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REVIEW

CHEMILUMINESCENCE DETECTION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOMEDICAL SAMPLES

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CONTENTS

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LIST OF ABBREVIATIONS

ABEI	N-(4-Aminobutyl)-N-ethylisoluminol
ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
BL	Bioluminescence
CIEEL	Chemically initiated electron exchange luminescence
CK	Creatine kinase
CL	Chemiluminescence
DNPO	Bis(2,4-dinitrophenyl)oxalate
EPA	Eicosapentanoic acid
FIA	Flow injection analysis
FMN	Flavin mononucleotide
HPLC	High-performance liquid chromatography
NAD	Nicotinamide-adenine dinucleotide, oxidized
NADH	Nicotinamide-adenine dinucleotide, reduced
2-NPO	Bis(2-nitrophenyl)oxalate
TCPO	Bis(2,4,6-trichlorophenyl)oxalate
TDPO	Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate

1 INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) has become a powerful tool for the analysis of a wide variety of samples However, the sensitivity and selectivity of detection in HPLC often do not meet the requirements of modern trace-level determinations in biomedical and environmental samples. For this type of analysis fluorescence is often chosen as the detection method. Since the range of compounds displaying strong native fluorescence is relatively small, derivatization plays an important role in HPLC with fluorescence detection. However, in many cases, even the detection of suitable derivatives is not sensitive enough for analysis in the low-ppb and ppt range, while the reliable determination of these compounds is more and more required The limiting factor in the detectability of fluorescence is the stray light that increases the background signal and the noise This problem can be solved by chemical excitation, i e., chemiluminescence (CL) If the reaction used for excitation occurs in a living system or is derived from one, the process is called bioluminescence (BL). During the last decade, CL has also been applied for detection in HPLC. The sensitivity of such a detection system is often 10–100 times higher than that of fluorescence detection Various reactions have been used on-line for the excitation of analytes eluting from an HPLC column

This paper describes some theoretical aspects of CL, especially of CL in

dynamic systems The mechanisms of the most important CL reactions used for HPLC and schemes of detection systems are discussed. Related flow injection analysis (FIA) methods are also mentioned. Biomedical applications of several systems are presented and the possibilities and limitations discussed.

2 THEORETICAL ASPECTS

2 1 Chemiluminescence

A few chemical reactions can produce excited states of compounds Light emission in the visible region of the electromagnetic spectrum (400–800 nm) corresponds to reaction energies of ca 200–400 kJ/mol. Therefore, it is not surprising that many CL reactions involve molecules with high internal energy, such as singlet oxygen, peroxides and strained rings. A part of the excited molecules releases their energy as light Therefore, the efficiency of a CL reaction can be described by the equation

$$\Phi_{CL} = \Phi_R \Phi_L \qquad (1)$$

where Φ_R is the number of molecules in the excited state per number of molecules reacted and Φ_L is the number of molecules emitting light per number of excited-state molecules (luminescence efficiency). This means that the emitted light can be used for the determination of low concentrations of one of the reactants (including catalysts) if the CL efficiency is high enough Another interesting possibility is that an excited molecule transfers its energy to a so-called acceptor molecule with an efficient luminescence efficiency In this case the total CL efficiency can be described by.

$$\Phi_{CL} = \Phi_R \Phi_{ET} \Phi_L \qquad (2)$$

where Φ_{ET} is the number of excited acceptor molecules per number of molecules directly excited by the reaction (donor molecules) This indirect (sensitized) CL extends considerably the number of types of molecule that can be determined by CL (see below) The CL efficiency is dependent on the reaction conditions This is true not only for Φ_R but also for Φ_{ET} and Φ_L For example, it is well known that Φ_L strongly depends on the solvents in which the luminescence process takes place If CL is measured in a dynamic system, i e for FIA or HPLC detection, the kinetics of the reaction are also very important. This will be discussed in the next section

2 2 Coupling of chemiluminescence reactions to flow systems

The CL reaction starts when the sample is mixed with the reagent(s) It proceeds at a rate characteristic of the reaction As a consequence, a luminescence growth curve is observed The signal reaches a maximum, and subse-

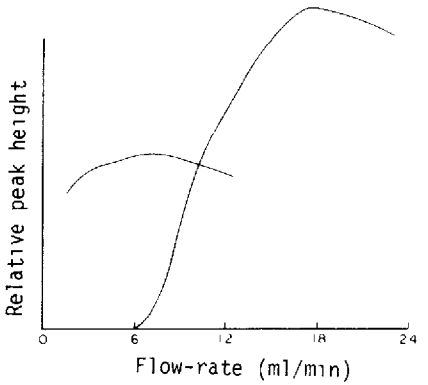


Fig 1 Peak height versus flow-rate for a kinetically fast reaction and a kinetically slow reaction The slow reaction data are on the left-hand side Both curves are for peroxide measurement using a $Cu^{(II)}$-catalysed luminol reaction with conditions manipulated to adjust the kinetics [1]

quently decays because of the exhaustion of the light-generating agent(s) Therefore, in a dynamic system, the part of the emitted light that is measured is dependent on the flow-rate and the form of the CL curve. Fig. 1 shows the influence of the flow-rate on the peak height of the FIA determination of peroxide using the $Cu^{(II)}$-catalysed luminol reaction [1]. The conditions were changed to adjust the kinetics. For a slow reaction, the signal is less dependent on the flow-rate In the case of a fast reaction, no signal is observed at low flow-rates because the reaction goes to completion before the sample is in the detector cell On the other hand, a fast reaction and a high flow-rate lead to a higher sensitivity than a slow reaction That is, the half-life of the CL signal is a very important parameter For given values of the flow-rate in the reaction system, the dead volume between the mixing tee and the flow-cell, and the volume of the flow-cell itself, the CL half-life determines the percentage of the emitted light that is measured.

