$Ru(bpy)_3^{3+}$ Electrochemiluminescence Detection of Aliphatic and Aromatic Amines with Diketene

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A new derivatization method for the detection of aliphatic and aromatic amines, based on electro-chemiluminescence (ECL) with $Ru(bpy)_3^{2+}$ is proposed. Sample amines were derivatized to acetoacetylamide-type derivatives using diketene. The derivatives were detected by reversed-phase HPLC with ECL detection using $Ru(bpy)_3^{2+}$.

Amines are probably the most heavily studied group of reagents for chemical derivatization because of their biological, industrial, and environmental importance.[1] The development of new method for their quantitation is therefore necessary. Chemiluminescence is an attractive detection technique for HPLC due to its very low detection limits and wide linear working range, whilst using relatively simple instrumentation. It has been found to be an important new detection system for analytical applications. The tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$) ECL system is useful as a selective and sensitive detection method for oxalate,[2] indoles,[3] diketones,[4,5] amino acids[6,7] and trialkylamines.[8] We previously reported on the ECL detection of alicyclic tertiary amines[9] at the pmol level using $Ru(bpy)_3^{2+}$, although the ECL intensity of aliphatic and aromatic primary amines could only be detected at a level approximately 1000-times lower than that of tertiary amines. We have reported that β-diketone[4] was detectable with a high degree of sensitivity and the detection of carbonyl compounds[5] with methylmalonic acid has been developed. Recentry, a labeling reagent for carboxylic acid and amines, having tertiary amine moiety, was developed.[10] As the background noise increased with increasing pH, it is desirable for it to be derivatized to emit ECL at a low pH range.

In this paper, we report a new method for the detection of amines after derivatization. Sample amines were modified to acetoacetylamide-type derivatives using diketene. The derivatives were detected by reversed-phase HPLC with ECL using $Ru(bpy)_3^{2+}$.

Chemicals and standard solutions used were as follows. $Ru(bpy)_3Cl_2$ was obtained from Sigma (St. Louis, M.O., U.S.A.) and used without further purification. Diketene was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sample amines were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All of the other chemicals and solvents were of guaranteed grade. Water was de-ionized and distilled using a hard-glass vessel. A stock standard solution (100 mM) of the amines was prepared by dissolving them in methanol. A working solution was prepared by appropriate dilution of the stock solution before use with a mixture of water and methanol (1 : 1, v/v).

The derivatization procedure was as described below. Five ml of a mixture of 0.1 M borate buffer (pH = 7.0) and acetonitrile (1 : 1, v/v), 10 microliters of the sample amines and 10 microliters of diketene were added in a tube and mixed. The mixture was left to stand at room temperature for 5 min for aliphatic amines and 90 min for aromatic amines. Twenty microlitres of the reaction mixture was injected onto the HPLC apparatus. The ECL intensity was measured using a $Ru(bpy)_3^{2+}$ ECL detection HPLC system[4] consisting of an intelligent micropump (Model-301, FROM, Tokyo, Japan), a Rheodyne sample-injection valve (Cotati, C.A., U.S.A.), an ECL detector (COMET-3000KANAGAWA, Kawasaki, Japan) and a recorder (Chromatopac CR-5A, Shimadzu, Kyoto, Japan). During current controlled (galvanostatic) electrolysis, the current was maintained at 80 μA. These components were connected with stainless steel tubing (0.3 mm i.d.). The mobile phase was a mixture of 0.1 M acetic acid and acetonitrile (7 : 3, v/v), and the flow rate was 0.5 ml/min. The reagent solution was 0.1 M sulfuric acid containing 0.3 mM $Ru(bpy)_3Cl_2$, and the flow rate was 0.3 ml/min. The column was a Spherisorb ODS 5 (150 mm × 0.4 mm).

The derivatization reaction for the primary amine with diketene is shown in Scheme 1.

$$R\text{-}NH_2 + (CH_2CO)_2 \rightarrow R\text{-}NHCOCH_2COCH_3$$

Scheme 1.

The conditions of the derivatization reaction, such as the pH, diketene concentration, reaction temperature and reaction time were optimized in terms of the ECL intensity of samples of cyclohexylamine and 4-chloroaniline. The effect of pH on the derivatization reaction was investigated over a range of pH values from 6.0–13. In the pH range 9.0–13, diketene was degraded to an ECL active substance that interfered with the measurements. The amount of degradation decreased with a decrease in the pH, and accordingly the pH 6.0–8.5 was perferred. The derivatization reaction of cyclohexylamine with diketene apparently occurred even in the pH range from 6.0 to 8.5 at room temperature. The reaction time and temperature was investigated (Figure 1). A higher temperature allowed the ECL intensity to develop slightly more rapidly. The ECL intensities reached a plateau at 5 min for cyclohexylamine and at 90 min for 4-chloroaniline. The effect of the concentration of the diketene was also investigated. The amount of derivative produced increased with increasing diketene concentration till it was over a three–fold molar excess with the amine, when it reached a plateau. The pH of the ECL reaction had an effect on the ECL intensity in the $Ru(bpy)_3^{2+}$ ECL system. The effects of pH on the ECL intensity and noise were investigated for a pH range from 1–2.5, which was controlled by the addition of sulfuric acid (Figure 2). The ECL intensity increased with decreasing pH, but the noise was found to be a minimum at pH 1.5. The inclusion of an organic modifier also affected the ECL intensity of all the amines investigated. It increased with increasing concentration of acetonitrile. The ECL intensity of amines was measured by FIA on an HPLC minus the column. The ECL intensity of amines is shown in Table 1. The intensity of n-

