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Liquid chromatography with luminol-based electrochemiluminescence detection Determination of histamine

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Abstract

The liquid chromatographic determination of histamine was achieved by precolumn derivatization with N-(4-aminobutyl)-N-ethylisoluminol isothiocyanate and electrochemiluminescence detection. Detection was carried out using postcolumn on-line electrochemical reagent generation and oxidation of the derivatized analyte at a gold gauze working electrode built in the flow cell of the detector. With this on-line method a detection limit of 1.5 pmol ($S/N=3$) of histamine was obtained. The method was linear in the range 0.5–10 nmol and the repeatability of the method was satisfactory (R.S.D.=13.7% and 3.7% at the 0.5 and 5 nmol level, respectively). © 1998 Elsevier Science B.V.

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1. Introduction

Luminol-based chemiluminescence (CL) detection is the most widely used CL detection method, especially for immunoassays and flow injection methods. Recently there is an increasing interest in liquid chromatography (LC) combined with luminol-based CL detection. Luminol analogues which were used for derivatization of analytes for their determination by LC–CL are N-(4-aminobutyl)-N-ethylisoluminol (ABEI) for the determination of carboxylic acids and amines [1–5], 6-isothiocyanatobenzo[*g*]phthalazine - 1,4(2H,3H) - dione for amines [6,7], isoluminol isothiocyanate for amino acids [8] and 4,5- diaminophthalhydrazide for α-keto acids and α-dicarbonyls [9–13].

Normally, a rather complex LC system is needed for the determination of compounds derivatized by a luminol analogue, because the CL reagents, hydrogen peroxide and a catalyst should be added separately to the effluent, just before detection. In a previous study, instead of the postcolumn addition of hydrogen peroxide, the on-line electrochemical generation of hydrogen peroxide was investigated to simplify the LC system [14], which was used for the determination of carboxylic acids after derivatization with ABEI [5].

To create a fully on-line LC–CL system, electro-

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chemiluminescence (ECL) of luminol can be carried out. To generate an ECL reaction, oxidation of the compound is achieved at an electrode, after which light is emitted. A major advantage of ECL detection instead of normal CL detection is that the ''catalyst'', the electrode, is present on-line and no pumps are necessary for the addition of reagents.

ECL of luminol was first described by Harvey in 1929 for a batch system [15]. In the 1960s Kuwana and coworkers intensively studied the electrooxidation of luminol in the presence of oxygen [16–19]. In the 1980s, the first applications were reported by Haapakka and coworkers viz. for the determination of copper(II) and cobalt(II) using a rotating ring–disk electrode system [20,21]. More recently, the ECL of luminol was also used as detection mode in flowing systems, especially in flow injection analysis (FIA) for the determination of (generated) hydrogen peroxide [22–26] and hydroperoxides [27,28], but also in capillary electrophoresis for the determination of ABEI derivatized amines [29].

In this study the ECL of luminol is studied for use in LC. Hydrogen peroxide is generated on-line at a porous graphite electrode just before detection takes place, and the electrooxidation of luminol is effected by a three-electrode system placed in the flow cell of the detector. This LC–ECL method has been used to determine histamine derivatized by ABEI-ITC [N-(4-aminobutyl)-N-ethylisoluminol isothiocyanate]. Histamine, a biogenic amine, plays an important physiological and pathological role in various tissues and cells, and a sensitive analytical method is necessary to investigate its role. Histamine is only used as a test compound to study ECL detection in LC and the utility of the new label ABEI-ITC for derivatization of amines.

2. Experimental

2.1. Chemicals and solutions

Luminol (3-aminophthalhydrazide) and thiophosgene were purchased from Janssen (Beerse, Belgium), ABEI and histamine from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade quality.

Standard solutions of histamine were prepared in methanol. The solution of ABEI-ITC (0.3 m*M*) was prepared in a mixture of acetonitrile–water–freshly distilled triethylamine (TEA) (88:10:2, v/v/v).

