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CHEMILUMINESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING N-(4-AMINOBUTYL)-N-ETHYLISOLUMINOL AS A PRE-COLUMN LABELLING REAGENT

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SUMMARY

N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) was used as a pre-labelling reagent for primary and secondary amines and carboxylic acids. Chemiluminescence generated by the reaction of ABEI–hydrogen peroxide–potassium hexacyanoferrate(III) was applied to a detection system for high-performance liquid chromatography. The combination of 2.2 ml/min of 0.3 *M* hydrogen peroxide, 3.9 ml/min of $1.25 \cdot 10^{-2}$ *M* potassium hexacyanoferrate(III) in 2.6 *M* sodium hydroxide and 1.5 ml/min of eluent (methanol–water) using a reversed-phase column was optimum for the detection of femtomole amounts of ABEI derivatives. The detection limit for the ABEI derivative of cholic acid was 20 fmol.

INTRODUCTION

In recent years, there has been increasing interest in development of high-performance liquid chromatography (HPLC) for the analysis of various biological substances. However, the sensitivity of HPLC was too low because of the use of a refractive index or UV detector. Therefore, a major thrust in the development of modern HPLC has been towards improved sensitivity and selectivity. The best HPLC detectors currently available are fluorimetric and electrochemical detectors. Chemical derivatization in HPLC[1,2] has become a popular means of increasing the sensitivity and selectivity of detection for trace components in complex matrices such as biological specimens, pharmaceutical preparations, etc. In previous work[3–6], we developed the fluorescence HPLC method for the determination of steroids using dansylhydrazine as a pre-labelling reagent.

Recently, the use of chemi- and bioluminescence analysis[7–9] has been introduced into biochemistry and clinical chemistry because of its high sensitivity. Several applications of chemiluminescence detection in HPLC have been reported over the last 5 years using oxalate–peroxide[10,11], firefly luciferase[12] or lucigenin[13] chemiluminescence reaction system.

Luminol (3-aminophthalic hydrazide)[14] reacts with hydrogen peroxide in the presence of a metal catalyst in alkaline solution to produce luminescence. This re-

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action is extremely sensitive and has been used for the determination of low concentrations of peroxide[15,16] and metals[17]. In this study, we investigated the chemiluminescence HPLC based on the isoluminol derivative–hydrogen peroxide–potassium hexacyanoferrate(III) reaction system, in which N-(4-aminobutyl)-N-ethylisoluminol (ABEI) was used as a pre-labelling reagent for a wide variety of compounds that have primary and secondary amino groups or carboxylic groups.

EXPERIMENTAL

Reagents

ABEI was purchased from LKB Wallac. N,N'-Disuccinimidyl carbonate (DSC) was obtained from Chemi Science. Isoluminol, 2-chloro-1-methylpyridinium iodide (CMPI), 3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one (DPP) and all other amine and carboxylic acid standards were obtained from Tokyo Kasei. Cholic acid, chenodeoxycholic acid, deoxychoic acid, lithocholic acid and ursodeoxycholic acid were obtained from Sigma or PL Biochemical. H_2O_2 (30%) was purchased from Mitsubishi Gas Kagaku. All other reagents and solvents were of analytical-reagent grade from commercial sources.

Instruments and chromatographic conditions

A schematic diagram of the apparatus for chemiluminescence HPLC is shown in Fig. 1. Chromatography was carried out with a Hitachi Model 635 liquid chromatograph, a Kyowa Seimitsu KHP-UI-130 injection valve and a Resolve column (5 μm Spherical C_{18}, 150 × 3.9 mm I.D.; Waters Assoc.) or a Radial-Pak NVC 18 column (5 μm, 100 × 8 mm I.D.; Waters Assoc.). The flow-rate of the mobile phase was 1.5 ml/min. The eluent from the column was mixed with 0.3 *M* H_2O_2 and 0.0125 *M* $K_3Fe(CN)_6$ in 2.6 *M* sodium hydroxide solution with use of two pumps (reciprocating type; Kyowa Seimitsu). The flow-rates of H_2O_2 and $K_3Fe(CN)_6$ solutions were 2.2 and 3.9 ml/min, respectively. The generated chemiluminescence was monitored with an Aminco Chem-Glow photometer equipped with a flow cell (cell volume *ca.* 60 μl).

Labelling reaction

The reactions were performed in screw-cap mini-vials (2 ml). Portions of 10–20 μl of the reaction mixture solutions were injected into the chromatograph.

