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Indirect chemiluminescence detection of amino acids separated by capillary electrophoresis

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Abstract

An indirect chemiluminescence (CL) detection method for amino acids following capillary electrophoretic separation is described. Since amino acids can form stable complexes with Cu(II) and complexed Cu(II) is a much poorer catalyst for the CL reaction between luminol and H_2O_2, indirect detection is based on the measurement of the decreasing catalytic activity of Cu(II) in the presence of amino acids. The degree of CL suppression is proportional to the amino acid concentrations. The optimal conditions for the indirect CL detection were determined with regard to buffer composition and reagent concentration. However, the sensitivity of the indirect CL signal to buffer additives limits the buffer composition and, hence, the resolution of complex mixtures. Due to the sigmoid nature of the calibration curves, a limited working range ($\leq$2 orders of magnitude) was generally obtained. Detection limits for the unlabeled amino acids were in the 100–400 fmol range, depending upon the magnitude of the complex formation constants. Precision was between 3 and 6%. Applicability of this method to the analysis of amino acids in relatively simple samples was demonstrated.

Keywords: Detection, electrophoresis; Chemiluminescence detection; Amino acids; Luminol; Copper(II); Hydrogen peroxide

1. Introduction

During the past several years, capillary electrophoresis (CE) has been shown to be a fast, powerful and efficient analytical separation technique [1]. One of the major areas of research is the development of sensitive detection methods. Owing to the small capillary dimensions encountered and the minuscule sample zone generated in CE, on-column optical detection modes, such as UV absorption and fluorescence detection, are the most commonly used.

Chemiluminescence (CL) has been shown to be a highly sensitive detection method in both flow injection analysis (FIA) and liquid chromatography (LC) [2–4]. Due to its simple optical system and low background nature, CL is expected to be an ideal detection method for CE as well. Recently, the feasibility of using CL detection in CE has been successfully demonstrated [5–12]. Several CL reactions, such as the luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) [5–7], acridinium [8], and peroxyoxalate [9–12], have been utilized. Because only a few substances show the native CL characteristic, derivatization of non-CL analytes prior to detection is generally required. However, derivatizing processes are often tedious and time-consuming. Quantitative labeling of low-concentration analyte is also difficult [6]. On the other hand, if the non-CL analyte will interfere with or suppress a CL reaction, then the analyte can be indirectly detected as an

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inverted peak where the CL intensity decreases from a normally high background level.

It is well known that the luminol CL reaction can be catalyzed by several transition metal ions including Cu(II) [13]. Besides, Cu(II) can form stable complexes with many amino acids and proteins. Hara et al. [14] first developed the FIA–indirect CL detection procedure for proteins using the Cu(II)-catalyzed luminol CL system. Based on the formation of a Cu(II)–protein complex, which lowers the available amount of catalyst [viz., free Cu(II) ion], protein may then be indirectly detected from the decreased CL emission as a negative peak. A similar detection scheme has also been utilized in FIA and LC of amino acids [15,16] and proteins [17–19]. Indirect CL detection, to our knowledge, has not been applied to CE yet.

In a recent communication [20], we briefly described the results of a preliminary investigation on the CE–indirect CL detection. The Cu(II)-catalyzed luminol CL system was used for monitoring several unlabeled amino acids and peptide. In this paper, a detailed strategy is presented for the optimization of the overall system. Results for the sensitivity and linearity with this detection scheme are presented. In addition, feasibility and limitation of this method for routine analysis of amino acids are discussed.

2. Experimental

2.1. Apparatus

The CE–CL detection system used in this study was similar to that described by Dadoo et al. [5] with some modifications. A schematic diagram of the apparatus is shown in Fig. 1. The capillary used for separation was 50 μm I.D., 150 μm O.D. and 50 cm long (Polymicro Technologies, Phoenix, AZ, USA). A 0–30 kV power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) provided the separation voltage.

The interface for the CE–CL detector was constructed by inserting a 3-cm section of one end of the electrophoretic capillary directly into the reaction/detection capillary which was 180 μm I.D., 360 μm O.D. and 30 cm long. The two capillaries were held in place by a PEEK (polyether ether ketone) tee connector (Upchurch, Oak Harbor, WA, USA). The $CuSO_4$ solution needed to catalyze the CL reaction was delivered by gravity through a reagent capillary of 180 μm I.D., 360 μm O.D. and 100 cm long. The outlet of the reagent capillary was also led to the tee connector. The end of the reaction/detection capillary was immersed in a grounded buffer reservoir to complete the CE electrical circuit.