For the peroxyoxalate chemiluminescence reaction (see Section 3), much attention has been paid to the half-life of the reaction and its influence on the sensitivity of an HPLC detection system based on this reaction It has been demonstrated that the half-life is dependent on the oxalate structure, the solvent composition, the pH, the water content, the catalyst concentration and the temperature [2–5] Fig 2 shows schematic drawings of the chemiluminescence decay curves of two oxalates, bis(2,4,6-trichlorophenyl)oxalate (TCPO) and bis(2,4-dinitrophenyl)oxalate (DNPO) (see Section 3), and the period in which the light-emitting compound is in the flow-cell The regions a and b indicate this period and, thus, the part of the CL decay curve that is measured, for a conventional and a miniaturized HPLC system, respectively With the exception of region a (conventional HPLC) of the TCPO curve, the regions correspond to 26–42% of the total emitted light For a rapid reaction, i e with

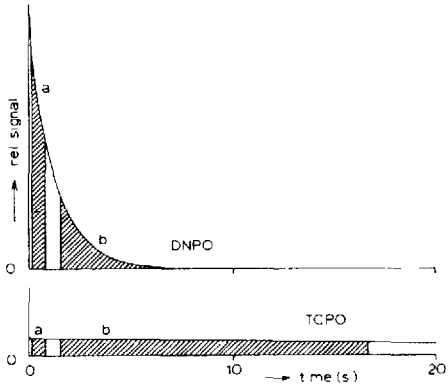


Fig 2 Schematic drawing of the decay curves of TCPO and DNPO and the period (a and b), in which the light-emitting compound is in a 50-μl flow-cell if the dead volume is 5 μl (a) Conventional HPLC system, total flow-rate, 67 μl/s, (b) miniaturized HPLC system, total flow-rate, 3 3 μl/s It should be noted that the curve of TCPO also shows an exponential decay [3]

DNPO as oxalate, the dead volume in front of the flow-cell was found to have a considerable influence on the percentage that is measured. All the percentages decrease, if the volume of the flow-cell decreases Generally, however, a 50-μl cell is less suitable for the miniaturized system because of the band broadening in the cell. As for the band broadening, it should be noted that this is less than for, e g., fluorescence detection because of the decrease of the CL signal in the flow-cell [6] This also means that an oxalate with rapid kinetics is more suitable for miniaturized HPLC. Hanaoka et al [5] used stopped-flow measurements in conjunction with the 'time-window concept' for optimization of FIA and HPLC systems based on peroxyoxalate CL The peak heights in the dynamic systems were in good agreement with the calculated data In conclusion, it can be stated that the form of the CL signal is very important for optimization of the pertinent analytical method, especially if a dynamic system is used Therefore, study of the influence of the reaction conditions on the kinetics of the reaction is needed in order to design an optimal detection system

Finally, the appropriate choice of the apparatus used for measurement of the emitted light is essential The advantage of CL in comparison with fluorescence is that no light source is used and, so, excitation optics are superfluous Emission light can be collected more efficiently because stray light is not present A theoretical calculation by Seitz and Neary [7], based on the assumption that the sensitivity of a CL method depends only on the light detection, revealed that sub-attomol detection limits could be achieved If the flow-cell is placed in an integrating sphere, very efficient collection of the light can result [3,6,8] The Kratos (Schoeffel) fluorescence detector with a 2π steradian mirror in front of the flow-cell also is very suitable Positioning of the cell in close proximity to the photomultiplier also seems very attractive Gandelman and

Birks [9] have optimized the detector by placing the detector cell (PTFE coil, 60 μl) within a ellipsoidal mirror The cell is positioned near the first focus of the mirror and the photomultiplier cathode is placed on the second focus Use of a photon counter can often improve the sensitivity of the detection system, unless the CL background is too high Generally, apparatus developed for fluorescence detection is not optimal for CL detection

3 PEROXYOXALATE CHEMILUMINESCENCE

3 1 Mechanism

The first example of peroxyoxalate CL was reported in 1963 by Chandross [10], who studied the reaction of oxalyl chloride with hydrogen peroxide in the presence of a fluorescent compound Rauhut et al. [11] further investigated the reaction by synthesizing and testing several substituted aryl oxalates They proposed the energy-rich intermediate 1,2-dioxetanedione as a possible chemical excitation source for the fluorophore, producing its excited singlet state and leading to a typical fluorescence emission process (see Fig. 3). The CL efficiencies are in the range 1–23% for several substituted oxalate esters [12] Lechtken and Turro [13] studied several fluorophores and concluded that it was possible to generate electronically excited states with an energy of up to 430 kJ/mol

The CL decomposition of dioxetanes has been studied by several groups [14–16], and Schuster [17] and Koo and Schuster [18] introduced the chemically initiated electron exchange luminescence (CIEEL) mechanism The chemiexcitation step is the electron back-transfer from the intermediate to the fluorophore, resulting in its excited state Peroxyoxalate CL is also thought to follow a CIEEL-type mechanism as shown in Fig 3, and this means that there should be a good correlation between the oxidation potential of the fluorophore and the CL efficiency This was confirmed by Catherall et al [19], who thor-

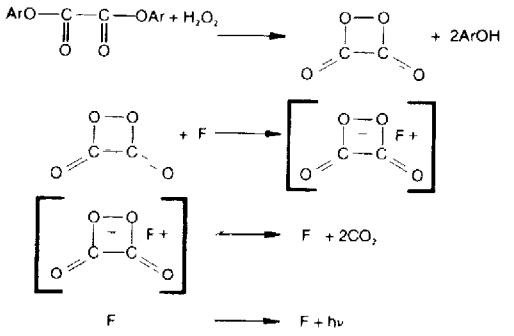


Fig 3 Peroxyoxalate reaction scheme

oughly investigated the kinetics and the mechanism of this CL reaction. They found that the CL intensity was strongly dependent on the electronegativity of the aryl group of the oxalate ester and that a common intermediate (the above-mentioned 1,2-dioxetanedione) was highly unlikely A key intermediate was proposed in which one of the aryl groups is still present. Recently, Alvarez et al [20] found a biphasic intensity–time profile for the reaction of hydrogen peroxide with TCPO in ethyl acetate catalysed by triethylamine A multiple intermediate mechanism was proposed in which more light-producing pathways are possible

In conclusion it can be stated that peroxyoxalate CL is a convenient tool for the detection of hydrogen peroxide and fluorophores In the latter case the analytes themselves should posses good CL characteristics (e.g. aromatic hydrocarbons [21,22]) or they should be labelled with a suitable CL tag [23], as will be discussed in Section 3.2.2.

3 2 Detection systems and biomedical applications

3 2 1 Reaction system

In order to obtain a high sensitivity in an HPLC system, the CL reagents (oxalate and hydrogen peroxide) should be mixed with the column eluate just before the photomultiplier (see Fig 4). Several oxalates have been investigated for their solubility in common LC solvents, stability in the presence of hydrogen peroxide and CL intensity [2,24,25]. Unfortunately, none of these oxalates combines all the required characteristics and often compromises have to be made Usually TCPO or bis(2-nitrophenyl)oxalate(2-NPO) are used, in the concentration range 1–10 m*M*, because of their stability in the presence of hydrogen peroxide DNPO is more soluble in common LC solvents, such as acetonitrile and methanol, but has a limited applicability owing to very fast reaction kinetics Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]-oxalate (TDPO) is soluble in acetonitrile up to 1 *M* [24], but cannot be used at these concentrations because of CL quenching by the phenolic reaction product [4] Usually the concentration of hydrogen peroxide is higher than the oxalate concentration Typically, 10–500 m*M* solutions are mixed with oxalate solutions.