Labelling of carboxylic acids with ABEI. To a solution of 0.1–5 nmol of carboxylic acids in 100 μl of methanol, 10 nmol of ABEI in 50 μl of methanol was

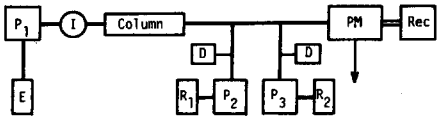


Fig. 1. Schematic flow diagram of the chemiluminescence HPLC system. P = Pump (P_1 = Hitachi Model 635; P_2, P_3 = 0986-554 reaction motor); I = injection valve (Kyowa Seimitsu KHP-UI-130); PM = detector (Aminco CHEM-GLOW photometer); Rec = recorder (Yokogawa Type 3066); D = damper; E = eluent (methanol–water), flow-rate 1.5 ml/min; R_1 = 0.3 *M* H_2O_2, 2.2 ml/min; R_2 = $1.25 \cdot 10^{-2}$ *M* $K_3Fe(CN)_6$ in 2.6 *M* NaOH, 3.9 ml/min.

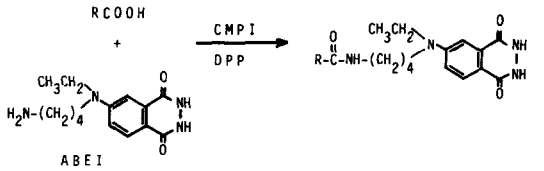


Fig. 2. Labelling reaction of carboxylic acids with ABEI. CMPI = 2-chloro-1-methylpyridinium iodide; DPP = 3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one.

added. The mixture was evaporated to dryness under a stream of nitrogen gas, then 20 nmol of CMPI in 0.5 ml of acetonitrile and 40 nmol of DPP in 0.5 ml of acetonitrile were added and the mixture was heated at 60°C for 2 h in a water-bath. We developed this method (Fig. 2) based on the work of Bald *et al.*[18].

Labelling of amines with ABEI. The labelling of amines with ABEI was carried by two steps (Fig. 3). To a stirred solution of 5 μmol of ABEI in 1 ml of methanol were added 5 μmol of DSC in 1 ml of acetonitrile and the mixture was allowed to stand at room temperature for 2 h. To a resulting solution (50 μl), 1–50 nmol of amines in 0.5 ml of methanol were added. After vortex mixing, the mixture was allowed to stand at room temperature for 2 h.

RESULTS AND DISCUSSION

Recently there considerable interest has been shown in using chemiluminescence detection systems with HPLC[10–13], because the chemical reactions offer a high degree of selectivity and the detector may consist only of a photomultiplier. As this type of system does not require an external light source, no stray radiation will reach the photomultiplier and therefore greater sensitivity is feasible. Kobayashi *et al.*[19] have shown that the sensitivity of a bis(2,4,6-trichlorophenyl) oxalate–H_2O_2 chemiluminescence detection system with a detection limit of 25 fmol was about 20 times higher than that of conventional fluorescence detection for fluorescamine-labelled catecholamines. Luminol was first reported by Albrecht[14] in 1928 and can be oxidized in simple reactions. It produces light with a high quantum efficiency, which can be measured at low picomole levels. Schröeder and Yeager[20] studied the chemiluminescence yields and detection limits of luminol, isoluminol and isoluminol derivatives in various oxidation systems and investigated chemiluminescent immunoassay.

In this study, we used ABEI as a pre-labelling reagent in chemiluminescence HPLC. Optimum conditions were determined first for the chemiluminescent reaction

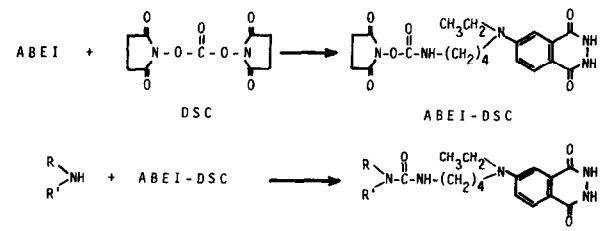


Fig. 3. Labelling reaction of primary and secondary amines with ABEI. DSC = N,N'-disuccinimidyl carbonate.

