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Luminarin 4 as a labelling reagent for carboxylic acids in liquid chromatography with peroxyoxalate chemiluminescence detection

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ABSTRACT

Luminarin 4 is a labelling reagent with a quinolizinocoumarin structure, which reacts with carboxylic acids after their activation by N-hydroxysuccinimide for 12 h at 20°C and dicyclohexylcarbodiimide for 60 min at 70°C. Small fatty acids were derivatized and separated by reversed-phase liquid chromatography. The fluorescence detection threshold was 300 fmol injected. The limit of chemiluminescence detection, which required a post-column reaction with an oxalic ester and hydrogen peroxide, was 50 fmol injected. The reaction was used to measure prostaglandin E_2, whose detection limit was 32 fmol injected, but the derivatization limit was 60 pmol. Linearity was observed in the range 0.1–10 nmol of prostaglandin E_2.

INTRODUCTION

Peroxyoxalate chemiluminescence (CL) detection was first used in liquid chromatography by Kobayashi and Imai [1] and further investigated by a number of others [2–11]. An oxalic ester reacts with hydrogen peroxide to form a proposed energetic intermediate, peroxyoxalate, which transfers its energy to a fluorescer, resulting in the emission of a photon. This mechanism has been applied to polycyclic and aminopolycyclic aromatic hydrocarbons [2,3], fluorescamine-labelled catecholamines [4], dansylated amino acids [5] and steroid [6], rhodamine-labelled chlorophenols [7] and coumarinic derivatives [8]. The main reason for the interest in this technique is that, for certain fluorophores, the CL detection limits are lower than those obtained with photoexcitation, but only a few fluorophores are better detected by chemiexcitation. In previous studies [9–11], we showed that, among various amino coumarins, the chemiluminescent intensity of the quinolizinocoumarin moiety was stronger [10], and the molecule was suitable for both normal and reversed-phase liquid chromatography with CL detection [11], affording femtomole detection limits.

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Quinolizinocoumarin served as the basis for the development of a series of chemiluminescent labels, called luminarins. In this study, luminarin 4 was investigated as a derivatizing reagent for carboxylic acids.

Many kinds of precolumn fluorescent derivatization reagents have been developed for the determination of carboxylic acids [12–25] by high-performance liquid chromatography (HPLC). They can be subdivided into two classes: those involving direct reaction between the acid and the labelling reagent, and those requiring the activation of the carboxylic acid before reaction with the fluorescer. To the first category belong halogenomethyl aromatics such as 4-bromomethyl-7-methoxycoumarin [12] and related compounds. These compounds had high limits of derivatization (L.D.) (about 200 pmol) and lacked selectivity, because they reacted with phenols, thiols and imides [16]. Aryldiazoalkanes were highly reactive (L.D. = 1 pmol) but were usually unstable when exposed to heat and light, with the exception of 1-pyrenyldiazomethane [14]. Derivatization with *o*-diamines, *e.g.* 9,10-diaminophenanthrene, to form 2-substituted phenanthrimidazoles [15] afforded a favourable L.D. (10 pmol) but necessitated the preparation of methylpolyphosphate as the solvent and the derivatives were quenched by dissolved oxygen.

To the second category belong the carboxylic acids activated by 1-methyl-2 halogenopyridinium iodide [16], N,N'-carbonyldiimidazole [16] and carbodiimides [17,18]. The activated acid was then condensed with an alcohol, *e.g.*, hydroxymethylanthracene (16), or a hydrazine, 2-nitrophenylhydrazine [17]. Among these methods, activation by 1-methyl-2-bromopyridinium iodide proved to be useful, with an L.D. in the subpicomole range. On the other hand, although activation by carbodiimides has been widely used in synthesis [19], application to the derivatization of amounts of carboxylic acids smaller than 1 nmol has not been reported. Owing to the attractive features of this activation reaction, including mild derivatization conditions and stability of the reagent and the derivatives, its application to fatty acids was investigated using luminarin 4 (Fig. 1) as a luminophore.

EXPERIMENTAL

Reagents

Isovaleric and nonanoic acid were purchased from Prolabo (Paris, France). Prostaglandin E_2 was a gift from Upjohn (Kalamazoo, MI, U.S.A.). N-Hydroxysuccinimide (NOHS) and bis-(2,4,6-trichlorophenyl) oxalate (TCPO) were bought from Fluka (Buchs, Switzerland). Dicyclohexylcarbodiimide (DCC), tetrahydrofuran (THF), imidazole, methyl acetate, dimethylformamide (DMF) and dimethyl sulphoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Coumarin 102, laser grade, was obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and luminarin 4 from Eurobio (Les Ulis, France). Hydrogen peroxide (30% aqueous solution) was supplied by Janssen (Beerse, Belgium).