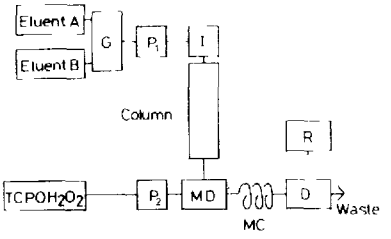


Fig 4 Scheme of a peroxyoxalate CL detection system [27]

TABLE 1

ANALYTES OF BIOMEDICAL INTEREST DETERMINED BY HPLC WITH PEROXYOXALATE CL DETECTION

Analyte	Label[a]	Sample[b]	Detection limit	References
Amino acids	Dansyl-Cl		1- 5 fmol	25-28
Bradykinine	Dansyl-Cl			29
Catecholamines, amino acids	Dansyl-Cl		5-10 fmol	30
Sec Amine drug	Dansyl-Cl	Serum	1-10 pg	6
Ketocorticosteroid	Dansyl-Hy	Plasma	7 5 pg	31
Secoverine	Dansyl-OH		500 pg	32
Estradiol	Dansyl-Cl	Serum	50 pg	33
Catecholamines	Fluorescamine	Urine	25 fmol	34
Carboxylic acids	Coumarins		50-75 fmol	35
Carboxylic acids	3-Aminoperylene		1-10 fmol	2
Urea, ouabain	–		2-20 pmol	48

[a]Dansyl = 5-dimethylaminonaphthalene-1-sulphonyl, Cl = chloride, Hy = hydrazine, OH = hydroxide

[b]If no sample is mentioned, only standard solutions have been measured

As most analytes of biomedical interest do not fluoresce, various labelling procedures have been applied. They are summarized in Table 1 and will be discussed in the next section

3 2 2 Derivatization

Among the known fluorescence derivatization reagents, dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride) has been widely used as a very sensitive CL label for primary and secondary amines and phenolic hydroxy groups Imai and co-workers [26–29] thoroughly investigated the CL detection of dansylated amino acids, and detection limits in the low femtomole region (20-μl injections of 10^{-9} M solutions) have been obtained. They were able to combine this detection system with gradient elution without major drift of the baseline [27] and with microbore HPLC [28] The latter system was applied to the determination of the N-terminal group of bradykinin after dansylation [29]. The low recovery of ca 10% was due to an incomplete separation of the dansyl–bradykinin derivative and reagent by-products, and to the peptide hydrolysis step (110 °C for 20 h) Mellbin [30] reported the detection of dansylated adrenalin, noradrenalin and some amino acids in the low femtomole region However, the method was carried out only with standard solutions, thus only illustrating the potential sensitivity of the CL method instead of the practical applicability De Jong et al [6] described the detection of a dansylated drug with a secondary amino group. For the analysis of serum samples a detection limit in the low picogram range was obtained (see Fig 5) This

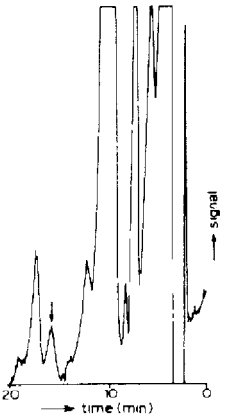


Fig 5 Determination of a dansylated secondary amine by HPLC with peroxyoxalate CL detection Analysis of serum sample spiked with 4 8 ng/ml (injected amount 24 pg) [6]

detection method was also applied to an HPLC system with a packed capillary column [8] By using a specially designed mixing device the reagents and the HPLC eluate were mixed in the flow-cell placed in an integrating sphere, a detection limit of ca 100 fg (0 5-μl injection) was obtained

Another application of derivatization with a dansyl label was reported by Koziol et al [31], who used dansyl hydrazine for the carbonyl group of fluocortin butyl, a 3α-keto corticosteroid The authors were able to detect as low as 100 pg/ml of blood plasma (or 7 5 pg injected) A chromatogram of a plasma sample is shown in Fig 6 Tertiary amines, which lack normal derivatization possibilities, can be sensitively detected by a post-column extraction system, the protonated amines form an ion-pair with negatively charged fluorophores, and the ion-pair is extracted on-line by segmentation with an organic solvent After phase separation the detection takes place in the organic phase This detection principle was modified to CL detection by Kwakman et al [32], using the hydrolysed form of dansyl chloride (dansyl-OH) as chemiluminescent counter-ion Hydrogen peroxide was added by pumping an apolar solvent (1,2-dichloroethane) through a column packed with perhydrit (hydrogen peroxide on a urea support) The detection limit for secoverine, a tertiary amine drug, was in the sub-nanogram range The limitation still is the extraction of the counter-ion itself, which increases the background signal

Recently, Nozaki et al [33] described the first combination of normal-phase LC with peroxyoxalate CL detection Estradiols were derivatized with dansyl chloride and separated on a silica column with hexane–chloroform–ethanol (70 30 0.1) as the eluent Solutions of TCPO and triethylamine in chloroform and of hydrogen peroxide in methanol were added post-column The overall recovery of estradiol from serum was ca 90%, with a detection limit of ca 50

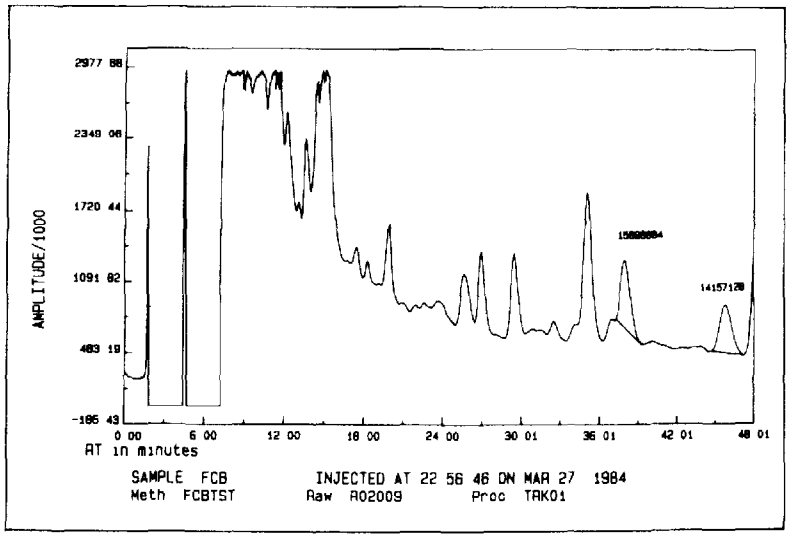


Fig 6 Determination of a dansylated ketocorticosteroid in dog plasma by HPLC with peroxyoxalate CL detection The two dimers formed by derivatization are indicated by integration numbers [31]