2.2. Synthesis of ABEI-ITC

An amount of 110 mg ABEI (0.4 m*M*) was dissolved in 500 ml of a 0.1 *M* sodium carbonate solution. After the dissolution of ABEI, 100 μl of thiophosgene (1 mmol) was added and the mixture was stirred for 2.5 h at room temperature, during which time carbon dioxide was released. Subsequently, 100 ml of a 1.0 *M* hydrochloric acid solution was added to the yellow coloured solution in order to adjust the pH to about 1. The solution was transferred to a one liter separatory funnel, after which the product ABEI-ITC was extracted with 2×250 ml of ethyl acetate. The combined extracts were evaporated to dryness under vacuum at 30–35°C. The remaining fine yellow powder was dissolved in 4 ml of dimethylformamide and after the addition of 100 ml of ice-cold water, fine yellow crystals precipitated during overnight storage in the refrigerator at 4°C. After filtration (0.45 μm) the crystals were dried for 16 h under vacuum and stored at 4°C protected from light.

The identity of ABEI-ITC was confirmed by mass spectrometry (Finnigan Mat 90, Bremen, Germany) using desorption chemical ionization with ammonia as reagent gas. The label ABEI-ITC gave a signal at m/z 319 [M+H] (Fig. 1).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile–aqueous 10 m*M* sodium carbonate buffer, pH 10.5 (30:70, v/v) containing 5 m*M* tetraheptylammonium bromide (THAB, Aldrich, Milwaukee, WI, USA). The mobile phase was delivered by a gradient high-pressure system (Gilson Model 305, Villiers-le Bel, France), at a flow-rate of 0.8 ml/min, a laboratory-made six-port injector with a 100-μl sample loop which was used to introduce the samples, and a 5-μm Asahipak ODP-50 column (250×4.0 mm I.D.; Hewlett-Packard, Amstelveen, Netherlands) was used for separation. A laboratory-made column (10× 2.0 mm I.D.) packed with 10-μm PLRP-S material

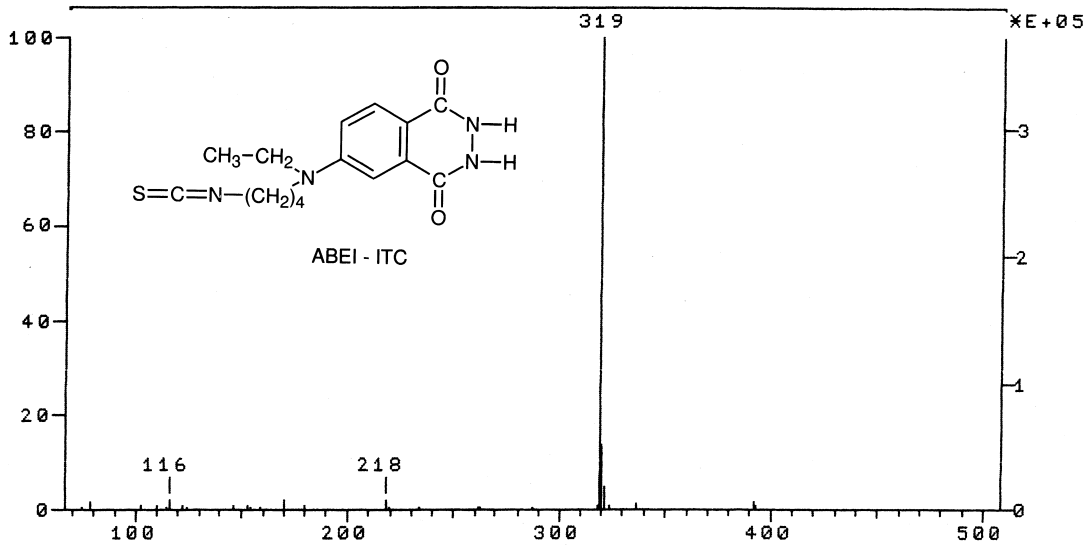

Fig. 1. Desorption chemical ionization mass spectrum of the label ABEI-ITC.

(Polymer Labs., Church Stretton, UK) was used as guard column.

