potential of the working electrode, resulting in extension of the linear dynamic range of the transducer by two orders of magnitude. A linear dynamic range of 5 1/2 orders of magnitude was found (50 pA → 2.5 μA) using picric acid as a model compound with the detector set at -0.8 V vs Ag/AgCl. The upper limit

of the linear dynamic range was not caused by the IR drop, but was due to saturation of the operational amplifier in the current-to-voltage converter. The modified transducer also allows the use of mobile phases with low ionic strength and high concentration of non-aqueous solvents such as acetonitrile, THF, or propanol.

It is very important to achieve a good amalgam formation around the edge of the gold substrate in order to minimize random noise spikes. It is not necessary to remove the old amalgam surface every time a new mercury surface was required. Usually an old amalgam surface was used 5 or 6 times as a substrate for the electrode surface regeneration, before it had to be removed with nitric acid. Slightly lower noise and long term stability at high detector sensitivity (especially at potentials greater than -0.8 V) was observed when the electrolytic method (Method II) was used to prepare a new electrode surface. Nevertheless,

TABLE 1

Typical Detection Limits of Easily Reduced Organic Compounds at S/N = 3 on a Au/Hg Electrode

Compounds	Detection Limits, picomoles
Picric Acid	0.13
2,5 - Dinitrophenol	0.16
p-Nitrophenol	0.72
RDX (Cyclonite)	0.76
Isosorbide dinitrate	1.3
Nitrazepam	1.8
Chlordiazepoxide	3.3
Diazepam	7.0
N-Nitrosopyrrolidine	10

Method I was generally preferred because it was less time consuming.

Representative detection limits for several easily reduced compounds are listed in Table 1. As expected, the detection limits for nitroaromatic compounds (especially for polyaromatic compounds) are lower because they undergo multielectron reduction processes, typically 4-12 electrons depending on the number of nitro groups, pH of the mobile phase, and the nature of the other substituents on the ring. Higher detection limits were observed for compounds with fewer electrons transferred and/or a higher reduction potential (diazepam and n-nitrosopyrolidine). Lower detection limits were obtained when the chromatographic and detector conditions were optimized for detection of a specific compound of interest. An LCUV system usually offers adequate sensitivity for nitro aromatic compounds, but the determination of several nanograms of nitrate esters per injection is difficult as illustrated in Figure 4. Crouthamel and Dorsch (22) reported LCUV detection limits of 30 ng for nitroglycerin. The UV detector performed better than expected in the case of TNT and RDX explosives as evident from a healthy response to 6 ng of RDX and 1.4 ng of TNT.

It was clearly evident from our determinations of the minimum detectable quantities that the background current and the quality of amalgam surface strongly affects the noise level of the detector. Two types of noise typically observed with a mercury film electrode were high and low frequency noise and random "spike" noise. As expected, the high frequency repetitive noise had a good correlation with the level of the background current as illustrated in Figure 5. The random spike noise had no correlation with the background current and was difficult to reproduce on a day-to-day basis and almost impossible to eliminate. Occasionally, when noise levels (typically due to spike noise) were unacceptably high (>2 nA), a new mercury