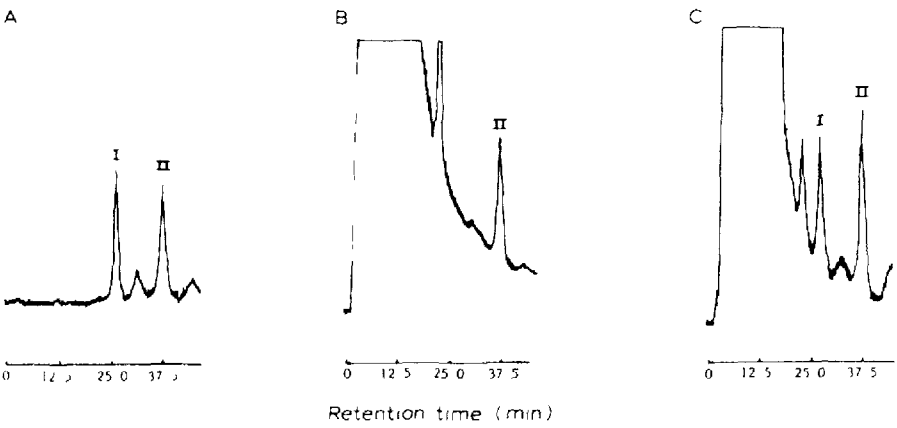


Fig 7 Normal-phase HPLC of dansylated steroids with peroxyoxalate CL detection (A) authentic dansyl estradiols (150 pg each), (B) serum sample (500 μl), (C) pooled serum sample spiked with 17α-estradiol (200 pg) and 17β-estradiol (300 pg) Peaks I = dansyl-17α-estradiol, II = dansyl-17β-estradiol [33]

pg Chromatograms of a standard solution and of a serum sample are shown in Fig 7

Kobayashi et al [34] derivatized the primary amino group of several catecholamines with fluorescamine The sensitivity of this method is ca 25 fmol,

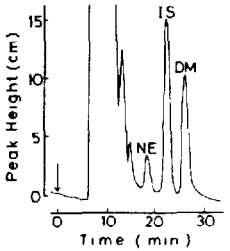


Fig 8 HPLC of fluorescamine derivatives of catecholamines with peroxyoxalate CL detection Sample corresponding to 17 5 μl of human urine was injected onto the column NE = norepinephrine, I S = internal standard, DM = dopamine [34]

which is about the same order of magnitude as for the dansyl derivatives studied by Mellbin [30] Fig 8 shows a chromatogram of a urine sample

Another important functional group for derivatization is the carboxyl group (fatty acids and prostaglandins). Grayeski and De Vasto [35] described the application of new coumarin labels for carboxylic acid detection. The most important result was that changing the methoxy group of the well known 4-bromomethyl-7-methoxycoumarin into a diethylamino group significantly improved the CL sensitivity. The strongly electron-donating influence of the diethylamino group lowers the oxidation potential, which facilitates the energy transfer (CIEEL mechanism, see Section 3.1) Several straight-chained carboxylic acids were detectable in the 50–75 fmol range Perylene was converted into 3-aminoperylene by Honda et al [2] for the derivatization of carboxylic acids using a carbodiimide as a coupling reagent Detection limits were in the low femtomole range, but the derivatization conditions (50 °C, 2 h) were unfavourable. In both case standard solutions were used, and up till now no real samples have been analysed with CL detection In particular, the determination of prostaglandins in urine and serum needs further investigation because a very high sensitivity is necessary It might be advantageous to apply labels with an emission maximum above 550 nm The CL signal can then be clearly separated from that of the CL reaction product(s) and of other CL compounds or impurities emitting below 550 nm, thereby leading to an improved signal-to-noise ratio [36]

3 2 3 Determination of hydrogen peroxide generated by enzyme reactions

Some enzymes produce hydrogen peroxide if the right substrate is present These trace amounts of hydrogen peroxide can be determined with peroxyoxalate CL in the presence of excess fluorophore (chemiluminophore) In contrast to other CL reactions (involving, e.g., luminol, lucigenin or acridinium esters) peroxyoxalate CL can easily be carried out at pH 7, which is compatible with the optimal pH of most enzymatic reactions Table 2 summarizes the applications of peroxyoxalate CL for the detection of hydrogen peroxide pro-

TABLE 2

PEROXYOXALATE CL DETECTION OF HYDROGEN PEROXIDE PRODUCED BY ENZYMIC REACTIONS

Analyte	Enzyme	Detection limit	Mode	References
Cholesterol	Cholesterol oxidase	0 1 pmol	FIA	37
Lactose, glucose	β-Galactosidase	12 ng	FIA	38
Formaldehyde, formic acid	Aldehyde oxidase	1 pmol	FIA	39
L-Amino acids	L-Amino acid oxidase	2 pmol	FIA	40
(Acetyl)choline	Choline oxidase + cholinesterase	1-10 pmol	HPLC	41
(Acetyl)choline	Choline oxidase + cholinesterase	1 pmol	HPLC	42
Glucose	Glucose oxidase	2 pmol	HPLC	44
L-Amino acids	L-Amino acid oxidase	10 pmol	HPLC	45
Glucose	Glucose oxidase	2 pmol	FIA	47

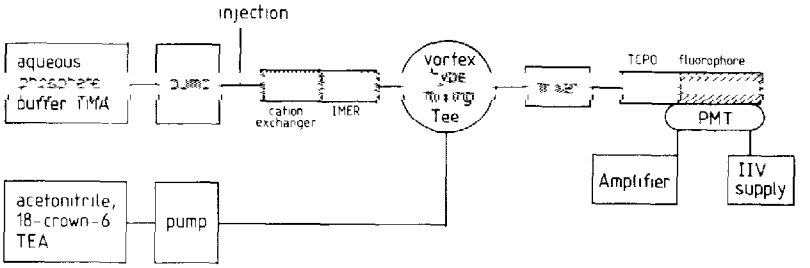


Fig 9 Scheme of the system for the detection of hydrogen peroxide produced by an enzymatic reaction of acetylcholine and choline [42]

duced by enzymatic reactions Rigin [37–40] detected several analytes in the flow-injection mode by immobilizing enzymes on porous quartz glass beads Hydrogen peroxide released from enzymatic reaction was detected by mixing with oxalate, fluorophore (9,10-diphenylanthracene) and catalyst (trimethylamine) right before the detector Thus very low amounts of cholesterol (enzyme cholesterol oxidase [37]), lactose and glucose (β-galactosidase [38]), formaldehyde and formic acid (aldehyde oxidase [39]) and L-amino acids (L-amino acid oxidase [40]) could be determined Honda et al [41] detected low picomole amounts of choline and acetylcholine using a post-column reactor containing immobilized choline oxidase and cholinesterase A buffer and a solution containing TCPO and the fluorophore perylene were added post-column Using the same enzymes, Van Zoonen et al [42] could detect choline and acetylcholine in urine and serum by applying a special flow-cell packed with a fluorophore and an oxalate (see Figs 9 and 10) The fluorophore, 3-amino-