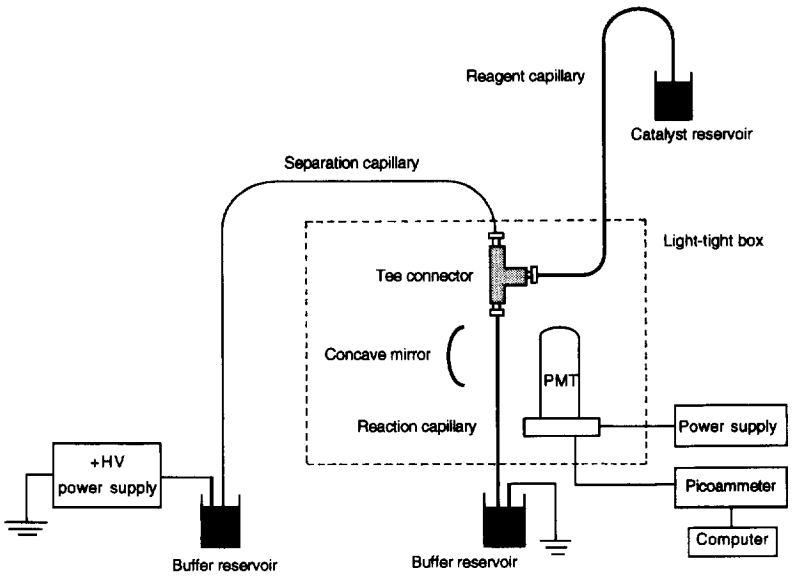

Fig. 1. Schematic diagram of the CE–CL detection system. PMT=photomultiplier tube; HV=high voltage.

A 5-mm detection window was formed on the reaction capillary (starting at the point where the inner electrophoretic capillary terminated) by burning off the polyimide coating. The detection window was situated at the focal point of a 2.5-cm diameter concave radius mirror (Edmund Scientific, Barrington, NJ, USA). The CL light emitted from the capillary window and collimated by the radius mirror were collected with a R-928 photomultiplier tube (PMT) (Hamamatsu, Hamamatsu City, Japan) 1 cm in front of the detection window. The PMT was operated at −900 V. The photocurrent was fed to a picoammeter (Model 485; Keithley Instrument, Cleveland, OH, USA) and the signal was recorded using a Macintosh SE computer equipped with a data acquisition interface. The whole CL detection system was held in a light-tight box constructed from black Plexiglas to avoid stray light.

2.2. Reagents

Luminol and 30% H_2O_2 solution were purchased from Fluka (Buchs, Switzerland). Amino acids were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade. Water purified by a Barnstead NANOpure II system (Dubuque, IA, USA) was used for all solutions.

2.3. Procedure

Electrophoretic separation was performed in 15 m*M* carbonate buffer (pH 10.0) containing 5 m*M* luminol and 25 m*M* H_2O_2. A catalyst solution of 30 μM $CuSO_4$ was prepared in 15 m*M* carbonate buffer which also contains 30 μM tartaric acid. All solutions were filtered through a 0.45-μm pore-size membrane filter before use. The separation capillary was initially filled with the electrophoretic buffer using a syringe. The reagent capillary and the tee connector were also filled with the catalyst solution by a syringe. The catalyst reservoir was placed 40 cm above the buffer reservoirs during the analysis. A high voltage was first applied for a short period, until the measured background CL intensity stabilized. The sample solution was then electrokinetically injected. In order to avoid siphoning air into the electrophoretic capillary, the catalyst reservoir was lowered to the same level as the buffer reservoirs during injection. The separation voltage was resumed after lifting the catalyst reservoir to the fixed height, and the detection system was turned on again for monitoring.

3. Results and discussion

The luminol–H_2O_2 CL reaction is known to be catalyzed by Cu(II) [2,13]. Besides, complexed Cu(II) is generally a much poorer catalyst than the uncomplexed form of Cu(II). The feasibility of indirect CL detection for CE of amino acids based on suppression of catalysis is demonstrated in Fig. 2. In the absence of amino acids, the Cu(II) catalyst was continuously mixed with the luminol and H_2O_2 in

