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# Short Communication

# Primary and secondary amine derivatization with luminarins 1 and 2: separation by liquid chromatography with peroxyoxalate chemiluminescence detection

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ABSTRACT

Luminarins 1 and 2 are labelling reagents with a quinolizinocoumarin structure and an N-hydroxysuccinimide ester reactive function. They react with primary and secondary amines under relatively mild conditions (50–80°C, 20–180 min) without a catalyst and yield fluorescent and chemiluminescent derivatives. Luminarin 1 was more reactive than luminarin 2, but required an anhydrous medium to perform the derivatization. Small alkylamines were derivatized and separated by reversed-phase liquid chromatography. The fluorescence detection limit was 100 fmol injected. The limit of detection with peroxyoxalate postcolumn chemiexcitation was in the low femtomole range. Both methods were used to measure histamine with luminarin 2. Linearity of derivatization was obtained for amounts of histamine ranging from 0.5 to 5 nmol. The possibility of chemiluminescence detection in highly aqueous mobile phases (76%) was demonstrated. The results compared favourably with those of *o*-phthalaldehyde–mercaptoethanol derivatization of histamine.

## INTRODUCTION

Precolumn derivatization and chemiluminescence detection by high-performance liquid chromatography (HPLC) have been developed for the determination of trace amounts of analytes. When the analyte contains an amine functional group, several chemiluminescence derivatization reagents, such as dansyl chloride [1], fluorescamine [2], 4-chloro-7-nitrobenzo-1,2,5-oxadiazole [3], *o*-phthal-

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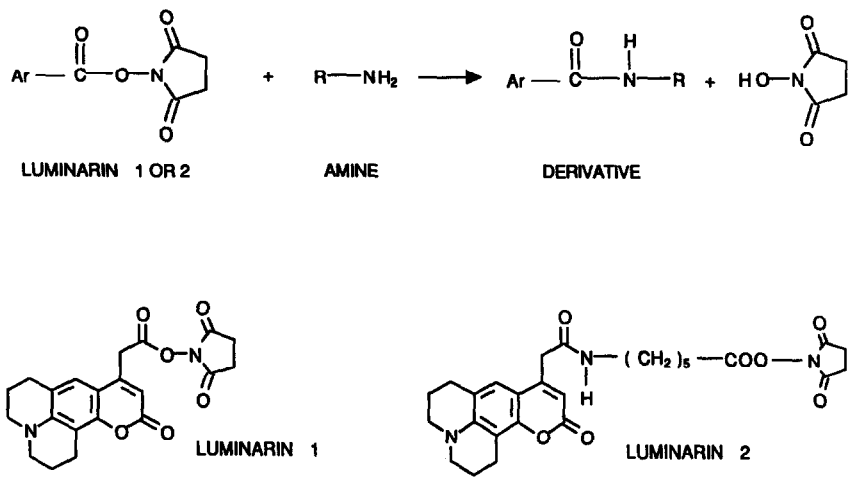


Fig. 1. Derivatization of amines by luminarins 1 and 2.

aldehyde [3], naphthalene- and anthracene-2,3-dialdehyde [4] and N-(4-aminobutyl)-N-ethylisoluminol [5] can be used.

Aminocoumarin derivatives are excellent chemiluminogenic reagents for many compounds, such as fatty acids [6] and fluoropyrimidines [7]. In previous papers we described the development of the luminarins, which are labelling reagents derived from quinolizinocoumarin [8–10]. This nucleus offers the advantage of a high chemiluminescence yield in various solvents, which results in low limits of detection (LOD) in normal- or reversed-phase chromatography. In this study, the ability of luminarins 1 and 2 (Fig. 1) to derivatize primary and secondary amines was investigated. Both reagents have an N-hydroxysuccinimide ester reactive function, which was chosen for its reactivity with [11,12] and its selectivity towards amines [13]. Alkylamines were studied as model compounds and an application to histamine was developed.