All reagents and solvents were of analytical-reagent grade, unless stated otherwise, and were used as supplied. N-Succinimidoxyisovalerate was synthesized by dissolving 57.5 mg of NOHS and 123.6 mg of DCC in 2 ml of DMF and mixing with 55 μl of pure isovaleric acid. The solution was left for 1 h at 4°C and then for 1 h at room temperature. Precipitated dicyclohexylurea was removed by centrifugation and the solution was used diluted 1:100 in DMF.

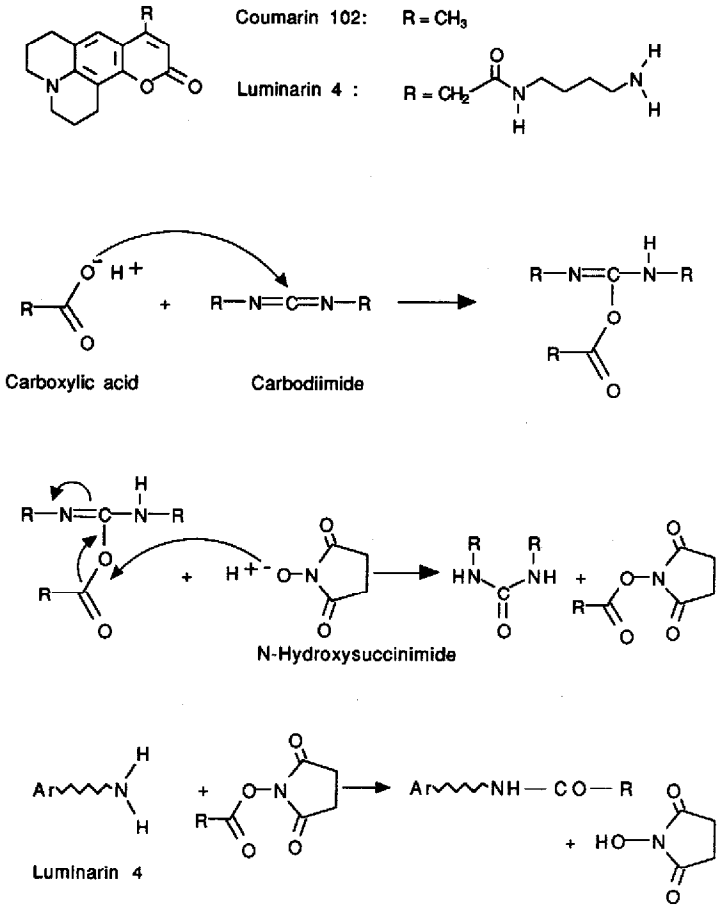

Fig. 1. Derivatization of carboxylic acids by luminarin 4.

Instrumentation

The liquid chromatograph consisted of a Chromatem 380 pump (Touzart et Matignon, Vitry, France), a Rheodyne Model 7125 injector with a 20-μl sample loop, a Kratos FS970 fluorescence detector (Applied Biosystems, Rungis, France) and a Shimadzu CR3A integrator (Touzart et Matignon). For chemiluminescence measurements, a Shimadzu CTO-6A column oven (Touzart et Matignon) and a Kratos URS051 post-column reactor (Applied Biosystems) were added, with a 292-μl capillary placed in the oven for mixing TCPO and hydrogen peroxide, and a 60-μl capillary for mixing these reagents with the eluent before entering the detector.

For on-line absorbance measurements, a Shimadzu diode-array SPD-M6A detector was used. Excitation, emission and synchronous excitation spectra were recorded on a Model LS-5 spectrofluorimeter (Perkin-Elmer, Norwalk, CT, U.S.A.), with the slits adjusted to 2.5 nm. All spectra were fully corrected.

For the determination of the relative fluorescence quantum yield, a Shimadzu

SPD2A absorbance detector was connected to the outlet of the fluorescence detector. The signal from each detector was recorded on a double-trace integrator.

