Solution Chemiluminescence—Some Recent Analytical Developments*

Plenary Lecture

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Some recent developments in analytical solution chemiluminescence (CL) are described. These include various approaches for monitoring CL, especially flow injection, applications based on luminol and peroxyoxalate CL, direct determinations based on the CL of the analyte, and the uses of CL for liquid chromatographic detection.

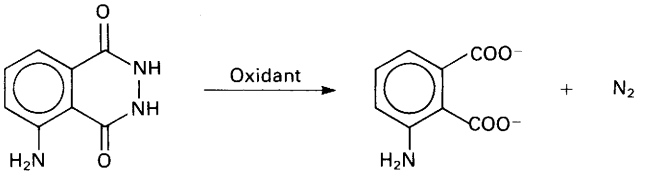
Keywords: *Chemiluminescence; flow injection; liquid chromatographic detection; luminol; peroxyoxalate*

Chemiluminescence (CL) is the emission of radiation, usually in the visible or near infrared region, as a result of a chemical reaction. One of the reaction products is formed in an electronic excited state, and emits the radiation on falling to the ground state. A general description of the reactions is

$$A + B \rightarrow C^* + D$$
$$C^* \rightarrow C + h\nu$$

where * indicates an electronic excited state.

The best known example of such a reaction is the oxidation of luminol (**I**) to produce excited 3-aminophthalate (**II**):



(I) (II)

The spectrum of the CL of this system is identical with the fluorescence spectrum of 3-aminophthalate.[1] Much of the current analytical interest in CL arises from this means of exciting molecules without the need for sample irradiation and the consequent problems of light scattering, unselective excitation and source instability.

In some instances, the excited product (C*) is an ineffective emitter, but by transfer of the excitation energy to an efficient fluorophore (F) added to the system, a considerable increase in luminescence may be achieved:

$$C^* + F \rightarrow C + F^*$$
$$F^* \rightarrow F + h\nu_1$$

The emission is now identical with the fluorescence of F, so that this process, known as sensitisation, enables the luminescence of F to be stimulated without the need for irradiation. The peroxyoxalate CL systems described below are good examples of this type of process.

Chemiluminescence can be produced in the gas phase,[2] including flames,[2,3] and on solid surfaces (*e.g.*, candoluminescence[4] and electroluminescence[5]). These phenomena have all been used analytically, often with great sensitivity, but will not be discussed here. This review will be concerned with CL in solution, and even here some considerable restrictions will be applied. For example, bioluminescence (BL) is the CL produced by a wide variety of organisms (fireflies, bacteria, etc.).[2] The reactions provide extremely sensitive analytical procedures, but are not discussed here. The uses of CL or BL reactions in immunoassays are being shown to provide extreme sensitivity, but again are not included. Finally, electrogenerated CL (*i.e.*, electrochemical reactions producing solution CL), although the subject of renewed interest,[6] remains of rather restricted application, and is omitted.

Chemiluminescent reactions can occur very rapidly (<1 s) or can be long lasting (>1 d). Although the duration is influenced by the reaction conditions, such a wide range presents a challenge to the development of instrumentation for CL monitoring. The light intensity produced is dependent on the CL quantum yield. For BL systems this is often high (*e.g.*, 0.88 for firefly luciferin[2]), resulting in detection limits down to 1×10^{-21} mol in the most favourable instance.[7] For non-biological systems, however, the most efficient system (peroxyoxalate) has a quantum yield of up to 0.50,[2] but other common systems, such as luminol or lucigenin, have quantum yields of only 0.01,[2] and many of the less well known systems that will be dealt with later in this review have quantum yields many orders of magnitude less. The highly selective nature of CL reactions, and the frequent, almost complete absence of background emission, however, means that it is possible to monitor even such very inefficient reactions without difficulty, thus allowing them to be used for analytical purposes.