After the elution of the derivatized histamine (ABEI-ITC-HIS) (at t_R=20 min) the mobile phase is switched to a mobile phase containing a higher percentage of acetonitrile (70%) without THAB to accelerate the elution of the excess of the label ABEI-ITC. After 6 min the mobile phase is switched back to the first one and the system is stabilized for 30 min before the next injection.

2.4. Detection system

The mobile phase exiting from the LC column was led through an ESA Coulochem guard cell, Model 5020 (Bedford, MA, USA) inserted between the column outlet and the detector. The electrochemical guard cell contains a porous graphite working electrode at which hydrogen peroxide is generated from the oxygen present in the mobile phase. The reference electrode was constructed from a proprietary material and was typically placed within a millimetre of the working electrode [14].

The second electrochemical reaction took place at a gold gauze (diameter=5.0 mm, d=0.3 mm) placed in a laboratory-made flow cell of polyether ether ketone (PEEK) (volume=37 μl) in the fluorescence detector (Spectroflow Model 970 or 980, Applied Biosystems, Foster City, CA, USA) which also contained a platinum wire (diameter=0.43 mm, length=6 mm) and a palladium/palladium monoxide electrode (Pd/PdO) (diameter≈0.6 mm, length=6 mm) as auxiliary and reference electrode, respectively, as shown in Fig. 2. Only the flow cell of the detector was changed; all other parts were standard. The potential of both working electrodes (−600 and +600 mV, respectively) was controlled by laboratory-made potentiostats.

To detect ECL, the lamp of the fluorescence detector was disconnected and the light generated at the electrode was directly recorded by the photomultiplier of the detector, which was operated at 900 V using a cut-off filter of 389 nm.

2.5. Voltammetric procedures

Voltammetric curves in relation to the current (i–E) and the ECL intensity (I_{ECL}–E) were obtained at ambient temperature by passing a 10 μM solution of luminol in a 10 μM carbonate buffer through the system with the LC column disconnected, i.e., in the FIA mode, at a flow-rate of 0.8 ml/min and scanning the potential (Model 175 Universal Programmer, Princeton Applied Research, Princeton, NJ, USA) at the gold working electrode from −0.80 to +0.80 V at a scan rate of 5 mV/s. The influence of the pH, the

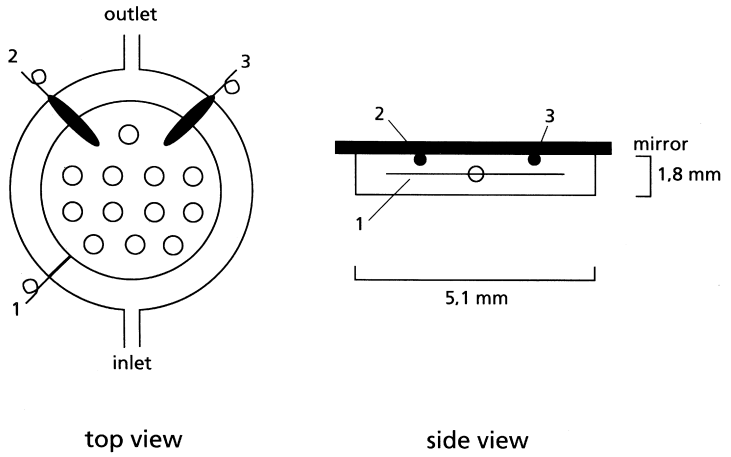

Fig. 2. Schematic presentation of the flow cell showing the gold gauze working (1), the Pd/PdO reference (2) and the Pt auxiliary (3) electrode.

percentage of modifier (methanol and acetonitrile), the scan range and the presence or absence of hydrogen peroxide (switching the ESA cell on/off) on the CL and current intensity was studied. The electrode potential was cycled until the residual curves were reproducible. Data were recorded using a Model BD30 *x–y* recorder (Kipp and Zonen, Delft, Netherlands). The current output for the *i–E* curves was 1 mA full scale, while the ECL output for the I_{ECL}–*E* curves was arbitrary; however in the latter instance the configuration of the detector remained the same and values of I_{ECL} can be compared with each other.