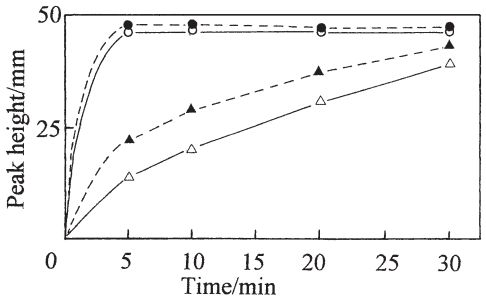

Figure 1. Effect of temperature and reaction time on reaction yield represented by peak height of cyclohexylamine at 20 °C (◯) and at 40 °C (●) and 4-chloroaniline at 20 °C (△) at 40 °C (▲).

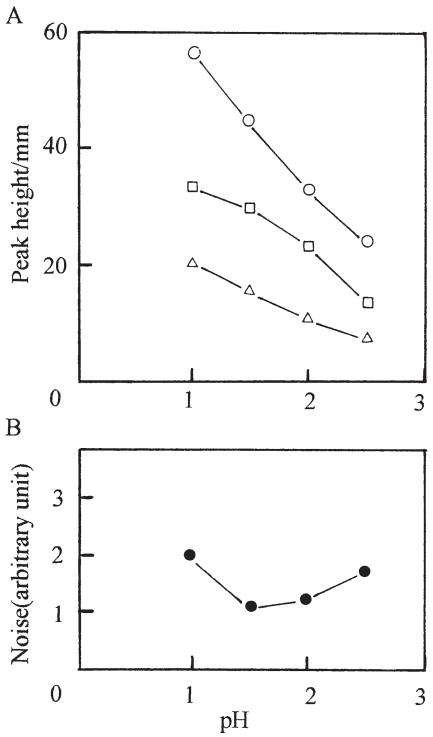

Figure 2. Effect of pH on (A) ECL intensity represented by peak height for cyclohexylamine (◯), 4-heptanamine (□) and heptylamine (△) and (B) noise.

Table 1. ECL intensity of derivatives of amines

Amine	ECL intensity (arbitrary unit)
Propylamine	224.1
Butylamine	161.8
Pentylamine	95.9
Hexylamine	80.0
Heptylamine	63.8
Cyclohexylamine	120.1
4-Heptanamine	76.6
Dipropylamine	875.0
Dibutylamine	658.0
Aniline	78.5
4-Chloroaniline	80.4
4-Methylaniline	78.0

alkylamines decreased with increasing alkyl chain length and the ECL intensity of a secondary amine, dipropylamine, was about four times as high as that of a primary amines, propylamine. Aromatic amines could be detected at the same sensitivity as alkylamines. AT pH 1.5, the ECL intensity of the tertiary and secondary amines are a very weak, therefore, it is thought that the ECL is based on the formed acetoacetylamide rather than the amines themselves.

HPLC using a pre-column derivatization technique was demonstrated. The derivatives were separated by HPLC (Figure 3). The linear range for cyclohexylamine was from 10 pmol to 20 nmol and detection limits (s/n = 3) were at a level of about 10 pmol for aliphatic and aromatic amines. The coefficients of variations (%, 5 nmol, n = 5) were 1.6 and 2.1 for cyclohexylamine and aniline respectively.

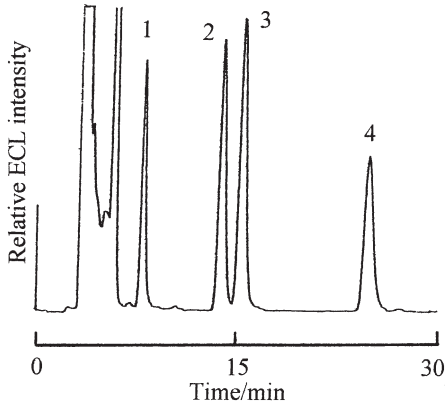

Figure 3. Chromatogram of the derivatives of aliphatic amines. Peaks (10 nmol each on column) 1 = cyclohexylamine; 2 = 4-heptanamine; 3 = hexylamine; 4 = heptylamine.

In conclusion, a detection method for primary and secondary aliphatic, and aromatic amines was developed. This method should prove to be useful for the selective and sensitive detection of amines.

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