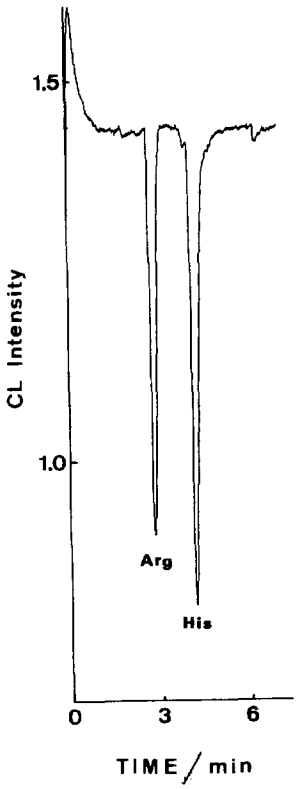

Fig. 2. Electropherogram of arginine and histidine with indirect CL detection. Conditions: separation capillary, 50 μm I.D., 50 cm long; buffer, 15 m*M* carbonate containing 5 m*M* luminol and 25 m*M* H_2O_2 (pH 10.0); separation voltage, 21 kV; catalyst, 30 μM $CuSO_4$; sample injection, 2 s at 21 kV; sample concentration, 1 m*M* of each analyte.

the reaction/detection capillary, resulting in a high, constant CL background emission. The observed decrease of the CL intensity in the presence of amino acids is attributed to the lowering of the catalytic activity of Cu(II) due to postcolumn formation of Cu(II)–amino acid complexes.

The luminol CL reaction requires an alkaline pH, preferably pH 9 to 11 [2]. The selection of an electrophoretic buffer which is compatible with the detection reaction is important in the development of the method. If the separation of amino acids can be carried out at an alkaline pH, then the separation and detection are readily compatible. Among the electrophoretic buffers we tested (e.g. borate, phosphate, ammonium and carbonate), 15 m*M* sodium carbonate at pH 10.0 was found to be the optimal, judging from the stability of background CL emission and the detection sensitivity of amino acids.

Optimizing the background CL emission is critical because the sensitivity of the indirect detection increases with background intensity if the catalyst flow is held constant. The background CL intensity was found to increase with increasing H_2O_2 concentration in the electrophoretic buffer. However, bubble formation in the buffer reservoir, probably caused by electrolytic oxidation of H_2O_2 on the anodic electrode, became evident when the concentration of H_2O_2 was higher than 25 m*M*. Since it is difficult to degas the H_2O_2 solution and bubble formation in the capillary is problematic, a concentration of H_2O_2 higher than 25 m*M* should not be employed. The observed peak height of histidine as a function of the luminol concentration in a carbonate buffer containing 25 m*M* H_2O_2 is shown in Fig. 3. The optimal concentration ratio for luminol:H_2O_2 was found to be 5 m*M*:25 m*M*. This ratio is somewhat different from the optimal ratio (luminol:H_2O_2=10 m*M*:20 m*M*) observed in FIA and LC with luminol–indirect CL detection catalyzed by Co(II) [15]. One of the probable reasons is that in CE some H_2O_2 will be electrolytically consumed in the buffer reservoir, a higher than optimal amount of H_2O_2 in the electrophoretic buffer is therefore needed.

The detection sensitivity and the background noise as a function of catalyst concentration are shown in Fig. 4. The observed peak height of leucine increases with increasing Cu(II) concentration, reaching a

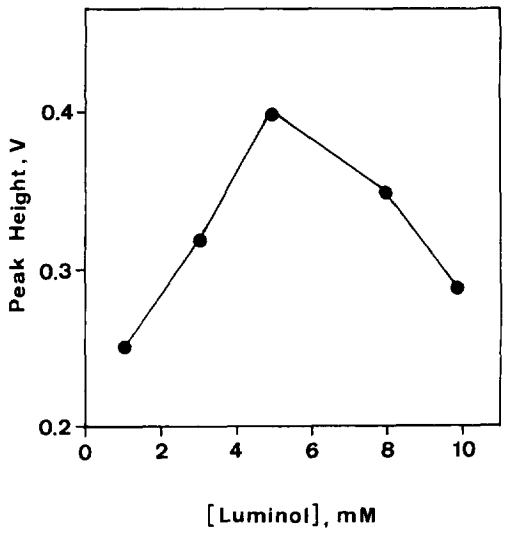

Fig. 3. Effect of the luminol concentration on the observed peak height of histidine in CE–indirect CL detection. Concentration of H_2O_2, 25 m*M*; analyte, 1 m*M* histidine; other conditions as in Fig. 2.

maximum at 0.1 m*M* Cu(II). However, the background noise level also increases with increasing Cu(II) concentration. From Fig. 4, the optimal Cu(II) concentration which generates the largest signal-to-