2.6. The Pd/PdO reference electrode

To manufacture the Pd/PdO reference electrode the method of Kinoshita et al. [30] was used. Therefore, a palladium wire was successively put for 20 s in aqua regia, demineralized water and a 50% sodium hydroxide solution. After drying by nitrogen the wire was placed for 20 min in an oven at 760°C. A black layer of palladium monoxide was formed on the wire. The potential of the Pd/PdO electrode, which is pH dependent, is about +250 mV in relation to the standard hydrogen electrode at pH 10.5 [30].

2.7. Derivatization procedure

One-hundred μl of a histamine solution was added to a 1.5 ml Model 3810 reaction vial (Eppendorf, Hamburg, Germany) and evaporated to dryness under nitrogen at ambient temperature. To the residue was added 100 μl of the ABEI-ITC solution. After 10 s of vortex mixing, the mixture was allowed to react for 1 h at 80°C. Next 600 μl of mobile phase were added to the reaction mixture and 100 μl of the resulting solution were injected into the LC system.

3. Results and discussion

For the LC system an on-line electrochemical flow cell (ESA Coulochem guard cell) equipped with a porous graphite working electrode was selected to reduce oxygen present in the mobile phase, to hydrogen peroxide, as was described in our previous work [5,14]. Because the electrode reaction of luminol is very fast, the electrode system which should effect the oxidation of luminol, was built in the flow cell of the detector.

Firstly, the influence of the nature of the electrode material of the working electrode and reference electrode was studied in the FIA mode. Therefore,

four wire electrodes were placed in the flow cell; one of these, the auxiliary electrode, was made of platinum. As reference electrode first of all an Ag/AgCl electrode, made by depositing a layer of AgCl on a silver wire, was used. To prevent dissolution of the AgCl layer, 100 m*M* of potassium chloride was dissolved in the carrier (10 m*M* carbonate buffer of pH 10.5). However, in this configuration, the I_{ECL} of luminol was not reproducible.

Replacing the Ag/AgCl reference electrode by a Pd/PdO electrode gave stable and reproducible signal intensities. The Pd/PdO electrode was therefore used in further measurements.

Two working electrodes placed in the flow cell, a gold and a platinum wire of about the same total area, were tested to find the best electrode material for the ECL of luminol. A 1 μ*M* solution of luminol was injected in the FIA system, with the ESA electrochemical flow cell being operated at −600 mV. The gold working electrode gave slightly better results (1.5- to 2-times higher I_{ECL} at +600 to +800 mV) than the platinum electrode, which was also found by Vitt et al. [31].

To enhance the efficiency of the oxidation of luminol a working electrode of gold gauze (diameter=5.0 mm, d=0.3 mm) was placed in the flow cell instead of the gold wire and the platinum working electrode was removed.

3.1. Voltammetric experiments

To investigate the influence of various parameters a carrier stream of the 10 m*M* carbonate buffer of pH 10.5, containing 10 μ*M* luminol was recirculated through the FIA system and the potential at the gold working electrode was scanned. In the basic carrier stream luminol is present as the luminol monoanion. Both I_{ECL} and the current (i) were measured as a function of the potential in all experiments.

First the influence of the scan range was investigated by cycling the potential between −0.80 and +0.60, +0.70 or +0.80 V, respectively. During the positive scan two peaks were observed for the ECL measurements, which were shifted during the negative scan (Fig. 3). The first ECL peak was of low intensity; it was observed at +0.02 V during the positive scan and shifted to about −0.15 V during the negative scan, independent of the scan range. This ECL peak is possibly caused by the reaction of the superoxide anion (O_2^-) and the luminol monoanion, which generates light. The presence of the superoxide anion is caused by the oxidation of the hydrogen peroxide anion (HO_2^-), which is formed at the electrode of the ESA cell in front of the detector flow cell [14].