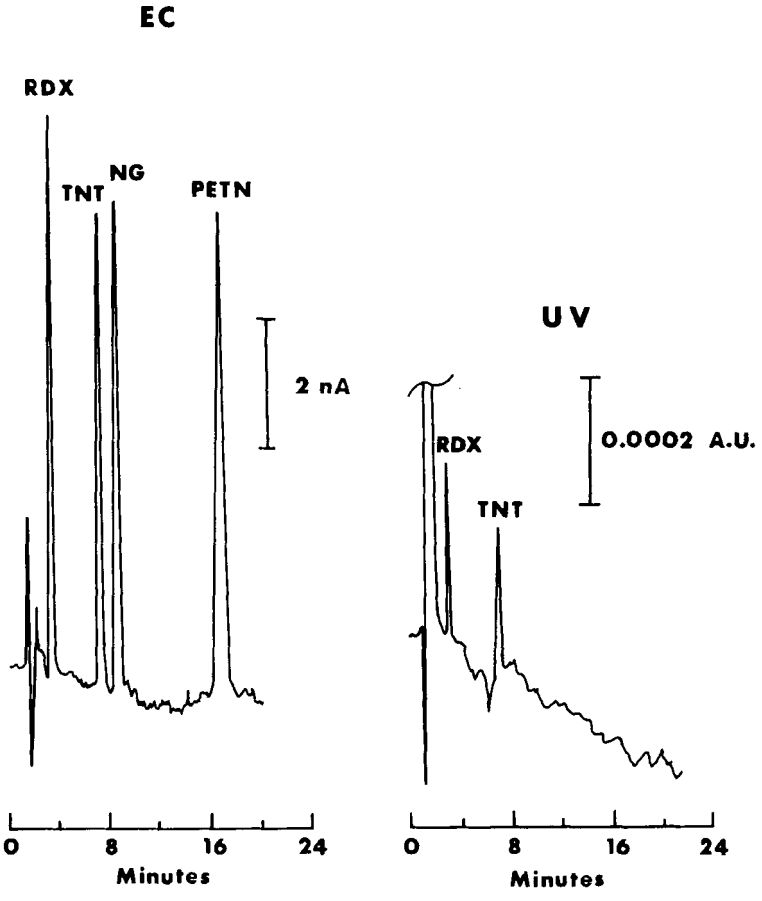

FIGURE 4. Comparison of EC (on the left, E = -1.0 V vs Ag/AgCl) and UV (on the right, at 254 nm) detectors for a mixture of explosive compounds. 27 pmoles of RDX, 6.2 pmoles of TNT, 41 pmoles of NG and 54 pmoles of PETN were separated on a 25 cm Biophase column. Mobile phase composition: 0.02 M monochloroacetic acid, 0.0146 M sodium acetate, 0.001 M EDTA, 16%(V/V) 1-propanol, and 5%(V/V) ethanol at 2 mL/min.

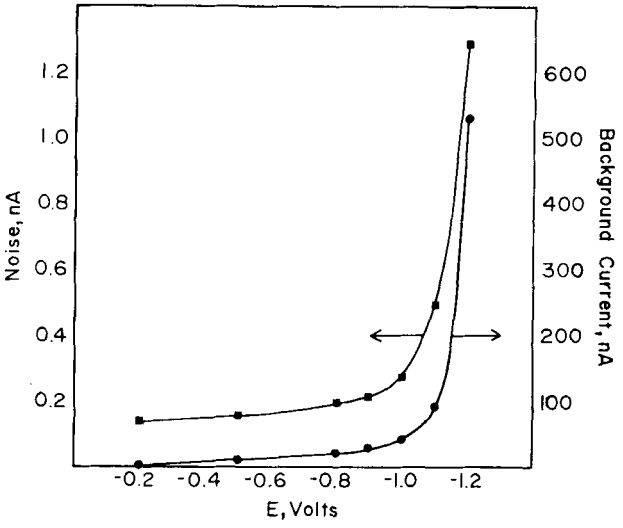

FIGURE 5. The dependence of the high frequency noise (■) and background current (●) on the applied potential of the Au/Hg electrode.

surface had to be prepared and extreme care was exercised in order to obtain a mirror-like gold substrate free of scratches and residue (formed during the nitric acid treatment of old amalgam). The lifetime of the electrode surface was dependent on the operating potential, nature of the sample, and amount of analyte injected.

Applications

Determination of Parathion and Methylparathion from Runoff Water

Parathion and *methylparathion* are organophosphorous insecticides which have been found to be effective replacements for extremely toxic organochlorine compounds such as DDT and Aldrin. Even though these pesticides accummulate very slowly in biological food chains because of their relatively rapid decomposition, they are still very toxic in their native form.