## EXPERIMENTAL

### *Reagents*

Pentylamine, nonylamine, dipropylamine, dibutylamine, bis-(2,4,6-trichlorophenyl)oxalate (TCPO) and bis-(2,4-dinitrophenyl)oxalate (DNPO) were purchased from Fluka (Buchs, Switzerland), histamine from Calbiochem (Meudon, France), luminarins 1 and 2 from Eurobio (Les Ulis, France), *o*-phthalaldehyde (OPT) and dimethylaminopyridine (DMAP) from Sigma (L'Isle d'Abeau, France) and hydrogen peroxide (30% aqueous solution) from Janssen (Beerse, Belgium). Imidazole, molecular sieves (0.3 nm) and solvents (UV or fluorescence grade) were purchased from Merck (Darmstadt, Germany). Stock solutions of luminarins 1 and 2 (0.01 *M*) were prepared in tetrahydrofuran (THF) previously dried with molecular sieves, then diluted as required and kept at −20°C.

### *Instrumentation*

For fluorescence measurements, a Chromatem 380 pump (Touzart et Matignon, Vitry, France), a Rheodyne Model 7125 injector with a 20-μl sample loop, a Shimadzu RF 530 fluorescence detector and a Shimadzu CR3A integrator (Touzart et Matignon) were used.

For chemiluminescence measurements, a Shimadzu CTO-6A column oven (Touzart et Matignon) set at 30°C and a Kratos URS051 postcolumn reactor including two pumps (Applied Biosystems, Rungis, France) were added, and the fluorescence detector was a Kratos FS970 (Applied Biosystems). With mobile phases containing a high proportion of acetonitrile (*e.g.*, 70%), the reagents and the eluate were mixed by flowing through a 60-μl PTFE capillary tube containing glass beads (Supelco, France) connected to a 120-μl delay coil placed in the col-

umn oven. With mobile phases containing a low proportion of acetonitrile (*e.g.*, 26%), TCPO and hydrogen peroxide were mixed using a 292-$\mu$l capillary tube placed in the oven, and a 60-$\mu$l capillary tube was used for mixing these reagents with the eluate before entering the detector.

*Chromatographic conditions*

For fluorescence measurements of alkylamine derivatives, an Ultrasphere ODS-2 (5 $\mu$m) column (250 × 2 mm I.D.) (Beckman, Les Ulis, France) was used and the mobile phase was acetonitrile–10 m*M* imidazole nitrate buffer (pH 7.0) (70:30, v/v) pumped at 0.3 ml/min. The excitation and emission wavelengths were 390 and 490 nm, respectively. For chemiluminescence detection, a 4.6 mm I.D. but otherwise identical column was used and the flow-rate was 1 ml/min. A 0.2 *M* hydrogen peroxide solution in THF and a 10 m*M* TCPO solution in methyl acetate were pumped at 0.25 ml/min each. The column oven was kept at 30°C and a 470-nm long-pass filter was used.

For fluorescence measurements of histamine derivatives, a Nucleosil $C_{18}$ (5 $\mu$m) column (150 × 4.6 mm I.D.) (SFCC, Neuilly-Plaisance, France) was used with a mobile phase consisting of acetonitrile-5 m*M* ammonium acetate (26:74, v/v) pumped at 2 ml/min. The excitation and emission wavelengths were 390 and 490 nm, respectively, for histamine–luminarin derivatives and 350 and 440 nm respectively, for histamine–OPT derivatives.

For chemiexcitation of the histamine–luminarin 2 derivative, a 0.4 *M* hydrogen peroxide solution in THF and a 1.1 m*M* TCPO solution in methyl acetate were pumped at 0.3 ml/min each, while the eluate flow-rate was 1.5 ml/min and the oven was set at 40°C.

