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Note

Electrochemiluminescence as a detection technique for reversed-phase high-performance liquid chromatography

IV. Detection of fluorescent derivatives

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Electrochemiluminescence (ECL) is light emitted during the electrolysis of solutions of (usually) organic compounds. We have been investigating the use of ECL as a detection technique for reversed-phase liquid chromatography (RPLC) using relatively simple electrode assemblies containing flow cell volumes of the order of 10 μl. We reported earlier[1,2] that a modest sensitivity could be achieved with a relatively simple two-electrode cell arrangement and non-deoxygenated eluents involving 10% water. More recently[3] we discussed a flow cell constructed from a Kel-F body housing two platinum working electrodes and a platinum wire reference electrode, and demonstrated that controlled potential a.c. electrolysis could be used to achieve reasonable sensitivities and reproducibilities for a range of aromatic compounds. We have also discussed results obtained from lumograph studies[4], *i.e.* the variations of emission intensity as a function of electrolysis potential and frequency, and shown that in most aqueous eluents ECL efficiency is optimised at relatively low frequencies (< 10 Hz).

Our experiments have demonstrated that ECL offers some attractions as a detection technique for reversed-phase high-performance liquid chromatography (RP-HPLC), in that many classes of compounds appeared to emit light during electrolysis and the sensitivities for detection are reasonable. However, some classes of compounds either do not produce ECL at all, or do so with relatively low efficiency, so that the limit of detection is poor. In this paper we report on some experiments carried out to study the effect of derivatisation on ECL detection. Our initial objective was to examine the standard dansyl and phenylthiohydantoin derivatives of amino acids, although we have also examined a range of phenolic compounds which appear to benefit from derivatisation.

EXPERIMENTAL

The chromatographic system and the experimental ECL detector have been described in ref. 3. For the present chromatographic separations Waters RCSS cartridges were used in either an RCM-100 or Z-module unit. The cartridges used were

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C_{18}. Resolve (5 mm I.D.) in the RCM-100, or C_{18} μBondapak (8 mm I.D.) in the Z-module. The eluent was prepared from acetonitrile and water and tetrabutylammonium perchlorate (TBAP) (all as described previously)[3].

The sample and reagent materials used were as follows: 4-acetamidophenol (paracetamol); ethyl *p*-hydroxybenzoate (ethyl paraben); *n*-propyl *p*-hydroxybenzoate (propyl paraben); *n*-butyl *p*-hydroxybenzoate (butyl paraben); dansyl-L-alanine, cyclohexylamine salt; dansyl-glycine; dansyl-L-glutamine; dansyl-L-leucine, cyclohexylamine salt; dansyl-L-methionine, cyclohexylamine salt; dansyl-L-phenylalanine; dansyl-L-proline; N-dansyl-L-serine, cyclohexylamine salt; dansyl-L-tryptophan, monocyclohexylammonium salt; dansyl-L-valine, monocyclohexylammonium salt; phenylthiohydantoin (PTH)-alanine; PTH-glycine; PTH-histidine; PTH-leucine; PTH-phenylalanine; PTH-tryptophan; PTH-valine; all 99% purity, from Sigma. Phenol, 2,4 di-chlorophenol, and 2,4,5-trichlorophenol, 99% purity, were supplied by BDH. 5-Dimethylamino-1-naphthalenesulphonyl chloride (dansyl chloride), 99% purity, was supplied by Lancaster Synthesis.

All test compounds were used without further purification. TBAP was prepared by neutralising a concentrated solution of tetrabutylammonium hydroxide (Aldrich) with AR perchloric acid (Fisons) and was purified by two recrystallisations from ethyl acetate–pentane (5:1, v/v). HPLC-grade acetonitrile (Rathburn) was used without further purification. Distilled water was further purified using a Water-1 purification unit (Gelman Sciences) and filtered through a 0.45-μm Millipore filter.

The dansyl derivatisation procedure was carried out using the method of Knapp[5]. The procedure requires that the reaction mixture is heated to 60°C for 20 min. Reaction mixtures were allowed to cool to room temperature before HPLC analysis.

RESULTS AND DISCUSSION

All the dansylated amino acids tested gave ECL emissions. A typical separation of dansylated amino acids is shown in Fig. 1. In this case the excitation potential was

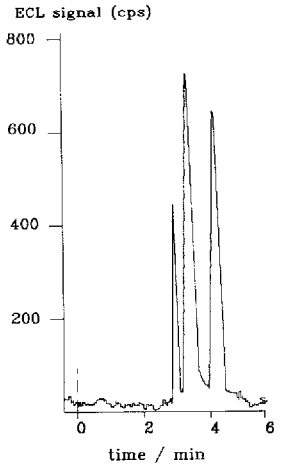

Fig. 1. Chromatogram showing the elution of dansyl derivatives of L-tryptophan, L-alanine and L-proline. See text for details.