Analytical interest in CL has increased considerably over the last decade. This is especially clear from the abundance of books and review articles that have appeared during this time,[2,8–20] and which the present author does not wish to duplicate. The present paper, therefore, is intended as a rather personal selection of recent developments and trends in analytical applications of solution CL, and of the instrumentation that has been developed for this purpose. The applications are divided into those procedures that involve established CL reactions, such as those of luminol or peroxyoxalates, and those that involve direct CL reactions.

Instrumentation

Until recently, the major application of CL has been the determination of adenosine 5′-triphosphate (ATP) in the clinical field, based on BL reactions. Such systems are highly sensitive, and the reactions can take several minutes. This has enabled very simple instrumentation to be used,[21] so that commercial luminometers have almost entirely been based on a direct injection batch procedure. The sample, in a cuvette, is placed in a light-tight box, the CL or BL reagent added (usually as drops from a syringe) and the light intensity monitored (usually over a pre-determined time interval) by an

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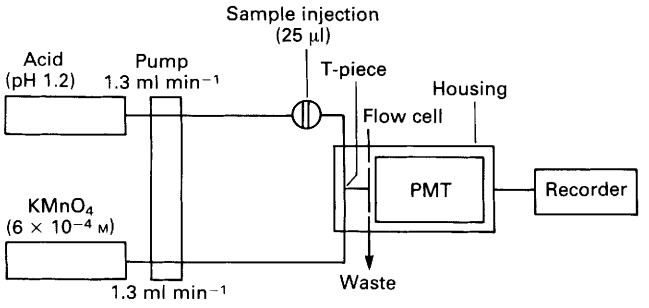


Fig. 1. Schematic diagram of a typical flow injection assembly for chemiluminescence monitoring (used for the detection of morphine)[88]

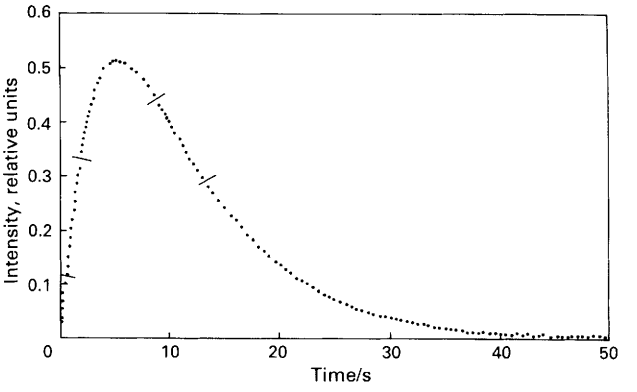


Fig. 2. Intensity - time profile obtained from the CL stopped-flow determination of 5.3×10^{-7} M H_2O_2 (3.2×10^{-4} M luminol and 4.0 µg ml^{-1} of cobalt). Reproduced, with permission, from reference 33

adjacent photomultiplier tube (PMT). Because there is only one emitting species, wavelength discrimination is not necessary. Recent developments in such commercial luminometers have been in the mechanisation or automation of the reagent addition systems, the addition of multi-cuvette holders and computerised data handling, but the basic concept is unchanged. Syringe-driven reagent-addition luminometers have been described, however, which perform very satisfactorily.[21,22]

The majority of the CL reactions discussed in this review are fast (*i.e.*, are complete in a few seconds) or can be made to be so. For the less efficient reactions, especially, a fast reaction is necessary in order to produce a reasonable burst of photons. Thus, the batch luminometer, which has a rudimentary reagent addition system and probably no mixing device, will not allow reproducible monitoring of fast CL reactions. The batch procedure also requires a separate cuvette for each sample, and the light-tight apparatus must be opened to insert each cuvette, or set of cuvettes, thus necessitating special precautions to protect the PMT. The recent modification of immunoassay-type microtitre plates to monitor CL or BL, whilst of considerable interest, does not solve these problems.[23]