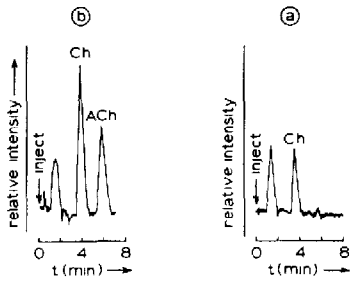


Fig 10 HPLC of choline (Ch) and acetylcholine (ACh) with an on-line enzymatic reaction and hydrogen peroxide detection by the peroxyoxalate CL reaction (a) Chromatogram of an undiluted urine sample, (b) chromatogram of an urine sample spiked with 20 pmol of choline and acetylcholine [42]

fluoranthene, was covalently bound to controlled-pore glass beads [43], and TCPO was simply packed in the solid state form. The same detection system was applied to the detection of glucose using a post-column glucose oxidase reactor [44] and to a post-column L-amino acid oxidase reactor, enabling the enantioselective detection of amino acids at picomole levels [45]. Recently other fluorophores, 8-hydroxyquinoline and Rhodamine B, have been immobilized in a flow-cell by Ding et al [46] for the single flow line detection of hydrogen peroxide Grayeski et al. [47] have designed a flow injection system for the determination of glucose by the detection of hydrogen peroxide generated by glucose oxidase A water-soluble oxalate was employed with Rhodamine B as fluorophore This oxalate, however, had to be dissolved in acetonitrile because of its limited stability in water (half-life ca 6 min)

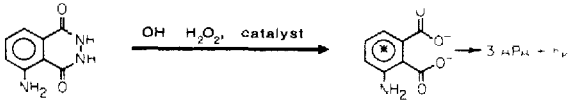
3 2 4 Other peroxyoxalate-based detection systems

A rather exceptional CL detection system was described by Capomacchia et al [48] The non-fluorophores urea and ouabain appeared to enhance the CL background of DNPO and hydrogen peroxide leading to detection limits of 20 and 2 pmol, respectively The mechanism of this phenomenon is not yet really understood and might lead to a new field of applications

4 LUMINOL CHEMILUMINESCENCE

4 1 Mechanism

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) reacts with hydrogen peroxide in basic media under the formation of an energy-rich intermediate with subsequent light emission of the excited aminophthalic acid (see Fig 11). The CL efficiency for this reaction is ca 1% [49] The exact mechanism of the reaction is still a matter of debate [49] The reaction is catalysed by metal ions



Luminol 3-Aminophthalate

Fig 11 Luminol reaction scheme

such as $Co^{(II)}$, $Cu^{(II)}$ and $Fe^{(III)}$ [50], and by enzymes such as horseradish peroxidase [51], microperoxidase [52] and myeloperoxidase [53]

In analytical detection systems luminol can be used for the determination of hydrogen peroxide generated by enzyme reactions, of certain metal ions or complexes containing metal ions, of compounds forming complexes with metal ions which, therefore, suppress the CL signal, and of analytes labelled with specially modified luminol reagents These systems will now be discussed in detail

4 2 Detection systems and biomedical applications

4 2 1 Detection of hydroperoxides and enzymatically generated hydrogen peroxide

Lipid hydroperoxides are formed during the oxidation of lipids Yamamoto et al [54] adapted the luminol–microperoxidase reaction to organic hydroperoxides and found a sensitivity similar to that of hydrogen peroxide. In an HPLC system a solution of isoluminol and microperoxidase was added to the column eluate resulting in detection limits of a few picomoles. The analysis of serum samples is under investigation Miyazawa et al [55] developed an assay for phosphatidylcholine hydroperoxides in human blood plasma The hydroperoxides were extracted from plasma with chloroform–methanol and injected onto a silica column with chloroform–methanol–water (1 9 0.1) as mobile phase Luminol and the catalyst cytochrome c (in borate buffer, pH 9 3) were added just before the detector Detection limits of 10 pmol in human plasma could be achieved Fig 12 shows the higher selectivity of CL in comparison with UV detection for the total lipid extract of plasma Nieman and co-workers [56,57] designed several systems using luminol CL A system containing the immobilized enzymes β-glucosidase and glucose oxidase on-line with the analytical column enabled the detection of β-D-glucosides and β-glucose with a detection limit of 10^{-7} *M* (2 pmol injected) [56] The generated hydrogen peroxide was mixed with a luminol–peroxidase solution and led through the detector This two-pump system is necessary because the pH of the enzymatic reaction (ca 6.5) is clearly different from the pH of the CL reaction (ca 10–11) Recently, a single-pump FIA system for hydrogen peroxide was described by the same authors [57] Both luminol and catalysts were immobilized on silica.

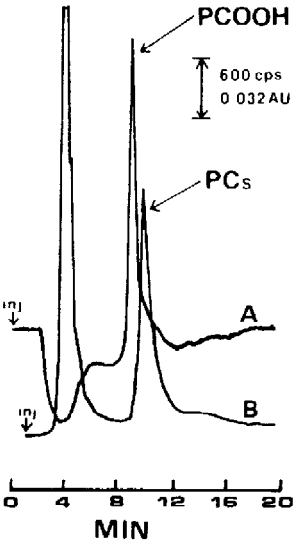


Fig 12 HPLC pattern of plasma phosphatidylcholine hydroperoxide (PCOOH) with luminol CL detection Total extracted from a healthy male plasma was diluted with 40 μl of chloroform–methanol, and 20 μl were injected (A) Chromatogram of CL detection, (B) chromatogram of UV detection (234 nm), PCs = phosphatidylcholine derivatives [55]

4 2 2 Direct and indirect detection using metal ions

Very few authors have discussed the mechanism of metal ion catalysis. The formation of a six-membered chelate ring by $Cu^{(II)}$ and luminol was suggested by Yu et al [58] to explain the difference between luminol and isoluminol Komatsu et al [59] demonstrated a very sensitive method for the determination of $Co^{(II)}$ with a detection limit of 0.06 pg, without being able to elucidate the exact role of the $Co^{(II)}$ ion Nevertheless, the detection of metal ions can be used for the selective determination of haem-containing proteins via the complexed $Fe^{(II)}$ ion. Maltsev et al [60] were able to detect 10 ng/ml myoglobin after HPLC separation of a diluted human serum sample (Fig 13).