Chromatographic conditions

Four sets of conditions were used, as follows. System 1 consisted of an Ultrasphere ODS-2 (5 μm) column (250 × 4.6 mm I.D.) (Beckman, Les Ulis, France) and the mobile phase was acetonitrile–DMSO–5 m*M* imidazole nitrate buffer (pH 6.5) (60:10:30, v/v/v) pumped at 1 ml/min. In system 2, a Spherisorb ODS-2 (5 μm) column (150 x 4.6 mm) [Société Française de Chromatographie sur Colonne (SFCC), Neuille-Plaisance, France] was used with a mobile phase of acetonitrile–DMSO–5 m*M* imidazole nitrate buffer (pH 7) (45:5:50, v/v/v) at a flow-rate of 1.5 ml/min. System 3 was identical with system 2 except that the buffer concentration was 10 m*M*. System 4 used a Spherisorb ODS-2 (5 μm) column (250 × 4.6 mm I.D.) (SFCC) with a mobile phase of acetonitrile–10 m*M* imidazole nitrate buffer (pH 7.7) (45:55, v/v) pumped at 1.2 ml/min.

For all systems, fluorimetric detection was performed with the excitation wavelength set at 390 nm and a 470-nm emission cut-off filter. When chemiluminescence detection was used, the lamp was turned off. The reagent solutions (1 mg/ml TCPO in methyl acetate and 0.4 *M* hydrogen peroxide in THF) were each pumped by the post-column system at a flow-rate of 0.25 ml/min and the oven was set at 40°C. The conditions were optimized as described previously [11].

The fluorescence quantum yield was calculated as the ratio of the total fluorescence intensity to the absorbance of the corresponding peak, in arbitrary units [20]. The two detectors were set at 360 nm for excitation and absorbance, respectively, whereas a 419-nm cut-off filter was used to collect all the emission bands of the fluorescers.

Derivatization procedure

Luminarin 4 derivatives were prepared by mixing 0.1 ml of 62.5 m*M* NOHS in DMF, 0.1 ml of 75 m*M* DCC in DMF and 0.05 ml of a solution of the carboxylic acid to be tested in DMF. The activation reaction was complete after 12 h at 20°C. Then 0.05 ml of a 2 m*M* coumarin 102 solution (internal standard) in DMSO and 0.5 ml of a 1 m*M* luminarin 4 solution in DMSO were added. After 60 min at 70°C, the derivatives were diluted 1:10 or more in DMSO before injection, so that the amount of derivative injected ranged from 0.5 to 20 pmol.

RESULTS AND DISCUSSION

Luminarin 4 isomerism

The first attempts to optimize the chromatographic conditions for luminarin 4 revealed an unusual feature of this product compared with other quinolizinocoumarins: the injection of luminarin 4 solutions, even when freshly prepared, resulted in several peaks of unequal heights when assessed by fluorescence detection. Various treatments of the luminarin 4 powder (recrystallization, acid–alkali extraction) yielded no modifications, and the luminarin 4 structure was confirmed by mass, ^{1}H NMR and IR spectrometry and C, H, O, N and S determinations [21]. Before trying purification by preparative chromatography, it was necessary to ensure that no peak was

retained on the column (*i.e.*, that recovery was complete), and that the fractions were stable. To achieve these goals, three chromatographic systems (1–3) were optimized and five peaks were detected as reported in Table I. Peaks 2 and 5 were the major components, representing 80% of the total area of the five peaks. A "recovery test" was made using system 1, by comparing the total areas of the absorbance signal with and without a column. The absorbance of the five peaks of luminarin 4 was not significantly different from that of the luminarin 4 solution when the mean areas of five injections were compared: 109 ± 11 *vs.* 127 ± 13 ($p > 0.05$), in arbitrary units.

TABLE I

RETENTION TIMES OF LUMINARIN 4 ISOMERS IN THREE CHROMATOGRAPHIC SYSTEMS

System	Retention time (min)				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
1	2.8	6.9	6.3	ND[a]	8.8
2	3.0	4.8	5.4	9.7	12.8
3	2.0	2.8	5.8	ND[a]	7.0

[a] Not detectable.

The stability of the fractions was then determined using system 2. Luminarin 4 (10^{-5} *M*) in DMSO was injected repeatedly and the five peaks were collected. Immediate reinjection of the fractions allowed the verification of their chromatographic purity and after 24 h of convervation at room temperature, each of them gave the same five peaks. This demonstrated the establishment of an equilibrium between the five components of luminarin 4 which are isomers and eliminated the possibility of preparing a "single peak" of luminarin 4.