Three other major approaches have been used to monitor CL reactions. Perhaps the most successful of these is based on flow injection (FI).[24] A typical instrumental set up is shown in Fig. 1. The sample is injected into one flowing stream of appropriate pH remote from the detector; the CL reagent flows in the other stream. The two streams meet head on at the T-piece, inside a light-tight casing, then flow through a flat coil placed immediately in front of a PMT. The coil serves to retain the solution in view of the PMT whilst it is emitting most intensely (*e.g.*, for 5–15 s). The external flow tubing can be of black, opaque plastic, thus preventing light piping, and the internal assembly can readily be made completely light tight, as it never has to be opened, except for repair. The whole assembly is very compact and inexpensive. It provides very rapid, reproducible mixing, thus giving reproducible emission intensities, and allows rapid sample throughput. The concept was introduced by Rule and Seitz[25] and has since been used by numerous workers, including ourselves,[26,27] with great effect.

The design of both the mixing device and of the means of retaining the emitting solution in view of the detector are important. Mixing is most effective at a T-piece, but a Y-junction can be used, and some workers have used conventional dispersion of an injected sample into the surrounding flowing reagent to achieve mixing. This last approach is reproducible, but not very rapid. A description of some of the retaining cells proposed has been given by Seitz.[10] Recently, the use of a flowing "film" of solution has been described for this purpose.[28] Petersson *et al.*[29] have designed an FI microconduit assembly for CL monitoring. Another major advantage of this flow approach is that the systems developed are readily useful for liquid chromatographic (LC) detection, and flow luminometers readily function as LC detectors, as will be described below.

Table 1. Detection limits (DL) for H_2O_2 by chemiluminescence

Reagents	DL/mol l^{-1}	System	Reference
Luminol/Co^{2+}	5×10^{-12}	FI	36
TCPO*	1×10^{-9}		37, 38
Luminol/haemin	5×10^{-9}	Continuous flow	39
Luminol/Cu^{2+}	3×10^{-8}	Batch	35
Imm.† luminol/haemin	1×10^{-6}	FI	40
Imm. TCPO	1×10^{-8}	FI	41
Solid TCPO	6×10^{-9}	FI	42

* TCPO = bis(2,4,6-trichlorophenyl) oxalate.
† Imm. = immobilised.

About a decade ago, attempts were made to use centrifugal analysers for CL monitoring[30–32] because of their proven advantages of rapid and convenient multi-sample processing. Although CL could be measured in this way, the optical design and the intermittent light collection meant a considerable loss of potential sensitivity which would have greatly restricted the applications of the technique had it been pursued.

The stopped-flow approach has many attractive features when applied to CL monitoring. This was used most recently by Perez-Bendito and co-workers.[33] Again, very rapid mixing is achieved, the emitting solution may be retained in the measuring cell for whatever time is desired and, unlike the FI approach, the intensity - time variation can be monitored, thus allowing kinetic measurements to be made. A typical intensity - time plot for luminol oxidation, obtained in this way, is shown in Fig. 2. Up to 60 samples per hour were monitored by this technique.

Uses of Established CL Reactions

Luminol

Luminol (**I**), when oxidised by most strong oxidants in alkaline solution, gives rise to a characteristic blue luminescence. The reaction is catalysed by a number of metal ions of which iron(II), copper and cobalt are particularly effective.[8] The most obvious use of the reaction has been to determine oxidants. Mayneord *et al.*,[34] for example, applied the reaction to H_2O_2 determination in irradiated water; a modification of this procedure, 10 years later in 1965,[35] gave a detection limit of 3×10^{-8} M H_2O_2, limited by the blank response. It is interesting that since that time the detection limit for H_2O_2, using luminol or other CL reagents, in a variety of experimental arrangements hardly changed until Abbott[36] achieved a considerable improvement, using equipment similar to that shown in Fig. 1. Some detection limits reported for H_2O_2 are