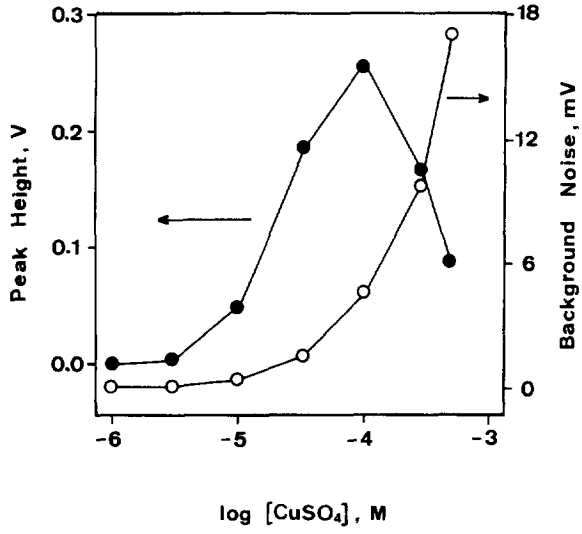

Fig. 4. Effect of catalyst concentration on the observed peak height of leucine and the background noise level in CE–indirect CL detection. Analyte, 0.5 m*M* leucine; other conditions as in Fig. 2.

noise (S/N) ratio is about 30 μM. In order to avoid formation of $CuCO_3$ or $Cu(OH)_2$ precipitate in the carbonate buffer (pH 10.0), which will interfere with the CL detection, a small amount (ca. 30 μM) of tartaric acid was included in the Cu(II) solution as an auxiliary complexing agent which complexes the Cu(II) until amino acid is introduced. The stability constant of the Cu(II)–tartrate complex ($\log K_1 = 3.2$) is much smaller than those of the Cu(II)–amino acid complexes ($\log \beta_2 = 14 \sim 17$ [21]), therefore, the tartrate ligand can be easily displaced by amino acid. The catalytic activity of Cu(II) was obtained with good reproducibility in the presence of tartaric acid without producing precipitate.

Fig. 5A shows the electropherogram of 10 amino acids with indirect CL detection under the optimal condition. Unfortunately, the best buffer composition for indirect CL detection may not be that needed for the optimal CE separation. Complete separation of the 10 amino acids was not achieved. Attempts to improve the resolution by adding organic modifiers into the electrophoretic buffer were not successful. As an example, Fig. 5B shows the electropherogram obtained by using the same buffer as in Fig. 5A but also containing 50 mM α-cyclodextrin (α-CD) and 5% methanol. Resolution was somewhat improved but at the expense of sensitivity, as evidenced by the scale difference between the ordinates of Fig. 5A and B. Besides, a system peak appears in Fig. 5B, but not in Fig. 5A. One cause for the poorer detection sensitivity is the increased buffer viscosity in the presence of α-CD, which retards the transportation of appropriate amounts of luminol and H_2O_2 to the reaction zone. A lower background CL intensity as well as a smaller peak size are the results. The system peak was probably caused by the slight mismatch between the composition of sample solution (without methanol) and electrophoretic buffer (containing 5% methanol). The migration velocity of this peak corresponds to that of the electroosmotic flow (EOF). The sensitivity of the indirect CL signal to buffer additives limits the buffer composition and, hence, the resolution of complex mixtures. A similar difficulty has been reported by Zhao et al. [6] in CE–direct CL detection of isoluminol thiocarbamyl-labeled amino acids. Poor resolution was also caused by low efficiency of the CE–CL detection system used here. The theoretical plate counts for the amino