Before applying luminarin 4 to carboxylic acid derivatization, it was necessary to ensure that its isomerism would not complicate the procedure unnecessarily. Three questions had to be answered: which of the isomers can derivatize the acids, do the isomers have similar excitation and emission spectra and does the derivatization lead to a single derivative? The first point was examined by looking for the isomers reacting with acetic anhydride, *i.e.*, containing a terminal amino group. The equimolar reaction of luminarin 4 with acetic anhydride for 60 min at 70°C in DMSO showed that peaks 1, 2 and 5 disappeared completely whereas peak 3 remained unchanged. No conclusion could be drawn regarding peak 4, which was too small. It follows that the two major components of luminarin 4 (peaks 2 and 5) are able to react with activated acids.

It was important to determine the excitation and emission spectra of the isomers because their positions are related to the singlet excitation energy, which is a major factor governing the chemiluminescent intensity [22]. Owing to the rapid interconversion of the isomers, these data could not be obtained in the usual way by spectrofluorimetry.

The absorbance spectra, determined using a diode-array detector, for the three

TABLE II

ABSORPTION SPECTRA[a] OF THREE LUMINARIN 4 ISOMERS AND COUMARIN 102

Parameter	Peak 1	Peak 2	Peak 5	Coumarin 102
λ_{max} (nm)	254 and 445	254 and 400	254 and 400	254 and 400
Peak-height ratio[b]	0.42	0.55	0.50	0.45

[a] In the mobile phase of system 2.
[b] Ratio of absorbance at 254 nm to absorbance at 400 (or 445 nm).

peaks giving sufficiently high absorbance (1, 2 and 5) and that of coumarin 102 are shown in Table II. It could be concluded that the two major components (peaks 2 and 5) probably contain the intact quinolizinocoumarin moiety, whereas peak 1 does not because its band at 400 nm is shifted to 445 nm. The occurence of distinct emission bands was then investigated using synchronous fluorescence spectrometry, with wavelength intervals between monochromators equal to those of the known intervals between excitation and emission bands of coumarin 102, *i.e.*, 80, 100, 125, 218 and 236 nm. The spectra of luminarin 4 and coumarin 102 showed no differences, and consisted of a single band in each instance. Hence the emission of the major components was similar to that of coumarin 102, with an emission maximum at 490 nm in the mobile phase of system 2. Finally, relative fluorescence quantum yields (another major factor controlling the chemiluminescence intensity) were similar for peaks 1, 2 and 5 and coumarin 102, whereas the yield of peak 3 was three times lower (Table III). Thus, in answer to the second question, the major components of luminarin 4 have similar fluorescence properties.

The third point concerned the derivatization of luminarin 4 isomers. With all the carboxylic acids labelled to date (fatty acids and prostaglandin E_2), a single peak of a derivative was obtained. It seems that the amidification of the terminal amino group supresses the interconversion between the isomers. Finally, ^{1}H NMR studies in several solvents gave an insight into the probable structure of four out of five isomers (Fig. 2) [21]. The structure of "peak 3" remains controversial, but the absence of reaction with acetic anhydride and the low fluorescence quantum yield could be explained by an interaction between the terminal amino group and the lactone ring. In conclusion, luminarin 4 isomerism is not a major obstacle to its use as a derivatization reagent.

TABLE III

RELATIVE FLUORESCENCE YIELDS OF LUMINARIN 4 ISOMERS AND COUMARIN 102

Parameter	Peak 1	Peak 2	Peak 3	Peak 5	Coumarin 102
Absorbance[a]	7	25	7	32	31
Fluorescence[a]	14	43	4.5	58	58
Ratio	2.00	1.72	0.64	1.81	1.87

[a] In arbitrary units. See text for details.

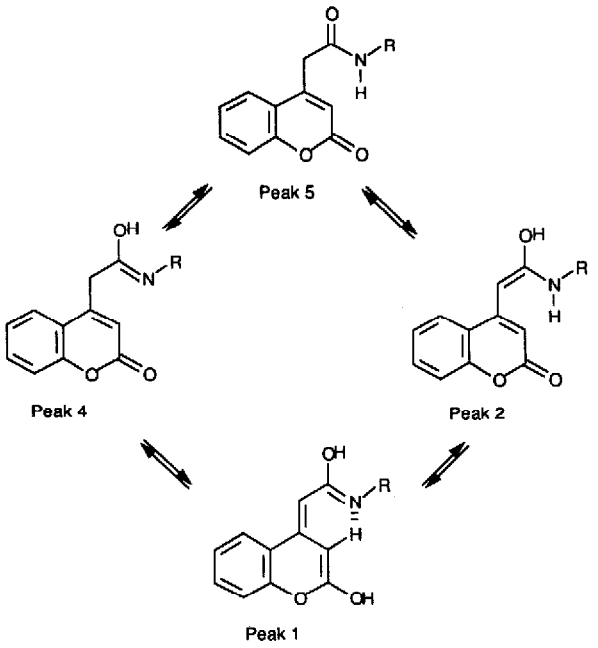

Fig. 2. Probable structures of luminarin 4 isomers. The quinolizine ring has been omitted for convenience.