A second, larger ECL peak was observed during the positive scan at a potential of +0.34 V. During the negative scan the potential was dependent on the scan range: for the scan range ending at +0.60 V the peak was observed at +0.42 V; it shifted to +0.48 and 0.52 V when the scan ranges ending at +0.70 and 0.80 V, respectively. The ECL intensity was higher for the longer scan ranges. These ECL peaks are caused by the oxidation of the luminol monoanion to the luminol radical at the gold working electrode, with the CL reaction subsequently taking place in the carrier stream as the result of reaction of the radical with an oxidator like oxygen or the superoxide or the hydrogen peroxide anion. The peak height difference and the shift of the ECL peaks are caused by the formation and removal of the oxide layer on the gold electrode during the scan. The oxidation of the gold electrode (peak potential at +0.80 V) is a reversible reaction; the reduction peak was observed at about +0.23 V. The oxidation of luminol, however, is a totally irreversible electron reaction. Both results are comparable with those reported by Haapakka and Kankare [32].

The influence of oxygen and the hydrogen peroxide anion was studied by switching the ESA electrochemical flow cell off or on. The I_{ECL}–E curves all had the same shape and differed only in intensity: at the peak potential, I_{ECL} was about 3-, 8- or 30-times higher with the ESA cell on than with the ESA cell off, at pH 10.5, 11.5 or 9.5, respectively. This means that, firstly, the ECL reaction is more efficient in the presence of the hydrogen peroxide anion than of oxygen. Secondly, the pH dependence of the ECL luminol reaction in the presence of oxygen obviously is different from the reaction in the presence of hydrogen peroxide. This can be explained by the fact that only the basic form of hydrogen peroxide (pK_a= 11.65) leads to ECL. The pH also affects the ECL luminol reaction because of the fact that the basic