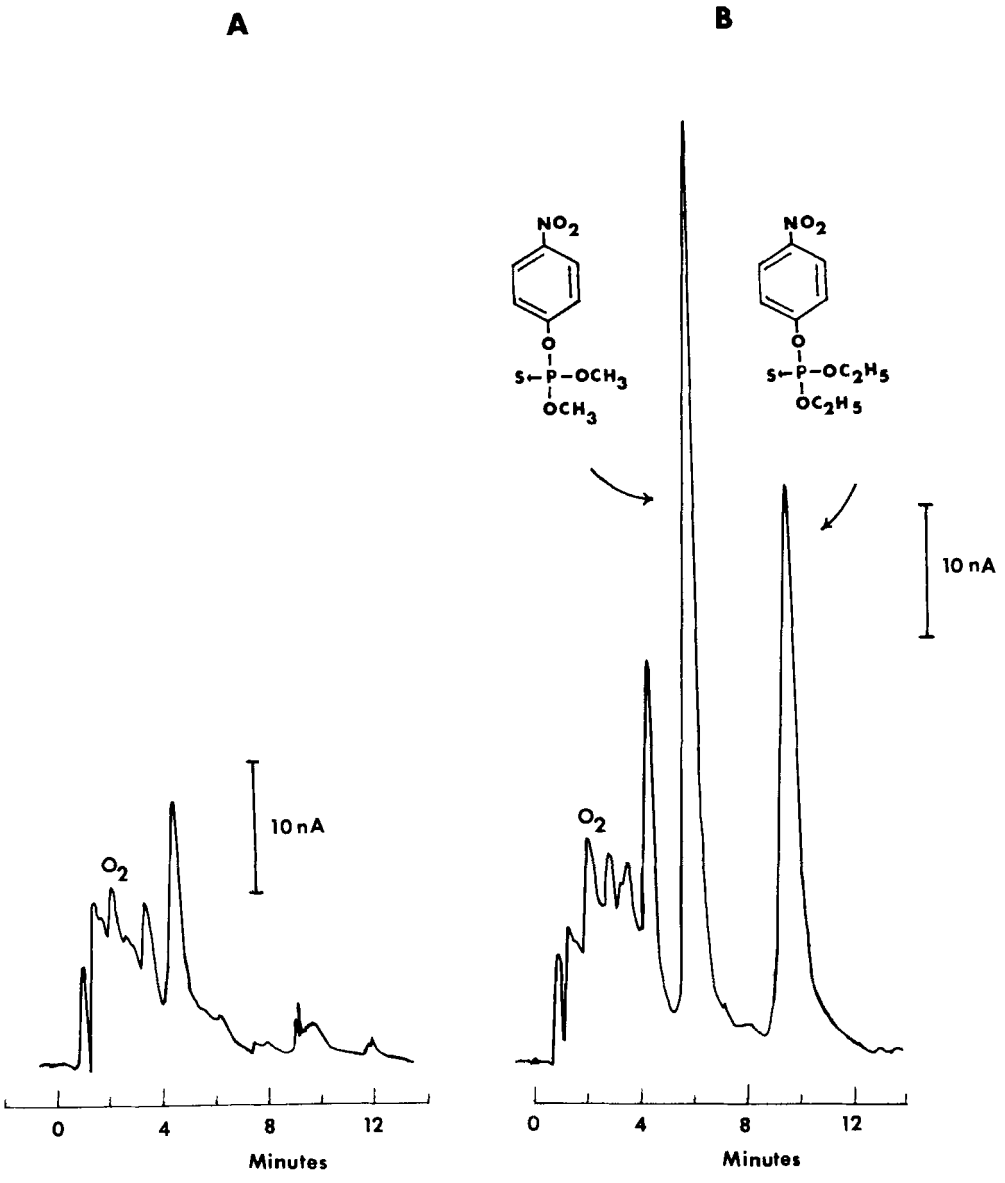

FIGURE 6. Representative chromatograms of runoff water. (A) Runoff water blank. (B) Runoff water spiked with 40 ng/mL of methyl parathion and parathion. Separation was achieved using a 15 cm column packed with 10 um LiChrosorb RP-2 material, mobile phase composition: 0.005 M acetate buffer pH 4 and 45% (V/V) acetonitrile at 0.4 mL/min. Au/Hg working electrode was set at -1.05 V vs Ag/AgCl.

The isolation and quantitation of parathion and methylparathion were chosen as an example for illustrating the utility of the thin-layer gold/mercury electrode as a reductive mode detector.