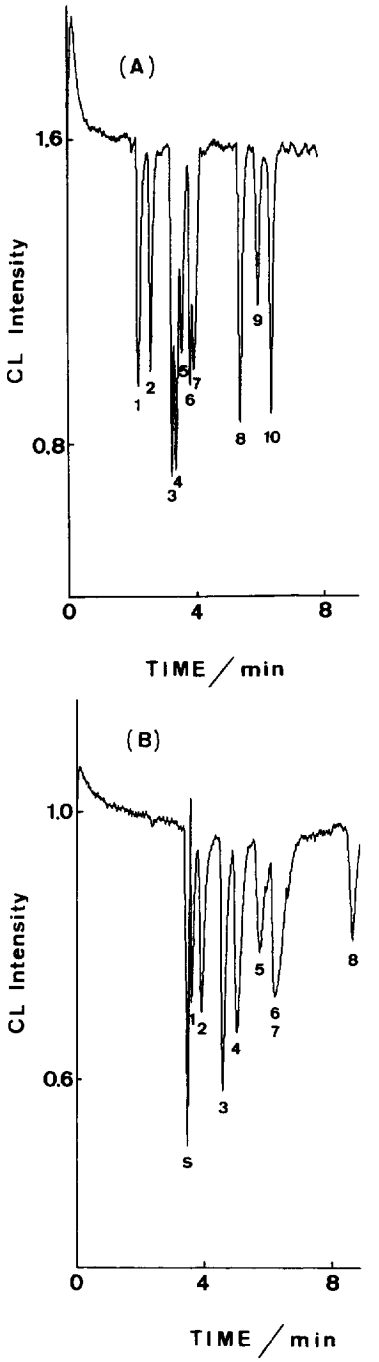

Fig. 5. Electropherogram of 10 amino acids with indirect CL detection. (A) Electrophoretic buffer: 15 mM carbonate–5 mM luminol–25 mM H_2O_2, pH 10.0; (B) same as (A), but also containing 50 mM α-CD and 5% methanol. Other conditions as in Fig. 2. Peak identities: 1=arginine, 2=proline, 3=valine, 4=leucine, 5=glutamine, 6=asparagine, 7=serine, 8=cysteine, 9=glutamic acid, 10=aspartic acid, s=system peak.

acids investigated are generally less than 5000. This poor efficiency was presumably due to turbulence created at the end of the separation capillary [7]. Separation of all 20 common amino acids by the present method is not possible.

The calibration curves for the 10 amino acids investigated are shown in Fig. 6. These curves are all nonlinear and are sigmoid in shape. The sigmoid nature of the calibration curve in indirect CL detection has been discussed by Nieman [2,16]. The maximum peak size is obtained when essentially all of the Cu(II) is in the complexed form; higher analyte concentrations yield the same peak height. As the analyte concentration decreases, the working curve should follow a curve defined by the fraction of free Cu(II) vs. analyte concentration. This curve is nonlinear and is sigmoid if the analyte concentration axis is logarithmic. In Fig. 6, the working curves are all similar in shape. The slight difference on the relative response to different analytes is explained when the relative magnitudes of the complex formation constants are investigated. The analyte detection limit is then determined by the smallest change in the Cu(II) concentration which is measurable [16]. Table 1 gives the linear ranges, complex formation constants and the detection limits for the 10 amino acids. Although the linear working range of the calibration curve is rather limited (≤2 orders of magnitude), the linearity is good ($r \geq 0.99$). The detection limit was determined from the peak height which equals 3 times the background noise level ($S/N=3$). This peak size corresponds to the smallest change in the Cu(II) concentration which is experimentally measurable. The detection limits range from 120 fmol (proline) to 440 fmol (glutamine). In general, the larger the formation constant the lower the observed detection limit. The detection limits found in this work are approximately 3 orders of magnitude lower than those obtained in LC of amino acids with luminol-indirect CL detection [16], but about 2 orders of magnitude higher than those reported in CE of labeled amino acids using direct CL detection [6]. The precision on peak height was about 3–6% with an injection amount of 3 pmol. A similar precision level was obtained with peak area measurement.

Given the limited range of separation buffers

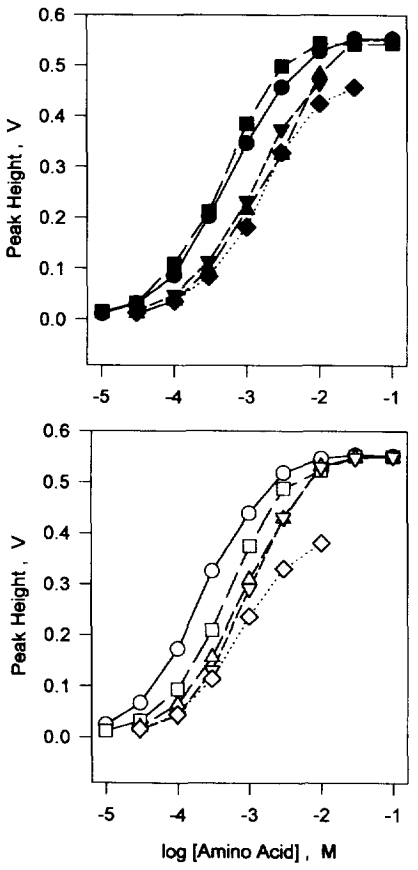

Fig. 6. Calibration curves of 10 amino acids. Symbols: ●=arginine, ■=leucine, ▲=serine, ▼=cysteine, ◆=aspartic acid, ○=proline, □=valine, △=glutamine, ▽=asparagine, ◇=glutamic acid.