TABLE I

ECL RESPONSE FOR DANSYLATED AMINO ACIDS

Amino acid	*Response (counts per μg)*		
	Compound	*Dansyl derivative*	*PTH dervative*
Alanine	0	9654	6685
Proline	0	8091	–
Tryptophan	839	8307	10 086
Leucine	0	5695	4393
Methionine	0	5854	–
Phenylalanine	0	3941	8530
Valine	0	6261	5974
Glycine	0	9672	4798
Glutamine	0	3772	–
Serine	0	6924	–
Histidine	0	–	513

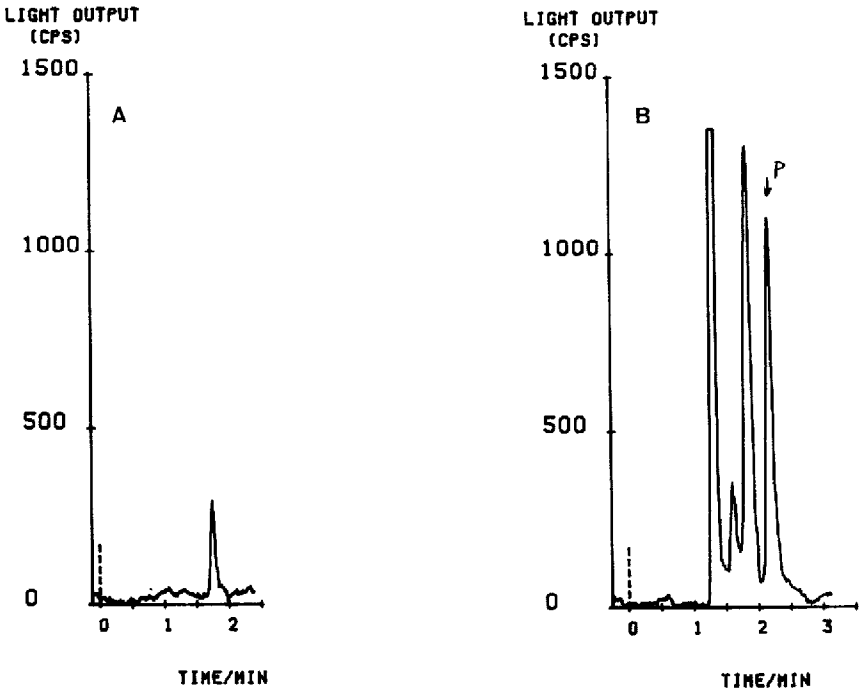

Fig. 2. Chromatographic peaks recorded from (A) paracetamol, and (B) dansylated paracetamol. In (B) the peak marked p is the paracetamol derivative; other peaks are excess dansylation reagents.

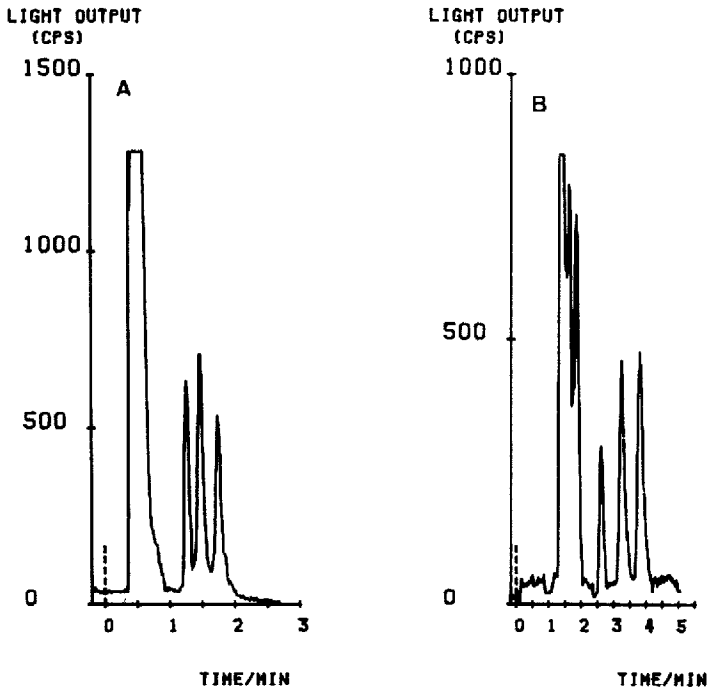

Fig. 3. Chromatograms for (A) dansyl derivatives of ethyl, propyl, and butyl paraben, and (B) dansyl derivatives of phenol, dichlorophenol and trichlorophenol.