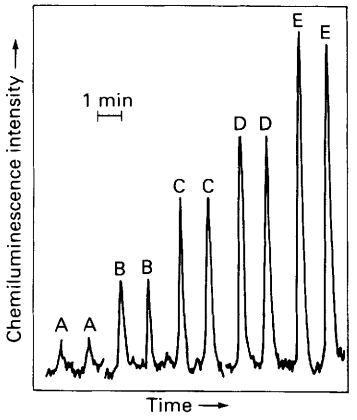


Fig. 3. CL - time responses for various concentrations of cobalt obtained after elution from a cation separation column with 3.0 mM $BaCl_2$ at pH 3.0. A, 1; B, 10; C, 20; D, 30; and E, 40 pg ml^{-1} of Co. Reproduced, with permission, from reference 48

summarised in Table 1. The procedure of Hool and Nieman[40] is unusual in that the reagents are released slowly into the solution by diffusion through a microporous membrane.[43] Phosphatidyl hydroperoxide (≥7 nmol) was detected by luminol - cytochrome C CL after LC separation.[44]

The luminol reaction has also been used to determine compounds which interact with the oxidant. For example, if hypochlorite is used as the oxidant, ammonia (0.1–5 mM) can be determined by an FI procedure by its reaction with the oxidant, thus decreasing the production of CL.[45] Alkyl phosphates or phosphonates react with H_2O_2 to give the corresponding peroxo compounds. These species oxidise luminol much more rapidly than H_2O_2 in alkaline solution, and can thus be determined very sensitively. By appropriate attention to the reaction conditions, a detection limit of 0.5 ng was obtained for the nerve gases DFP (diisopropyl fluorophosphate), sarin (*O*-isopropyl methylphosphonate) and soman (1,2,2-trimethylpropyl methyl fluorophosphate).[46]

Another ingenious application of the luminol reaction has been for the determination of thiols.[47] A thiol derivative of luminol (*N*-mercaptoacetylluminol) is bound to Sepharose 6B (a thiol polysaccharide) by an S–S bond. At pH 9.2, thiols exchange quantitatively with the luminol derivative on the Sepharose, and the released luminol is determined by its CL in a stopped-flow apparatus. The detection limit was 5×10^{-12} mol of thiol.

Determination of metal ions by their catalysis of the luminol or lucigenin reactions has been reported on numerous occasions.[8] Cobalt is particularly active but iron(II), copper, chromium and manganese are also good catalysts. The procedures for their determination are well known, and will not be elaborated here. A recent, interesting development has been the use of such reactions to detect metal ions separated by LC. Cobalt has received particular attention, with Sakai *et al.*[48] obtaining a detection limit of 0.1 pg of Co per 100 µl of sample, with ion-chromatographic separation and on-line luminol CL detection (Fig. 3). Jones *et al.*[49] obtained a detection limit of 0.1 pg of Co per 200 µl of sample after reversed-phase LC separation. Similar detection limits had earlier been obtained in an FI system.[26] The main problem with the chromatographic procedures is the need for better separations.

The effect of chelation of the catalytic metal ion has also been utilised. Polyamines such as ethylenediamine have been determined by reaction with salicylaldehyde to form a Schiff's base, which enhances the catalytic activity of manganese in the luminol - H_2O_2 reaction.[50] This type of reaction has also been used to distinguish between chelated and non- or weakly-complexed chromium(III). The strongly bound chromium has a much weaker catalytic effect.[51] Amino acids, separated by LC, have been detected by their effect on the copper catalysis of the luminol - H_2O_2 reaction.[52]

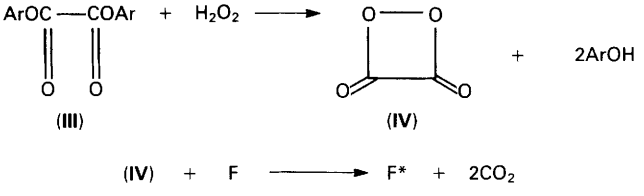
A major developing use of CL, mainly of luminol, or of firefly or bacterial BL, is to detect the products of enzyme-catalysed reactions.[2] Some luminol applications will be described here. The luminol is used to detect an oxidant, usually H_2O_2, produced in such a reaction. Glucose, for example, in blood or urine could be determined by oxidation in the presence of glucose oxidase to produce H_2O_2, which was detected by CL.[43, 53–56] Other substrates of oxidases can be determined similarly [*e.g.*, cholesterol,[57–59] uric acid[60] and reduced nicotinamide adenine dinucleotide (NADH)[61]] down to *ca.* 1×10^{-8} M.