*Derivatization procedure*

Alkylamines were derivatized by luminarin 1 by mixing 0.1 ml of an amine solution containing 0.2–20 nmol in THF (primary amines) or dimethyl sulphoxide (DMSO) (secondary amines) with 0.1 ml of a 1 m*M* luminarin 1 solution in THF. The solutions were heated on a water-bath for 20 min at 50°C (primary amines) or 180 min at 70°C (secondary amines). Care must be taken to avoid trace amounts of moisture. Solvents were dried using 0.4-nm molecular sieves. Alkylamines were labelled with luminarin 2 by mixing 0.1 ml of an amine solution containing 0.2–20 nmol in DMSO with 0.1 ml of a 2 m*M* luminarin 2 solution in THF. The solutions were kept at 70°C for 60 min (primary amines) or at 80°C for 180 min (secondary amines). After the reaction, the samples were diluted 1:50 (v/v) in mobile phase, in order to inject derivative amounts between 0.4 and 40 pmol.

Histamine was derivatized by luminarin 2 by mixing 0.5–5 nmol of histamine with 50 nmol of luminarin 2 in 0.5 ml of acetone and 0.1 ml of a 0.1 *M* solution of DMAP in acetone. The solution was evaporated to dryness and left for 90 min in the dark. Acetone (0.2 ml) was added just before injection.

OPT–mercapthoethanol derivatization of histamine was performed as described by Gupta and Lew [14].

## RESULTS AND DISCUSSION

*HPLC analysis*

Pentylamine, dipropylamine, dibutylamine and nonylamine were derivatized and separated according to the procedure described above, as shown in Fig. 2. Resolution between the peaks of homologous derivatives was most often complete. For example, the resolution between dipropyl- and dibutylamine–luminarin 1 derivatives was 3.5.

Other amines, such as octylamine, benzylamine and phenylethylamine, were also successfully labelled with luminarin 1 (data not shown). Luminarin 2 derivatives were less retained than luminarin 1 derivatives. The structures of pentylamine–luminarin 1 and dipropylamine–luminarin 1 derivatives were confirmed by mass spectrometry [15].

*Derivatization conditions*

The mechanism of the derivatization reaction is well known [16]. A basic dipolar solvent should *a priori* be appropriate to drive this reaction, and THF was chosen. Previously [11,12], chloroform was used as the solvent and the reaction was catalysed with triethylamine. In this study, three catalysts were tested with THF as the solvent: pyridine, triethylamine and dimethylaminopyridine. Pyridine (10 $\mu$l) inhibited the reaction; triethylamine (10 $\mu$l) accelerated the reaction but resulted in the formation of by-products; DMAP (100 $\mu$l of a 0.1 *M* solu-

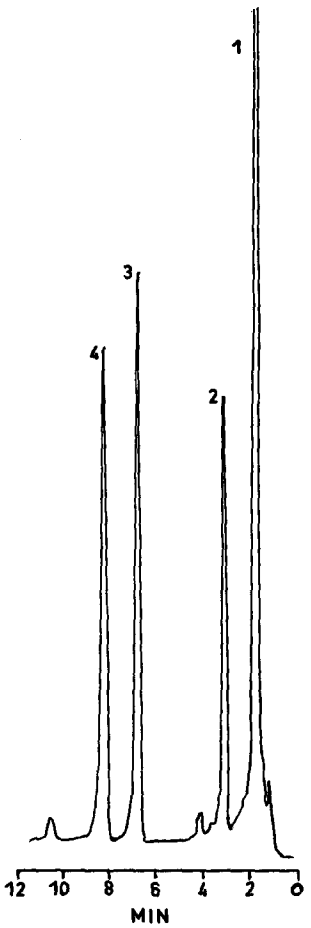


Fig. 2. Separation of luminarin 1 and 2 derivatives with fluorescence detection, 0.05 μA full-scale. Peaks: 1 = luminarin 1 (L1); 2 = pentylamine–L1; 3 = dibutylamine–L1; 4 = nonylamine–L2. 5 pmol of each derivative were injected on to the column.

tion) had no effect when THF was the solvent but increased the kinetics when histamine was derivatized by luminarin 2 in acetone or in the dry state. Accordingly, when a basic solvent such as THF is used, base catalysis has no effect. Otherwise, DMAP catalysis should be used.

Finally, alcohols as the solvent must be avoided, because they react with luminarins 1 and 2 to form the corresponding esters. This was verified with methanol, ethanol and isopropanol.