8.0 V at a frequency of 10 Hz. The sample contained dansylated tryptophan (300 ng), alanine (130 ng) and proline (170 ng) and was eluted from the μBondapak cartridge with acetonitrile–water (90:10) (made $5 \cdot 10^{-3}$ *M* in TBAP) at a flow-rate of 1.5 ml min^{-1}. The standard PTH derivatives of the amino acids gave similar ECL emissions. Dividing the integrated photon count by the mass of component injected yields the ECL response for a given eluent and electrical conditions. The figures obtained for the dansylated amino acids tested and for the PTH derivatives are collected in Table I. With the single exception of tryptophan, no ECL emissions have been detected from any of the non-derivatised amino acids tested.

Another example of the enhancement of ECL activity brought about by derivatisation is illustrated in Fig. 2, where chromatograms recorded from (a) paracetamol (512 ng), and (b) dansylated paracetamol (489 ng) are shown, both eluted at 2.0 ml min^{-1} from a C_{18} μBondapak cartridge, using the electrical and eluent conditions described for Fig. 1. The dansyl derivative yields approximately a four-fold increase in ECL emission.

Additional ECL chromatograms were obtained for a number of derivatised phenolic compounds, using the same excitation conditions as decribed above. Fig. 3A shows a chromatogram of dansyl derivatives of some commonly used food preservatives eluted from the C_{18} Resolve cartridge at 2.0 ml min^{-1} using the eluent described for Fig. 1. The sample contained dansylated ethyl paraben (E214) (567 ng), propyl paraben (E216) (729 ng), and butyl paraben (644 ng). Fig. 3B shows a sepa-

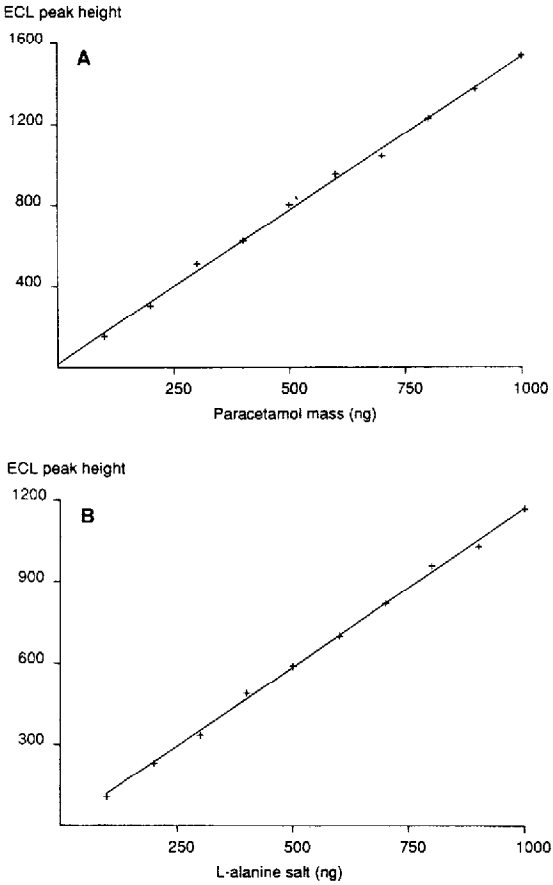

Fig. 4. Variation of ECL chromatographic peak height as a function of sample size for dansyl derivative of (A) paracetamol, and (B) alanine.

ration of dansylated phenol and chlorophenols eluted from the μBondapak cartridge using the same eluent and flow-rate as Fig. 2A. In this case the sample contained dansylated phenol (246 ng), dichlorophenol (419 ng), and trichlorophenol (669 ng).

The lincarity of thc chromatographic peak height with sample size was tested with a variety of derivatives. Typical results are shown in Fig. 4. Fig. 4A shows the peak height recorded from dansylated paracetamol as a function of mass injected, the sample being eluted from a C_{18} μBondapak cartridge at 2.0 ml min^{-1} using the eluent and electrolysis conditions as described for Fig. 1. It is clear that the response is linear over the range examined (up to 1 μg paracetamol), and this is consistent with most of our studies of linearity for ECL. However, it is an improvement over the results obtained with non-derivatised paracetamol, where a non-linear response is recorded. Fig. 4B shows the response from dansylated alanine as a function of sample size (eluted as for Fig. 4A), and again a linear response is recorded over the range 10–1000 ng.

Clearly the derivatisation of materials may significantly improve the efficiency with which a number of compounds may be detected using the electrochemiluminescence technique. We are now extending our experiments in the search for more convenient derivatisation agents.

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