The catalysing role of metal ions can also be used in an indirect way an analyte forming a complex with the catalysing metal ion will cause a drop in the CL intensity, and a negative peak will result. As proteins have some metal-complexing properties, this principle was applied to protein analysis by Hara and co-workers [61–65] They described the FIA of proteins based on the complexation of $Cu^{(II)}$ [61], $Fe^{(III)}$ [62] and $Co^{(II)}$ [63] In the last report the ultrasonically induced luminol CL catalysed by $Co^{(II)}$ (described by Komatsu et al [59]) was applied to the indirect measurements of proteins, resulting in detection limits of 0 2 ng. This detection principle was coupled with HPLC by the same authors [64,65]. In both papers $Cu^{(II)}$ was used because of its fast complexation kinetics with proteins MacDonald and Nieman [66] described a similar method for the determination of amino acids based on suppression

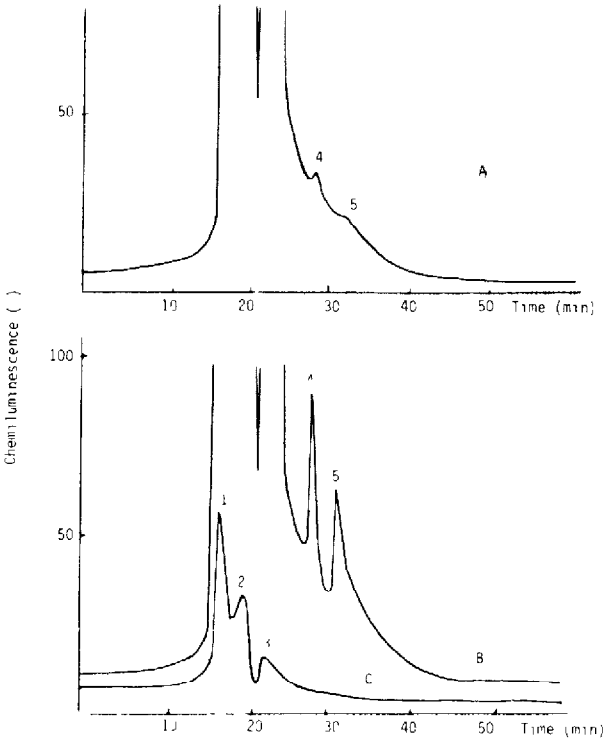


Fig 13 HPLC patterns of haem-containing compounds with luminol CL Chromatograms of (A) normal serum and (B and C) serum after myocardial infarction Peaks 1 = haemoglobin, 2, 3 and 4 = unidentified, 5 = myoglobin Dilution of serum (A, B) 1 10, (C) 1 1000 Sample B contained 2 6 μg/ml myoglobin [60]

of $Co^{(II)}$-catalysed CL with various detection limits for different amino acids (0 04 nmol for histidine to 20 nmol for aspartic acid) In all methods using suppressed CL measurements, great care has to be taken that no interfering metal ions influence the sensitivity A disadvantage of this, as of all indirect measurements, is the limited linear range

4 2 3 Luminol as a label

In principle the detection of luminol-labelled analytes can be carried out very sensitively and selectively. It is surprising that only a few papers have dealt with this detection method Kawasaki et al [67] were the first to describe the application of modified luminol as a label in liquid chromatography N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) was used for the labelling of fatty acids and several amines. The detection limit for cholic acid was ca 20 fmol Yuki et al [68] applied ABEI for the determination of eicosapentanoic acid (EPA), a prostaglandin precursor, in serum The recovery of EPA added to

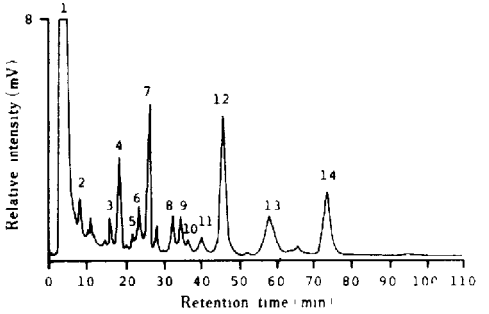


Fig 14 HPLC of ABEI-labelled fatty acids in human serum with luminol CL detection Peaks 1=ABEI, 2=lauric acid, 3=myristic acid, 4=linolenic acid, 5=eicosapentanoic acid, 6=palmitoleic acid, 7=unknown substance, 8=linoleic acid, 9=arachidonic acid, 10=docosahexanoic acid, 11=dihomo-γ-linoleic acid, 12=palmitic acid, 13=oleic acid, 14=margaric acid (ref 68)

human serum was nearly 100%, with a detection limit of 200 fmol Furthermore eleven fatty acids could be detected in human serum samples (Fig 14) Spurlin and Cooper [69] described another label, isoluminolisothiocyanate, for the derivatization of primary and secondary amino acids with detection limits of 10–20 fmol An off-line CL detection method for the determination of hormonal anabolics was presented by Jansen and co-workers [70,71] After selective fractionation by HPLC the fractions were labelled with ABEI and the CL intensity was measured This method should replace the more conventional radioimmunoassay method

5 LUCIGENIN CHEMILUMINESCENCE

5 1 Mechanism

Lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate) reacts in strongly alkaline media with oxidants [72] such as hydrogen peroxide and with organic reducing compounds [73] such as glucose, glucuronic acid, ascorbic acid and α-hydroxycarbonyls The mechanism in the oxidation reaction is likely to in-

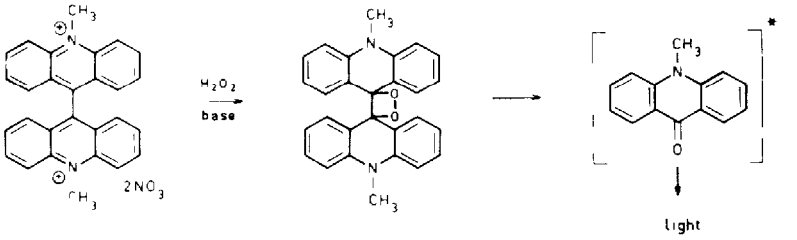


Fig 15 Lucigenin reaction scheme

volve a dioxetane intermediate (see Fig 15) The CL efficiency of ca 2–3% is slightly higher than in the case of luminol, but, generally, not as high as for the peroxyoxalate reaction. The mechanism involving organic reductants as analytes is not well understood. Obviously, lucigenin CL can be used in analytical systems for the detection of hydrogen peroxide or for the organic reducing compounds mentioned above