*Derivatization kinetics*

Kinetics of the pentylamine and dibutylamine reactions with luminarin 1 were determined at 50 and 70°C, respectively. The yield was measured by comparison with a standard obtained in a twofold molar excess of amine over luminarin 1. The reaction was complete (yield 95%) in 20 min for primary amines, or reached a plateau (yield 80%) in 180 min for secondary amines. As expected, the reaction was much slower with secondary amines, probably because of steric hindrance. The yield could not be improved by increasing the reaction temperature because of the degradation kinetics of luminarin 1 (see below).

Kinetics of the nonylamine and dibutylamine reactions with luminarin 2 were determined at 70 and 80°C, respectively, and the yield was measured as for luminarin 1. The plateau was reached in 60 min for primary amines (yield 88%) and 180 min for secondary amines (yield 75%). Hence the kinetics of the reaction were slower for luminarin 2 than luminarin 1.

*Hydrolysis kinetics*

The sensitivity of luminarins 1 and 2 to hydrolysis was examined, in order to determine if the reaction had to be carried out in an anhydrous medium. Hydrolysis was carried out in the reaction medium with 0.5% (v/v) water, at 50°C for luminarin 1 and 70°C for luminarin 2. Hydrolysis *vs.* time was almost linear and reached 50% in 180 min for luminarin 1 and 10% in 210 min for luminarin 2. A blank without water showed only a slight decrease. Thus, luminarin 2 is less reactive than luminarin 1 but also more resistant to hydrolysis.

*Detection and derivatization limits*

Limits of detection (signal-to-noise ratio = 3) of the pentylamine–luminarin 1 derivative with fluorescence or chemiluminescence detection was described previously [9] and were very similar for all other luminarin 1 and 2 derivatives: 100 fmol *vs.* 6 fmol injected for fluorescence *vs.* chemiluminescence detection, respectively. Hence, it should be possible to measure amounts of amines as small as 1 pmol (with fluorescence detection) if the derivatization has occurred. In fact, the derivatization limits were much higher, ranging from 200 pmol (primary amines with luminarin 1) to 500 pmol (secondary amines with luminarin 2). When the amount of amine was smaller, derivatization was poorly repro-

ducible and no linearity was observed. Hence, the limiting factor in the sensitivity of the method was not detection but derivatization. In this respect, fluorescence detection is valid for most applications, and chemiluminescence detection would be useful only when a greater selectivity of detection is required, or when the amounts of derivatives injected have to be reduced, *e.g.*, with small-bore columns.

*Application to histamine*

A classical method for histamine measurements is condensation with OPT [17], but this reaction suffers from several problems, *e.g.*, high chemical lability towards auto-oxidation and poor quantum yield of fluorescence of the isoindole derivatives (*ca.* 0.1) [18]. Condensation with OPT–mercaptoethanol [14] gives better results and was therefore compared with luminarin 2 labelling.

The kinetics of the histamine–luminarin 2 reaction in acetone were determined at 20, 60 and 80°C with a ten-fold molar excess of luminarin 2. The higher the temperature, the faster was the initial rate of the reaction, but the lower was the maximum yield; the latter then decreased. The kinetics of the reaction at 20°C in the dry state and with DMAP as the catalyst reached a plateau after 90 min with no subsequent decrease, and this procedure was used for further work. The reaction yield as a function of excess of luminarin 2 was studied in the range 1–1000. A plateau was reached for a molar ratio of 10. A higher excess of luminarin 2 resulted in the appearance of many by-products on the chromato-