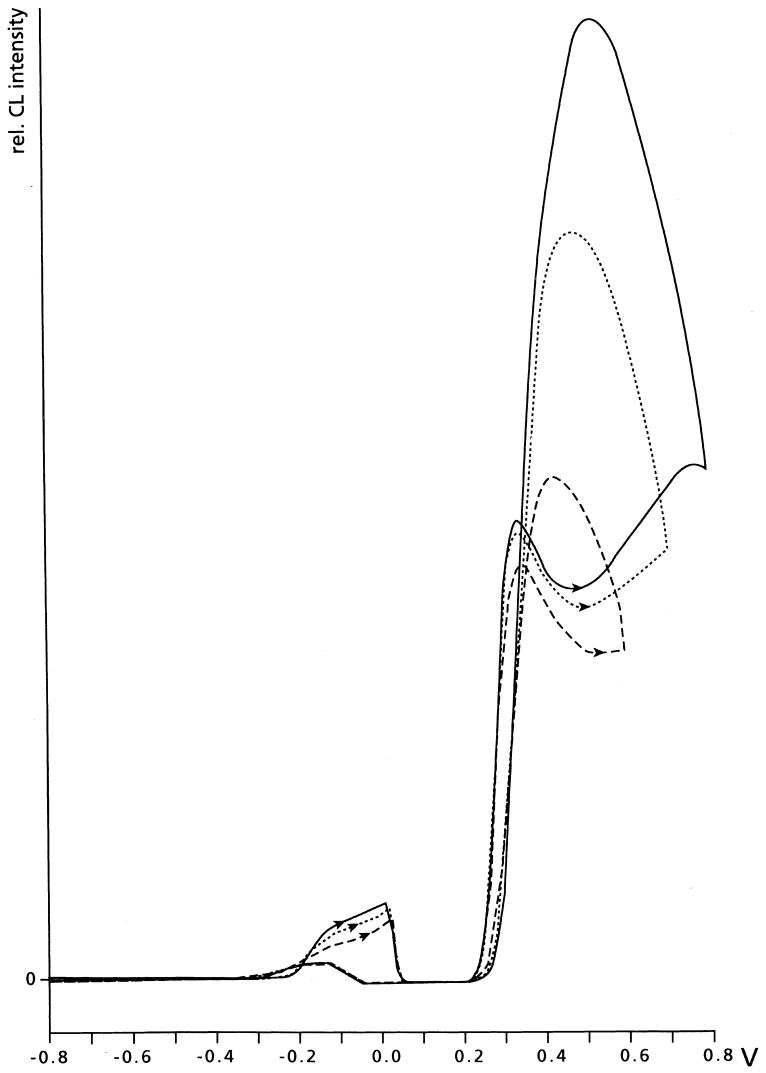

Fig. 3. I_{ECL}–E curves of luminol (10 μM) in a 10 μM carbonate buffer at a flow-rate of 0.8 ml/min at various scan ranges: from −0.80 to +0.60 (- - -); from −0.80 to +0.70 (· · ·); from −0.80 to +0.80 V (———). Scan rate, 5 mV/s.

form of the luminol intermediates (pK_a=11.1–12.1) leads to CL, and that the fluorescence quantum yield of 3-amino phthalate, which is the emitter of the CL reaction, decreases strongly above pH 11 [32].

The influence of the pH value is shown in Fig. 4. On going from pH 9.5 to 11.5 the peak potential shifts from +0.48 to +0.57 V. However, the optimum I_{ECL} is found at pH 10.5, which is in agreement with our previous study on the electrogenerated CL of luminol by using the ESA cell and microperoxidase as the catalyst [14].

The addition of a modifier to the carrier stream caused a decrease in I_{ECL}, but no shift in peak potential: the effect of methanol was much higher than that of acetonitrile (e.g., 20% modifier decreased I_{ECL} by a factor of about 80 for methanol and by a factor of 8 for acetonitrile). This effect was also found by Sakura [25]. Possibly, the reason of