5 2 Detection systems and biomedical applications

5 2 1 Detection of reductants

Veazey and co-workers [74,75] developed a detector for the determination of ppm amounts of ascorbic acid and dehydroascorbic acid Solutions of potassium hydroxide and lucigenin were added post-column. An interesting application for glucuronides was described by Klopf and Nieman [76]. Glucuronides were hydrolysed on-line to glucuronic acid by immobilized β-glucuronidase Subsequently, the glucuronic acid was separated from interfering compounds and mixed with chemiluminescent reagents just before the detector Thus, low concentrations (10 μM) in urine samples could be detected. However, as the enzymatic hydrolysis was followed by a chromatographic clean-up step, no individual glucuronides could be quantitated. For the determination of separate glucuronides, the HPLC separation should be carried out prior to the enzymatic reaction.

Another example of the detection of biological reductants was reported by Maeda and Tsuji [77]. Compounds containing an α-hydroxycarbonyl group gave intense CL with alkaline lucigenin solutions Based on that principle, the CL detection of corticosteroids (without derivatization) and carboxylic acids derivatized with *p*-nitrophenacyl bromide was developed. A system with just one reagent pump was described with detection limits of ca 500 fmol, which is similar to those obtained with fluorescence

5 2 2 Other applications of lucigenin

Maeda and Tsuji [78] developed a novel fluorescence and CL detection method for steroid and bile acid sulphates This flow injection system was based on the ion-pair formation of the negatively charged organic sulphates with lucigenin. In the fluorescence mode a detection limit of 25 pmol steroid sulphate was obtained In the CL mode hydrogen peroxide and base in methanolic solution were added in front of the detector, with detection limits of 0 5 pmol

6 BIOLUMINESCENCE REACTIONS

Many light-producing reactions are derived from living organisms Generally, BL reactions are catalysed by enzymes ('luciferases'). BL has been observed and studied for centuries [79] A main aspect of these reactions is that

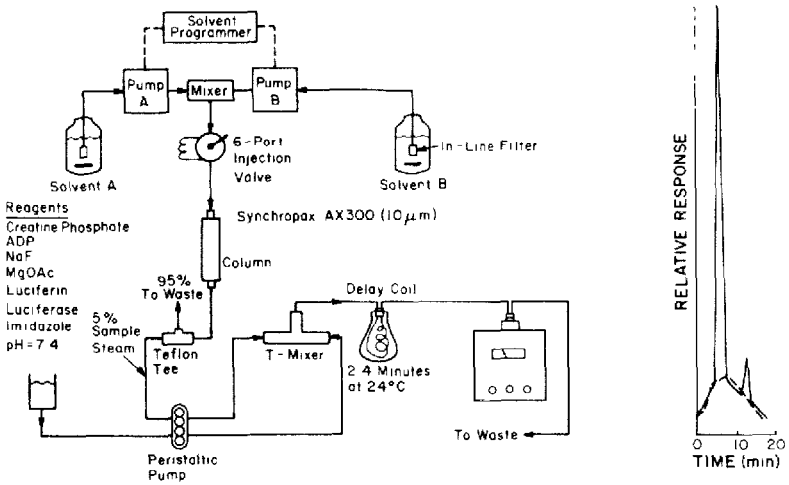


Fig 16 BL monitoring of creatine kinase (CK) isoenzymes separated by chromatography Schematic of the instrumentation and chromatographic separation and on-line BL monitoring of 50 μl of an authentic sample The dashed line represents a typical reagent blank and the solid line represents signals from creatine kinase with a trace of CK-MB isoenzyme from myocardial infarction [80]

they often possess a very high CL efficiency (see Section 2 1) Therefore, such reactions are highly interesting for analytical applications, and some examples of their use for HPLC detection have been published.

The isoenzymes of creatine kinase (CK), which are important for muscle contraction, have been separated by ion-exchange chromatography and detected by the so-called firefly luminescence [80]. The reaction scheme is·

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP} \quad (3)$$

$$\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{adenyloxyluciferin} + \text{light} \quad (4)$$

Picomole amounts of ATP can be detected, and this method was used for the analysis of blood samples from myocardial infarct patients. The scheme of the detection system and a chromatogram of a serum sample are shown in Fig 16 BL was also used for the detection of bile acids after a chromatographic separation [81] Bile acids react with nicotinamide-adenine dinucleotide (NAD) in the presence of 3α-hydroxysteroid-NAD oxidoreductase The product of this reaction, nicotinamide-adenine dinucleotide, reduced (NADH), can be detected by the following reaction with flavin mononucleotide (FMN)·

$$2\,\text{NADH} + \text{FMN} \rightarrow 2\,\text{NAD} + \text{FMNH}_2 \quad (5)$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{light} \quad (6)$$

Peroxyoxalate CL (see Section 3) is also suitable for the detection of NADH [2], but the sensitivity is not very high Oxidoreductase and bacterial luciferase have also been immobilized for the BL monitoring of reactions yielding NADH [82,83] Immobilized enzymes can also offer interesting possibilities for detection in HPLC [84]. Generally, such a system is cheaper and simpler than the addition of the enzymes by pumps

7 OTHER CHEMILUMINESCENCE REACTIONS

Some well known CL reactions that have been used in combination with HPLC have been discussed in the Sections 3–5 Some other reactions, based on the oxidation of organic compounds by hydrogen peroxide, have also been applied for detection in HPLC Hara and co-workers [85,86] have used the metal-catalysed reaction between 1,10-phenanthroline and hydrogen peroxide for the determination of proteins. Proteins such as albumin and γ-globulin decrease the catalytic activity of metal ions by complex formation and, in this way, can be determined in the picogram range (for a similar system, see Section 4 2 2) The system has also been used for the detection of proteins separated by immunoaffinity and metal chelate chromatography The solutions of

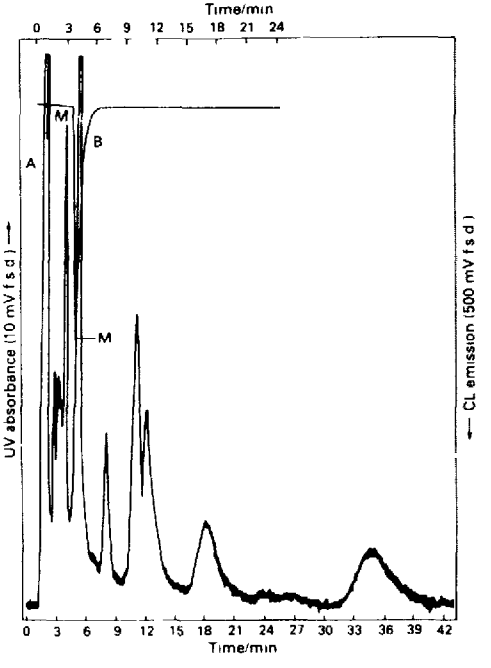


Fig 17 UV and CL chromatograms for morphine in urine 5 μg/ml spiked urine sample (500 ng on-column) (A) UV detection at 280 nm, (B) CL detection M=morphine [87]