HPLC analysis

Isovaleric acid, nonanoic acid and prostaglandin E_2 (PGE_2) were labelled according to the procedure described above, and were fractioned in different chromatographic systems, as shown in Table IV and Fig. 3. Derivatives of homologous fatty acids could be easily separated by varying the acetonitrile-to-buffer ratio.

Suprisingly, the isovaleric derivative was retained less than luminarin 4 itself, even though it is less polar. This was attributed to a strong interaction between the free silanol groups of the stationary phase and the terminal amino group of luminarin 4, by electrostatic attraction and/or hydrogen bonding. This hypothesis is supported by the strong effect of ionic strength on retention, as demonstrated by the comparison of the retention times in systems 2 and 3.

The mobile phase components (acetonitrile, imidazole nitrate buffer) were chosen according to their ability to promote a high chemiluminescence intensity and their miscibility with the excitation reagent solution.

Time and temperature of reaction

The kinetics of the first phase of the reaction were determined at −20, 4, 20 and 30°C. Carboxylic acids have often been activated by NOHS and DCC, or DCC alone, at low temperature [19], because the dicyclohexylurea thus formed is insoluble and precipitates, displacing the equilibrium to the right. However, when used for analytical purposes, dicyclohexylurea precipitation is not likely, because the reagent concentrations are low. In contrast, a higher temperature will promote a faster reaction. For these reasons, the reactions were carried out at the four temperatures mentioned

TABLE IV

RETENTION TIMES OF LUMINARIN 4 DERIVATIVES

System	Retention time (min)				
	Luminarin 4[a]	Luminarin 4–C 5[b]	Luminarin 4–C 9[c]	Luminarin 4–PGE_2	Coum 102
1	8.8	4.8	7.2	ND[d]	10.3
2	12.8	12.6	17.0	4.5	9.0
3	7.0	ND[d]	11.5	3.5	8.0
4	14.0	ND[d]	16.0	10.5	15.0

[a] Only peak 5 is given.
[b] Luminarin 4 derivative of isovaleric acid.
[c] Luminarin 4 derivative of nonanoic acid.
[d] Not determined.

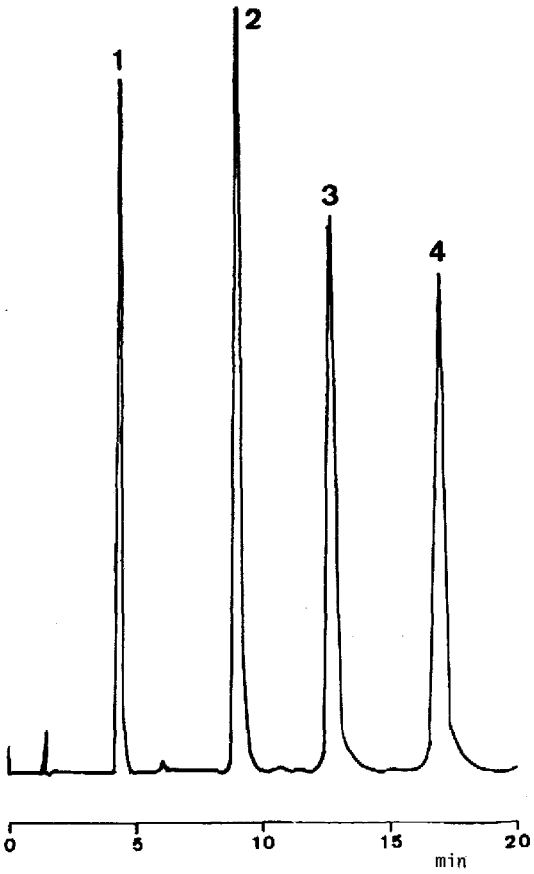

Fig. 3. Separation of some luminarin 4 derivatives with system 2 and fluorescence detection. Peaks: 1 = luminarin 4–PGE_2; 2 = coumarin 102; 3 = luminarin 4–isovaleric acid; 4 = luminarin 4–nonanoic acid. Peaks represent 10 pmol of each derivative injected onto the column. Range, 0.1 μA full-scale.