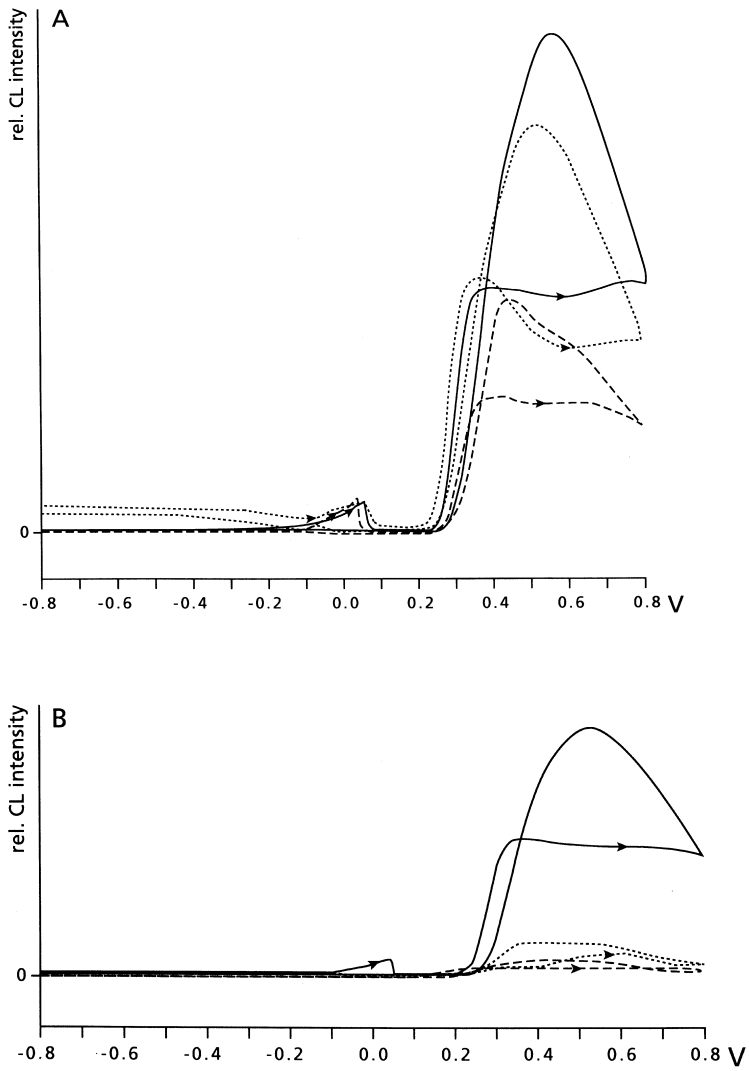

Fig. 4. I_{ECL}–E curves of luminol (10 μM) in a 10 μM carbonate buffer at a flow-rate of 0.8 ml/min at various pH values: 9.5 (· · ·); 10.5 (———) and 11.5 (- - -). Scan rate, 5 mV/s. (A) ESA cell on, (B) ESA cell off.

the dramatic decrease is that radicals, which are involved in the ECL reactions, are scavenged more easily by methanol than by acetonitrile.

3.2. Determination of histamine

The determination of histamine was selected to test the LC–ECL system. Therefore, histamine had to be derivatized with a luminol analogue. Derivatization of primary amines can be performed with many reagents e.g., sulphonyl halides, aryldialdehydes, chloroformates and isothiocyanates. In this study the isothiocyanate ABEI-ITC was used as the label for the derivatization of histamine. The conversion of ABEI to an isothiocyanate is a straightforward reaction which can be carried out by adding thiophosgene which is, however, extremely toxic.

Derivatization of amines by an isothiocyanate

should be carried out in a medium-alkaline environment. Spurlin and Cooper [8] used a solution of TEA in water to derivatize amino acids with isoluminolisothiocyanate. In this study acetonitrile was added to this mixture to dissolve ABEI-ITC. The optimum reaction mixture was found to be acetonitrile–water–TEA (88:10:2, v/v/v).

The derivatization yield at a reaction temperature of 80°C was about 90% after 30 min. In all experiments a 45-min reaction time was used to achieve nearly complete derivatization.

The chromatographic separation of ABEI-ITC-HIS from the excess of the ABEI-ITC label was first studied on a polystyrene–divinylbenzene polymer column (5 μm PLRP-S, 250×4.6 mm), which was also used in our previous studies [5,14]. However, Asahipak ODP-50, a poly(vinyl alcohol)-based polymeric column gave better efficiency than the PLRP-S column. Polymer columns offer the advantage that they can be used under the alkaline conditions is necessary for the luminol-based CL detection.

In order to increase the retention of ABEI-ITC-HIS, tetraalkylammonium bromides were added to the mobile phase, with THAB (5 m*M*) giving the best results for the separation of the compound and interfering components. The tetraheptylammonium cations form ion-pairs with both ABEI-ITC-HIS and ABEI-ITC, which causes an increase of their retention. THAB had no negative effect on the ECL detection.

A mobile phase containing 70% of a 10 m*M* carbonate buffer and 5 m*M* THAB was necessary to achieve a complete separation of ABEI-ITC-HIS from early eluting interfering compounds. However, with this mobile phase the retention time of ABEI-ITC was about 110 min and the total run time about 2 h. To decrease the total run time, the mobile phase was changed to a solution of 70% acetonitrile in water after the elution of ABEI-ITC-HIS (t_R=20 min). This caused a 2-fold increase of the speed of analysis. Typical chromatograms are shown in Fig. 5.

The linearity of the LC–ECL determination of histamine was investigated at seven levels (0.5–50 nmol histamine derivatized). Above an amount of 10 nmol of histamine there was no linear relation between amount and I_{ECL}, which means that at least a 3-fold excess of the ABEI-ITC is necessary for an