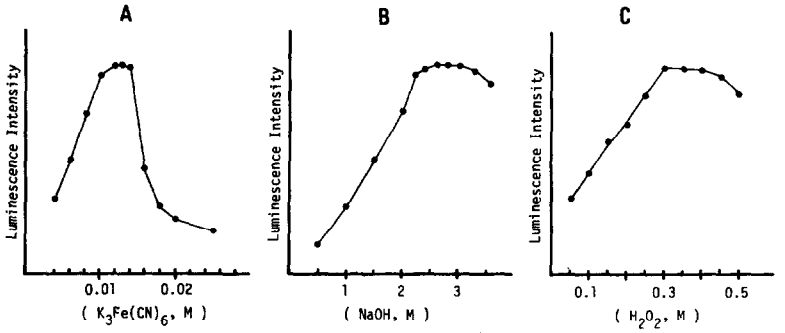


Fig. 4. Effect of reagent concentration on chemiluminescence intensity peak height. (A) $K_3Fe(CN)_6$; (B) NaOH; (C) H_2O_2.

using ABEI standards. For this study, the analytical column was removed from the HPLC system.

The luminol–H_2O_2 reaction is carried out in the presence of a metal catalyst in alkaline solution. Bostick and Hercules[16] adopted the $K_3Fe(CN)_6$ system, because $K_3Fe(CN)_6$ gave a linear chemiluminescent response and has good sensitivity. There-

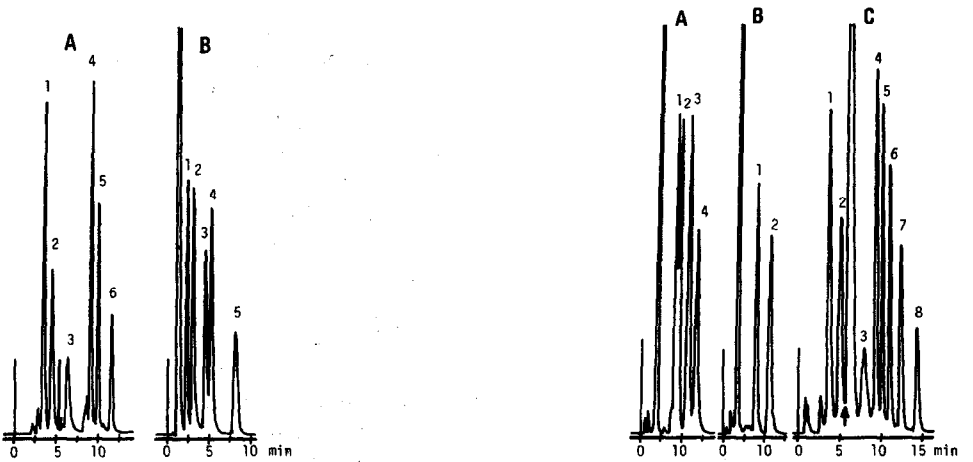
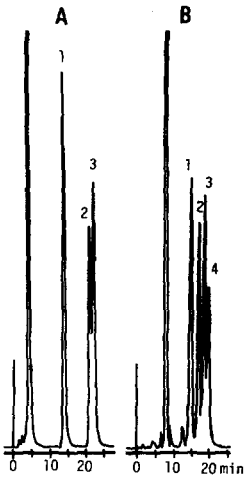


Fig. 5. Chromatograms of ABEI derivatives of a standard mixture of carboxylic acids. (A) Free fatty acids. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = *n*-butyric acid; 4 = caproic acid; 5 = caprylic acid; 6 = capric acid. Resolve column (5 μm Spherical C_{18}, 150 × 4.6 mm I.D.); mobile phase, 40 to 75% methanol, 1.5 ml/min. (B) Bile acids. Peaks: 1 = ursodeoxycholic acid; 2 = cholic acid; 3 = chenodeoxycholic acid; 4 = deoxycholic acid; 5 = lithocholic acid. Resolve column; mobile phase, 75% methanol, 1.5 ml/min.

Fig. 6. Chromatograms of ABEI derivatives of a standard mixture of amines. (A) Aryl-substituted amines. Peaks: 1 = aniline; 2 = benzylamine; 3 = α-phenylethylamine; 4 = β-phenylethylamine. Resolve column; mobile phase, 45% methanol, 1.5 ml/min. (B) Aromatic amines. Peaks: 1 = aniline; 2 = N-methylaniline. Resolve column; mobile phase, 45% methanol, 1.5 ml/min. (C) Aliphatic amines. Peaks: 1 = methylamine; 2 = ethylamine; 3 = *n*-propylamine; 4 = *n*-butylamine; 5 = *n*-amylamine; 6 = *n*-hexylamine; 7 = *n*-heptylamine; 8 = *n*-octylamine. Resolve column; mobile phase, 40 to 65% methanol, 1.5 ml/min.