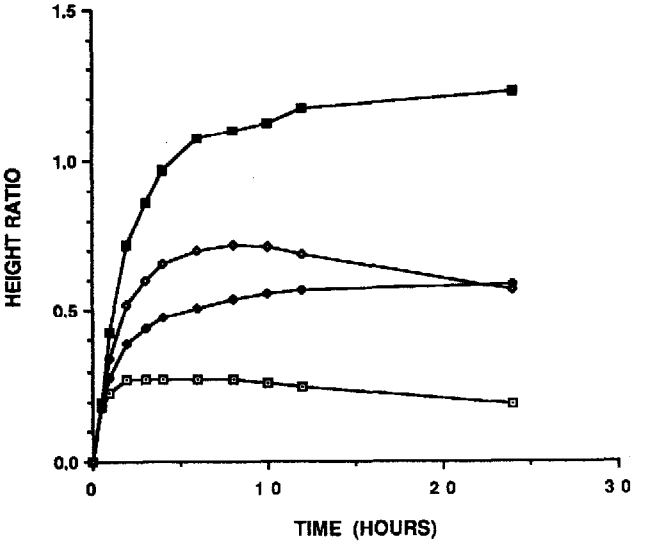

Fig. 4. Kinetics of derivatization (first phase: activation of the carboxylic acid) at four temperatures: □ = −20; ◆ = 4; ■ = 20; ◇ = 30°C. Ordinate is the ratio of the luminarin 4–isovaleric acid peak height to the coumarin 102 peak height.

above, and the results are shown in Fig. 4, with the highest yield being obtained at 20°C for 12 h.

The kinetics of the second phase of the labelling reaction were studied, using the N-succinimidoxyisovalerate prepared as described above. The formation of the luminarin 4 derivative was examined over 240 min at 50 or 70°C, as shown in Fig. 5.

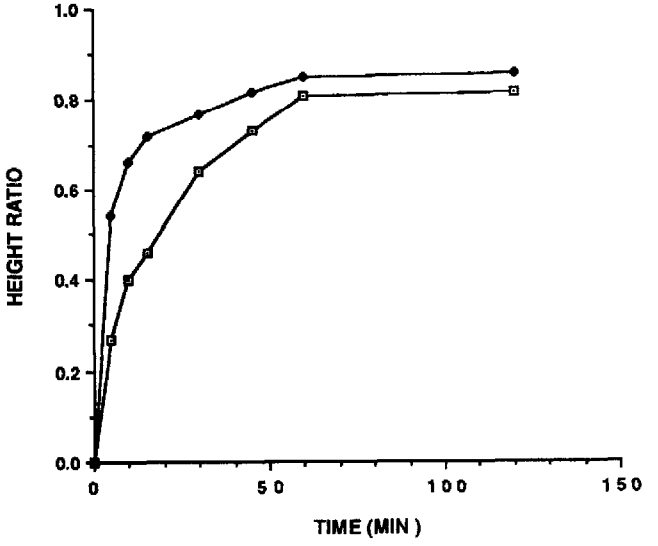

Fig. 5. Kinetics of derivatization (second phase: condensation with luminarin 4) at two temperatures: □ = 50; ◆ = 70°C. Ordinate is the ratio of the luminarin 4–isovaleric acid peak height to the coumarin 102 peak height.

Higher yields were obtained in shorter times at 70°C, the temperature at which the reaction was complete in 60 min. The overall yield, measured at a level of 50 nmol of nonanoic acid with reference to known amounts of the isolated derivative, was 91%. A larger excess of luminarin 4 or NOHS and DCC did not improve this yield but resulted in larger interfering peaks. As the linearity and reproducibility of the derivatization were found to be satisfactory, no further attempts were made to increase the reaction yield.