Table 1
Calibration data, detection limits and complex formation constants (log β_2 with Cu(II)) for the 10 amino acids investigated

Amino acid	Linear range (mM)	r^a	log β_2[b]	LOD[c] (fmol)
Arginine	0.1–3	0.990	14.57	150
Proline	0.03–3	0.994	16.40	120
Valine	0.1–3	0.989	14.90	150
Leucine	0.1–3	0.990	14.90	140
Glutamine	0.1–10	0.997	14.23	440
Asparagine	0.1–10	0.995	14.42	300
Serine	0.1–10	0.991	14.48	270
Cysteine	0.1–3	0.993	–	280
Glutamic acid	0.1–3	0.991	14.16	440
Aspartic acid	0.3–3	0.987	15.53	200

[a]Correlation coefficients.
[b]Source: Ref. [21].
[c]Limit of detection; $S/N=3$.

which can be used and the sigmoid nature of calibration curves, the present method will be useful only for relatively simple samples. As an example, the method was applied to the determination of glutamate content in a commercial health drink. Monosodium glutamate (MSG) is commonly used as a flavour enhancer in foods and drinks. Fig. 7 shows a typical electropherogram for the CE separation and indirect CL detection of glutamate in a health drink. Due to the limited working range of the calibration curve, an appropriate dilution of the sample which adjusts the concentration of glutamate into the linear calibration range is important. The sample was diluted 4:1 with the electrophoretic buffer and was filtered through a 0.45-μm membrane filter before analysis. The standard addition method was used for quantitative calibration. Based on peak height measurement and 4 replicate analyses, the glutamate concentration was found to be 0.58 ± 0.03 mg ml^{-1}, which compares favourably with the labeled MSG content, 0.55 mg ml^{-1}. Similar R.S.D. (~6%) was found with peak area measurement, but less accurate results (0.62 ± 0.04 mg ml^{-1}) were obtained, probably due to tailing nature of the glutamate peak.

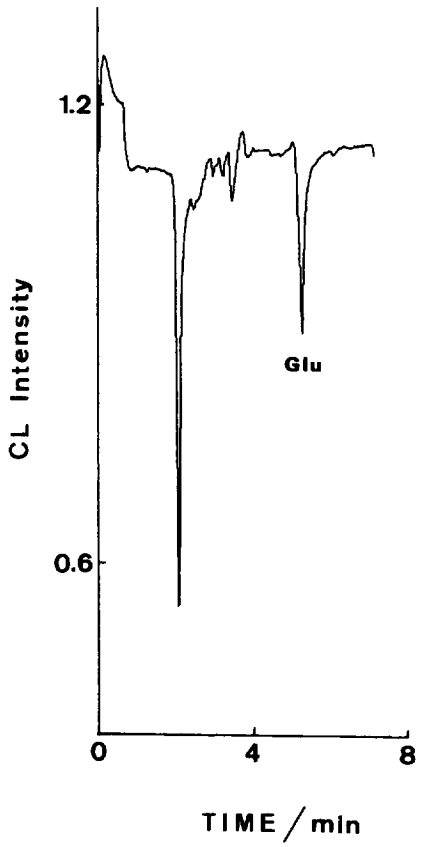

Fig. 7. Electropherogram of a commercial health drink. Sample diluted 4:1; other conditions as in Fig. 2.

4. Conclusion

We have demonstrated the feasibility of using the luminol-CL system for the indirect detection of unlabeled-amino acids following CE separation. In addition to its high sensitivity, the major advantage of indirect CL detection is that the tedious pre- or postcolumn derivatization of amino acids is not required. However, several drawbacks are also found in this new method, which include discrepancy between the buffer composition needed for the optimal CE separation and the best conditions for CL detection, limited working range of the calibration curve, and poor separation efficiency due to turbulent mixing at the end of the separation capillary. Some of these drawbacks are also found in various CE–direct CL detection systems [5–12]. Further studies, particularly on the design of an ideal interface between CE and CL detector, are needed in order to improve the performance of CE–CL detection. Applying indirect CL detection to CE of other organic species, e.g. catecholamines, is under study in our laboratory.

Acknowledgments

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