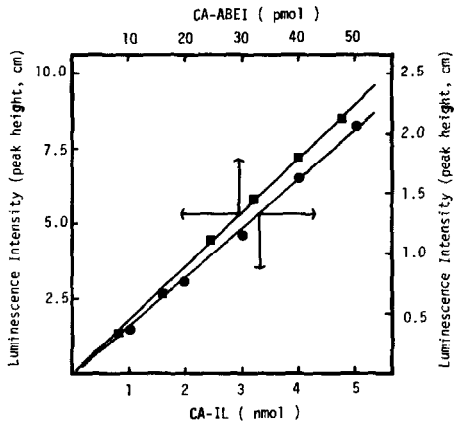


Fig. 7. Chromatograms of ABEI derivatives of a standard mixture of isomers of toluidine and butylamine. (A) *o*-, *m*- and *p*-isomers of toluidine. Peaks: 1 = *o*-toluidine; 2 = *p*-toluidine; 3 = *m*-toluidine. Radial-Pak NVC 18 column; mobile phase, 50% methanol, 1.5 ml/min. (B) *n*-, *sec*.-, *tert*.- and *iso*-isomers of butylamine. Peaks: 1 = *sec*.-butylamine; 2 = *iso*-butylamine; 3 = *n*-butylamine; 4 = *tert*.-butylamine. Radial-Pak NVC 18 column; mobile phase, 45% methanol, 1.5 ml/min.

Fig. 8. Comparison of calibration graphs for cholic acid labelled with ABEI (■) and IL (●).

fore, we also used $K_3Fe(CN)_6$ as the catalyst. The relative sensitivity of ABEI reaction was found to be markedly affected by the $K_3Fe(CN)_6$ concentration. As shown in Fig. 4A, maximum light emission occurred at a $K_3Fe(CN)_6$ concentration $1.25 \cdot 10^{-2}$ *M* and the sensitivity decreased sharply on either side of this concentration; therefore this concentration was adopted for the method.

The effect of pH on sensitivity was investigated, because the chemiluminescent reaction is pH dependent. The sensitivity was approximately constant between 2.5 and 3.0 *M* sodium hydroxide solution, as shown in Fig. 4B; 2.6 *M* sodium hydroxide solution was therefore used for dissolving $K_3Fe(CN)_6$.

The relative light emission increased with increasing H_2O_2 concentration, and the sensitivity was constant between 0.3 and 0.4 *M* H_2O_2 as shown Fig. 4C; 0.3 *M* was adopted for the method.

The optimal flow-rates of the H_2O_2 and $K_3Fe(CN)_6$ solutions were 2.2 and 3.9 ml/min, respectively.

The newly developed detection system was then applied to the separation of carboxylic acids and amino compounds. Many solvent systems and columns were examined in order to obtain complete separations. As shown in Fig. 5A, a good separation of ABEI derivatives of free fatty acids can be achieved with a Resolve column. The mobile phase was changed from 40% to 75% methanol 5 min after injection of sample solution. The five unconjugated bile acids were efficiently separated, as shown in Fig. 5B. Methanol–water (75:25) was found to be suitable when used on a Resolve column. Fig. 6 shows the separation of amine derivatives. Aryl-substituted amines and aromatic amines were completely resolved on a Resolve column using 45% methanol as the eluent (Fig. 6A and B). The eight aliphatic amines

were efficiently separated on a Resolve column using stepwise elution (Fig. 6C). Good separations of the *o*-, *m*- and *p*-isomers of toluidine and the *n*-, *sec.*-, *tert.*- and *iso*-isomers of butylamine as ABEI derivatives were achieved on a Radial-Pak NVC 18 column (Fig. 7).

The effect on the light yield of the alkyl bridge linking the chemiluminescent label isoluminol to cholic acid was investigated, because structural modification of the phthalhydrazine can greatly affect chemiluminescent quantum yields[21]. Fig. 8 compares the calibration graphs for cholic acid labelled with ABEI and IL. When cholic acid was coupled to the primary amino residue of IL, the maximum light intensity was markedly decreased compared with that of the ABEI derivative. Substitution of electron-withdrawing groups in the amino residue of IL decreased the light production.

The detection limit of cholic acid obtained with the present method was 20 fmol (signal-to-noise ratio 3.5) per injection, which is comparable to that of the fluorescence HPLC method reported by Goto *et al.*[22].

This paper represents a preliminary communication of our results and the first report of chemiluminescence HPLC using ABEI as a pre-labelling reagent and the H_2O_2–$K_3Fe(CN)_6$ system as a post-column reaction; further details will be published in the near future.

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