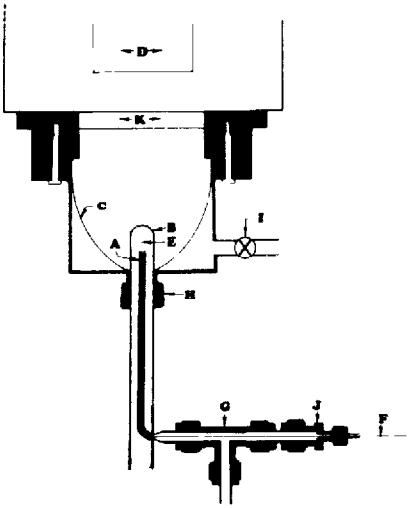


Fig 18 Scheme of the CL aerosol spray detector A=1 2 mm I D Pyrex tube (nebulizer), B=reaction cell, C=ellipsoidal mirror, D=photomultiplier tube, E=end of reaction cell (not painted black), F=polyethylene capillary tube, G=union-T, H=compression fitting, I=vacuum valve, J=reducing union [88]

1,10-phenanthroline, hydrogen peroxide and the $Cu^{(II)}$ salt used as a catalyst are added post-column

Abbott et al [87] have described a method of the determination of morphine in body fluid based on the CL reaction of this drug in the presence of permanganate in an acidic tetraphosphate (polyphosphoric acid) medium Fig 17 shows the chromatogram of a spiked urine sample with UV and CL detection The higher sensitivity and selectivity of CL detection is clearly demonstrated. Other opiates and related drugs can also be detected by this CL system

A CL aerosol spray detector for HPLC based on CL induced by ozone or singlet oxygen was described in 1980 by Birks and co-workers [88,89]. A schematic diagram of the detector is shown in Fig. 18. This detector responds to highly fluorescent compounds such as rhodamine and fluorescein derivatives, olefins, divalent sulphur compounds and certain nitrogen compounds such as hydrazines, azides and nitrogen heterocyclic compounds Detection limits were in the low microgram to low picogram range A similar principle has also been applied using a thermal energy analyser [90] or redox CL [91] for HPLC detection In the former system, nitric oxide is formed by pyrolysing nitrosyl-containing compounds, in the latter system organic species, such as sugars, are oxidized with dilute nitric acid, nitrous acid or nitrite ions In all systems, nitric oxide is detected by its CL reaction with ozone An interesting biomedical application of the thermal energy analyser as HPLC detector has been presented by Yu and Goff [90]. They used this detection system for the determination

of vasodilators such as glycerol trinitrate, isosorbide dinitrate and pentaerythritol tetranitrate, and their metabolites in plasma. Sub-nanogram detection limits have been obtained and ppb concentrations have been determined

A special mode of CL is electrochemiluminescence, i.e light emission caused by the electrolysis of solutions of analytes Malcolme-Lawes and co-workers [92–95] have published several papers on the optimization of the electrochemical cell coupled to HPLC. Many classes of aromatic compounds emit light during electrolysis, and detection limits in the nanogram range have been obtained Derivatization can extend the range of compounds detectable in this way [95]. So far, electrochemiluminescence detection has not been used for real samples

8 CONCLUSIONS

Various CL reactions have been applied for detection in HPLC and a high sensitivity (femtogram to picogram range) has been obtained for many compounds Detection systems based on the peroxyoxalate CL reaction seem the most versatile because many compounds can be excited by this reaction Several biomedical applications of such a detection system have been published. The mechanism and the kinetics of the reaction are still under investigation and seem complex The kinetics are especially important, because they influence the part of the CL decay curve measured in a flow system Derivatization of analytes is important for peroxyoxalate CL, because many non-chemiluminescent compounds can be converted into suitable derivatives The peroxyoxalate reaction can also be used for the determination of low amounts of hydrogen peroxide In biomedical analysis this can be applied for the detection of compounds that produce hydrogen peroxide via an enzymic reaction

The luminol and lucigenin reactions can also be used for the determination of hydrogen peroxide and related peroxides. The luminol reaction is very suitable for the detection of metal ions and metal-containing compounds because metal ions catalyse the reaction. The principle can be used in an indirect way for compounds, such as proteins, that form complexes with metal ions, thereby causing a decrease in the CL signal Luminol-type labels seem very promising for the detection of acids and amines. Detection limits of fatty acids and amino acids are in the femtomole range Probably, similar labels can be developed for compounds with other functional groups. The lucigenin reaction can be used for the detection of organic reducing compounds, e.g glucuronic acid obtained by enzymic hydrolysis of glucuronides Some other CL reactions with different mechanisms have also been successfully used for HPLC detection

Only a few applications of BL detection in HPLC are known, but this field has many possibilities, because BL reactions are very selective and sensitive Generally, the disadvantage of such systems is that they consist of several steps and, therefore, are relatively complex. The compatibility of enzymes with the typical constituents of HPLC mobile phases will require much attention

CL detection based on various reactions is now one of the most sensitive detection modes in HPLC and very suitable for biomedical analysis A more systematic optimization of several systems still is necessary and, as for the apparatus, there is a need for commercially available dedicated CL detectors The consequence should be that CL detection can be used for the routine analysis of complex samples in many laboratories

9 SUMMARY

During recent years, much progress has been made in the development of high-performance liquid chromatographic (HPLC) detection systems based on chemiluminescence (CL) CL is now one of the most sensitive detection methods in HPLC For many compounds detection limits in the femtogram to picogram range have been obtained Several on-line post-column reactions have been used for chemical excitation of the analytes Some theoretical aspects of CL detection are presented and special attention is devoted to the coupling of CL to flow systems The influence of the kinetics of the reaction on the sensitivity of the detection system is stressed The mechanisms and detection systems of the peroxyoxalate, luminol and lucigenin CL reaction are described A few examples of the use of bioluminescence for HPLC detection are given, and some less common CL reactions used in flow systems are also mentioned Many biomedical and related applications are shown Possibilities and limitations of the various reactions and detection systems are evaluated

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