Parathion and methylparathion were determined in spiked runoff water collected from a stagnant farm pond in West Lafayette. The preliminary isolation was according to Paschal et al. (21) with minor modifications. Final separation and quantitation was achieved using a microparticulate reverse-phase LC column with a TL-9A electrochemical transducer. Two 25 mL runoff water samples were analyzed in parallel; one was used as a sample blank and the second was spiked with 1 μg of each of the two pesticides (40 ng/mL). Each sample was passed through a 5 cm isolation column packed with XAD-2 resin. The pesticides were eluted from the resin with 25 mL of diethyl ether. After evaporating the ether under a stream of nitrogen gas at ambient temperature, the residue was dissolved in 400 μL of the mobile phase. A 20 μL aliquot was injected onto the LC column and the chromatograms of the blank (A) and spiked sample (B) illustrated in Figure 6 were obtained. Chromatographic and detector conditions are given in the figure caption.

Determination of Chloramphenicol in Serum

Chlorampenicol, a broad-spectrum antibiotic, was originally derived from Venezuelan strain of streptomycetes. This antibiotic is responsible for bringing thyphoid fever under the control since the end of World War II. More than fifty million people have been treated with this antibiotic in the past three decades. Many analogs of chloramphenicol have been synthesized leading to the observation that the nitro-group in the para position on the phenyl ring is essential to the antibacterial activity of the drug.

Chloramphenicol is easily reduced at a mercury film electrode. A chromatogram of a diethyl ether extract of plasma

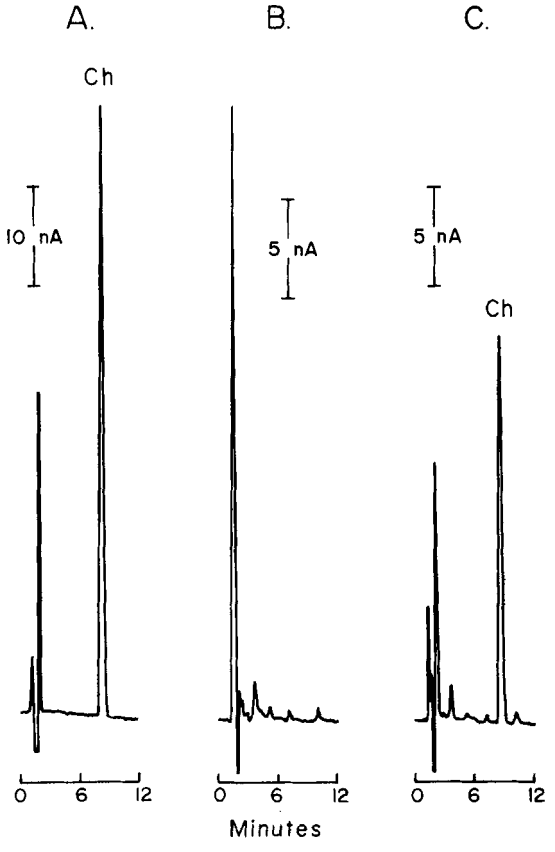

FIGURE 7. (A) Chromatogram of 295 pmoles of chloramphenicol (Ch), (B) Representative chromatogram of plasma blank, (C) Chromatogram of plasma spiked with 0.71 µg/mL of chloramphenicol. Chromatographic conditions: 25 cm Biophase column with C_{18} packing, mobile phase contained 0.02 M monochloroacetic acid, 0.001 M EDTA, and 0.0146 sodium acetate, and 9%(V/V) 1-propanol. 2 mL/min, Detector potential: -0.85 V vs AgCl.

is illustrated in Figure 7. One mL of plasma spiked with appropriate amounts of chloramphenicol was deprotenized with 50 µL of 4 M perchloric acid and centrifuged for 5 minutes at 2000 rpm. 300 µL of the resulting supernatant was loaded on an extraction column (Clin Elute Mode No. 1000-M). Chloramphenicol was eluted with 3 mL of diethylether. After evaporation of the ether, the residue was dissolved in 300 µL of a mixture of water: ethanol (80:20) and 50 µL of this reconstituted solution was injected onto a reverse phase column.

CONCLUSION

Reductive electrochemical detection has been developing very slowly over nearly 30 years. In the past several years progress has accelerated rapidly as applications to organometallic (19,20) and organic compounds (5,11-18) have been developed. It is now clear that reductive LCEC experiments can be made routine for a number of important problems. A new approach is therefore available in the arsenal of tools for trace determinations by liquid chromatography.

ACKNOWLEDGMENTS

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