Linearity and sensitivity

The calibration graphs obtained with this method showed good linearity; the equation for the calibration line was $y = ax + b$ with $a = 0.016 \pm 0.001$ (mean ± S.D.) and $b = 0.083 \pm 0.027$ ($r = 0.995$, $p < 0.01$) after analysing five samples of each standard containing 12.5–125 nmol of isovaleric acid, detected fluorimetrically. For PGE_2, the calibration graphs were linear from 0.1 to 10 nmol of PGE_2 ($r = 0.995$, $p < 0.01$). Under the prevailing conditions, the detection limits for the luminarin 4 derivatives of isovaleric and noanoic acid were 300 fmol injected (signal-to-noise ratio = 3) for fluorescence detection and 50 fmol injected for peroxyoxalate CL detection.

The former detection limit (300 fmol) was similar to that found in our previous study on luminarin 1 derivatives [11], whereas the latter was higher than that obtained previously (50 *vs.* 6 fmol). The main explanation lies in the difference between the excitation conditions. In the present investigation, the mobile phase was much more aqueous (50 *vs.* 30%). In order to prevent the precipitation of the oxalic ester TCPO in the system, three precautions were taken: the TCPO concentration was reduced (1 vs. 4.5 mg/ml), TCPO and hydrogen peroxide were mixed and heated at 40°C so that TCPO was almost completely transformed before being added to the mobile phase and the pH of the mobile phase was raised to 7.7 in order to accelerate oxalic ester hydrolysis and to increase the solubility of the trichlorophenol formed. The low concentration of TCPO resulted in a lower excitation level [9] and a higher limit of detection. Precipitation could also have been avoided if a more soluble oxalate ester such as TDPO, bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate, had been applied [5,23,24]. However, in this work TCPO was used to allow comparison with our previous results, and was found to work satisfactorily. Further, our results are in the same range as those reported by Grayeski and De Vasto [8] with aminocoumarinic derivatives.

For PGE_2, in order to improve the limit of derivatization (L.D.), only 0.05 ml of 0.5 m*M* luminarin 4 solution was added to the mixture after the activation step. This resulted in a higher concentration of activated PGE_2 in the reaction medium and a diminution in the amplitude of interfering peaks on the chromatogram. Further, the sample loop volume was reduced to 10 μl, to improve the separation (Fig. 6). The interfering peaks could also have been reduced by a preliminary extraction of the derivatives before injection onto the HPLC column, but this procedure was not investigated in this work. The L.D. was 60 pmol and the CL detection limit was 32 fmol injected (signal-to-noise ratio = 3).

Although there have been many papers [25–30] on the separation and measurement of prostaglandins by HPLC, it remains a difficult analysis, because physiological levels are very low; taking PGE_2 as an example, the levels are about 100 pmol/l

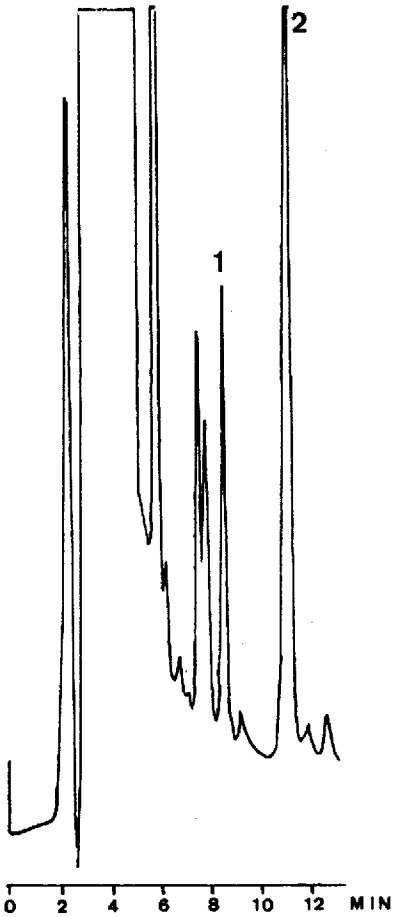

Fig. 6. Separation of luminarin 4–prostaglandin E_2 derivative with system 4 and chemiluminescence detection. Peaks: 1 = luminarin 4–PGE_2, 2 pmol injected onto column; 2 = luminarin 4 (last peak). See text for details. Range, 0.1 μA full-scale.

in human plasma and 0.3–40 nmol/l in human urine [25], the highest levels, 25–750 μmol/l, being in seminal fluid [26]. It is generally accepted that gas chromatography–mass spectrometry provides the most sensitive and specific method available, especially when using negative-ion chemical ionization [27], and that liquid chromatography lacks sensitivity. With UV detection in the 190–210-nm range [28,29], the limit of detection was about 60 pmol injected.