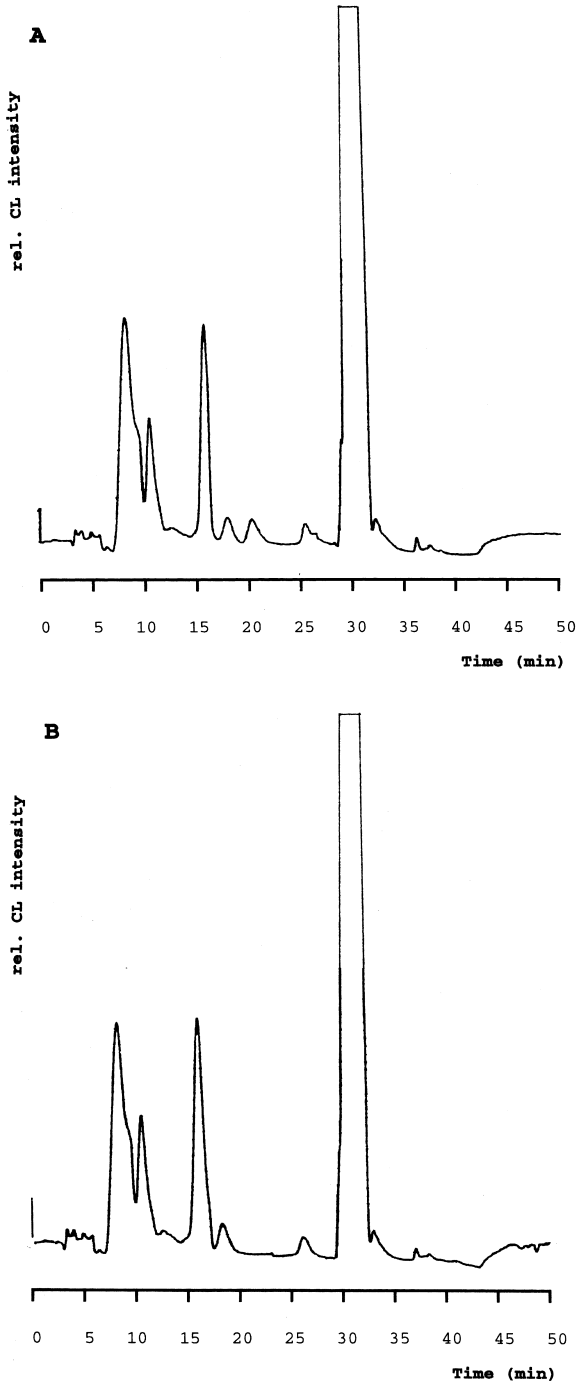

Fig. 5. LC–ECL chromatograms obtained (A) after derivatization of histamine at an amount of 0.4 nmol (0.06 nmol injected) and (B) a blank. Conditions as described in Section 2.

optimal derivatization yield. The linear range until 10 nmol of histamine gave the formula $y=221.3x+0.2720$ ($r=0.9997$, $n=5$). The repeatability of the total procedure was investigated by derivatizing of 0.5 and 5 nmol histamine; the R.S.D. values were 13.7% and 3.7%, respectively ($n=5$). The limit of detection was 1.5 pmol histamine injected at a signal-to-noise ratio of three.

4. Conclusions

Luminol-based ECL detection offers the possibility for a fully on-line LC–CL detection system. In conventional LC–CL the CL reagents have to be added to the system postcolumn; in ECL, however, reactions take place at electrodes which are included in the system on-line. Consequently, no additional pumps are required.

With the present LC–ECL method histamine can be determined at the low pmol level with is comparable with the results typically obtained in LC with fluorescence detection using o-phthaldialdehyde as the pre-, post- or oncolumn derivatization reagent [33–35], but up to 10-fold less efficient than other CL methods [36–38]. However, the present method uses a very simple derivatization reaction for both primary and secondary amines, and no postcolumn addition of reagents is necessary, which is a distinct advantage over conventional CL methods.

The sensitivity for amines, which do not have an acidic function like histamine will be better, because in that case a mobile phase without an ion-pairing reagent can be used. The result will be that the derivative will eluted after the excess of the label. The label ABEI-ITC and most of the side-products of the derivatization reaction, which have about the same retention as the histamine derivative, will be eluted in the dead volume.

In this study a new flow cell was constructed which houses a gold gauze as working electrode. However, the volume of the flow cell and the area of the electrode have not yet been optimized. It is our expectation that the luminol-based ECL detection can be made more sensitive by increasing the volume of the flow cell and the area of the working electrode [29].

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