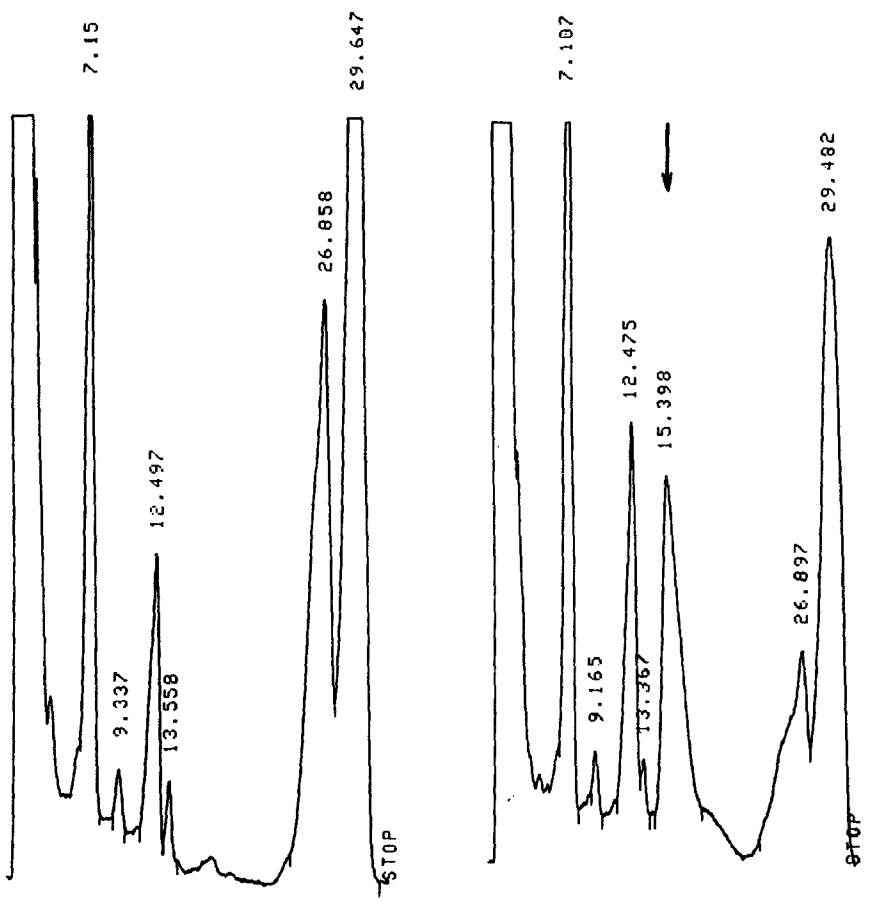


Fig. 3. Separation of the histamine–luminarin derivative with fluorescence detection, 0.5 μA full-scale. Left, blank; right, 0.5 nmol of derivatized histamine (50 pmol injected on to the column). Numbers at peaks indicate retention times in min.

gram. Under the conditions described (Fig. 3), the retention time of the histamine–luminarin 2 derivative was 15 min and that of luminarin 2 was 29 min. Peaks appearing at 7 and 12 min were impurities contained in the luminarin 2 powder. With fluorescence detection, the calibration graphs were linear for histamine amounts derivatized in each tube in the range 0.5–5 nmol ($r = 0.998$, $p < 0.01$), corresponding to 50–500 pmol of histamine derivative injected. Amounts up to 0.1 nmol of histamine could be derivatized, but the reproducibility and linearity were poor. The intra-run relative standard deviation was 8% for the 1-nmol histamine standard ($n = 5$). Once dissolved in acetone, the histamine–luminarin 2 derivative was stable for at least 4 h. Stability in the dry state was not investigated, but should be even longer. With chemiluminescence detection, the LOD was only two times lower than with fluorescence, 50 *vs.* 100 fmol (signal-to-noise ratio = 3), and was similar to that reported previously for luminarin 4 derivatives [10]. Hence luminarins, which are all derived from the quinolizinocoumarin nucleus, offer a wide range of chromatographic conditions for detection with peroxyoxalate chemiexcitation: polar mobile phases containing up to 75% of water or moderately polar mobile phases containing hexane–chloroform mixtures [9] can be used.

Derivatization of histamine by OPT (0.75 g/l) and mercaptoethanol (0.025%, v/v) in methanol was carried out for 30 min at 20°C. Under the conditions described, the retention time of the histamine–OPT derivative was 8.4 min. A linear calibration was obtained in the range 1–10 nmol of histamine, and the derivative was stable for at least 4 h in methanol. Hence both methods are similar, luminarin 2 being more sensitive but more time consuming than OPT.

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