CONCLUSIONS

Luminarin 4 appears to be a promising reagent for carboxylic acid derivatization. Luminarin 4 isomerism was not a real drawback, as a single derivative was formed and was easily separated from the excess of reagent. Linearity and quantita-

tive yields demonstrated the reactivity of luminarin 4 with activated acids. Activation by NOHS and DCC was not a fast reaction, but the mildness of the conditions allowed its application to unstable compounds, such as PGE_2. Chemiluminescence detection sensitivity was better than that of fluorescence, although the mobile phase was very aqueous (50%), and this depended on the properties of the quinolizinocoumarin structure of luminarin 4. A gap remains between the very low limit of detection and the much higher limit of derivatization, which is the limiting factor in the application of the method, but others have reported that sensitivity was increased by derivatization of the ketone [23] or carboxylic function [28], achieving a limit of derivatization of 0.3 pmol for PGE_2.

Work is in progress to improve the limit of derivatization, in order to take advantage of the low detection limit. Otherwise, CL detection could be applied in immunological assays, where the labelling reaction does not need to be carried out at a low carboxylic acid concentration.

REFERENCES

1 K. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
2 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
3 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (9184) 1096.
4 S. I. Kobayashi, J. Sekino, K. Honda and K. Imai, *Anal. Biochem.*, 112 (1981) 99.
5 K. Imai, Y. Matsunaga, Y. Tsukamoto and A. Nishitani, *J. Chromatogr.*, 400 (1987) 169.
6 T. Koziol, M. L. Grayeski and R. Weinberger, *J. Chromatogr.*, 317 (1984) 355.
7 P. J. M. Kwakman, J. G. J. Mol, D. A. Kamminga, R. W. Frei, U. A. Th. Brinkman and G. J. De Jong, *J. Chromatogr.*, 459 (1988) 139.
8 M. L. Grayeski and J. K. De Vasto, *Anal. Chem.*, 59 (1987) 1203.
9 M. Tod, R. Farinotti and G. Mahuzier, *Analusis*, 14 (1986) 271.
10 M. Tod, R. Farinotti, G. Mahuzier and I. Gaury, *Anal. Chim. Acta*, 217 (1989) 11.
11 M. Tod, M. Prevot, M. Poulou, R. Farinotti, J. Chalom and G. Mahuzier, *Anal. Chim. Acta*, 223 (1989) 309.
12 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
13 J. A. Secrist, J. R. Barrio and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 45 (1971) 1262.
14 N. Nimura, T. Kinoshita, T. Yoshida, A. Uetake and C. Nakai, *Anal. Chem.*, 60 (1988) 2067.
15 J. B. F. Lloyd, *J. Chromatogr.*, 189 (1980) 359.
16 H. Lingeman, A. Hulshoff, W. J. M. Hunderberg and F. B. J. M. Offermann, *J. Chromatogr.*, 290 (1984) 215.
17 J. Miwa, C. Hiyama and M. Yamamoto, *J. Chromatogr.*, 321 (1985) 165.
18 H. Miwa, *J. Chromatogr.*, 333 (1985) 215.
19 H. G. Khorana, *Chem. Rev.*, 53 (1953) 145.
20 C. A. Parker and W. T. Rees, *Analyst (London)*, 85 (1960) 587.
21 M. Tod, *Ph.D. Thesis*, Université-Paris Sud, Faculté de Pharmacie, Paris XI, 1990, No. 138.
22 K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta*, 177 (1985) 111.
23 K. Imai, H. Nawa, M. Tarraka and H. Ogata, *Analyst (London)*, 111 (1986) 209.
24 K. Imai, A. Nishitani and Y. Tsukamoto, *Chromatographia*, 24 (1987) 77.
25 K. Yamada, M. Onodera and Y. Aizawa, *J. Pharmacol. Methods*, 9 (1983) 93.
26 K. Svanborg, M. Bygdeman, P. Eneroth and E. Bendwold, *Prostaglandins*, 24 (1982) 636.
27 S. E. Barrow, K. A. Waddel, M. Enmis, C. T. Dollery and I. A. Blair, *J. Chromatogr.*, 239 (1982) 71.
28 A. Terragno, R. Rydzik and N. A. Terragno, *Prostaglandins*, 21 (1981) 101.
29 D. M. Desiderio, M. D. Cunningham and J. A. Trimble, *J. Liq. Chromatogr.*, 4 (1981) 1261.
30 K. Wessel, V. Kaever and K. Resh, *J. Liq. Chromatogr.*, 11 (1988) 1273.