Some compounds can be determined by a sequence of enzyme-catalysed reactions culminating in H_2O_2 production. A typical example is sucrose, which is converted in sequence to α-D-glucose (and fructose) and β-D-glucose before applying the glucose oxidase reaction. This sequence was used by Koerner and Nieman[62] in conjunction with their microper-oxidase reactor to determine 5–1000 µM sucrose.

Finally, mention must be made of the greatly enhanced CL of luminol, produced when horseradish peroxidase is used as the catalyst, in the presence of substituted phenols (*e.g.*, *p*-iodophenol) or naphthols or 6-hydroxybenzothiazoles (including firefly luciferin).[63,64] So far, this has been used for enhancing sensitivity in CL immunoassay, but it should also be useful for other applications.

Peroxyoxalate Systems

Certain aryl (Ar) oxalate esters (**III**) react with H_2O_2, and, most efficiently of all non-biological systems, produce CL if a sensitiser is present.[65,66] The reaction is suggested to proceed via a cyclic intermediate, 1,2-dioxetanedione (**IV**), which then interacts with the sensitiser (F) to excite it and liberate carbon dioxide:



Almost all the analytical applications of this type of reaction, with the exception of H_2O_2 determination (see below), are concerned with the determination of molecules which act as sensitisers. In such applications, the intermediate (**IV**) replaces irradiating photons in exciting the fluorophore, F. It is not surprising, therefore, that the applications of oxalate ester CL are to molecules that have previously been determined by spectrofluorimetry, or have been converted into species that could so be determined. Compounds that are highly fluorescent, therefore, are very sensitively determined by this CL procedure. They include polycyclic aromatic hydrocarbons,[67] of which perylene is generally the most sensitive. The great advantage of chemical excitation is the greatly decreased background signal, thus enabling much improved detection limits to be achieved, often in the sub-fmol region.[68]

As with spectrofluorimetry, direct application of CL measurements to real samples is often impossible or undesirable, because of the effect of other sample components. Therefore, the use of oxalate ester CL for LC detection is widely applied to systems where fluorescence detection has already been shown to be useful. Thus, the technique has been applied with advantage to coal tar extracts[69] (Fig. 4) and urinary porphyrins, which exhibit native fluorescence, and also, for example, to dansylated amino acids,[70] steroids[71–73] and aliphatic amines,[74] and to fluorescamine labelled cate-

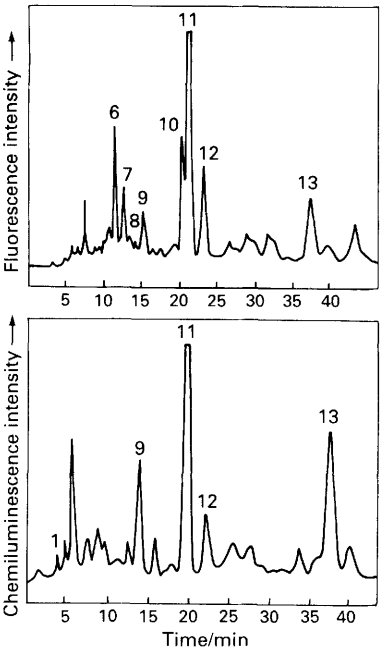


Fig. 4. Chromatograms of polycyclic aromatic hydrocarbons in a coal tar extract. Comparison of CL and fluorescence detection. Reproduced, with permission, from reference 69. 1, Indene; 6, fluoranthene; 7, pyrene; 8, 1,2-benzofluorene; 9, benz[*a*]anthracene; 10, benzo[*b*]fluoranthene; 11, perylene; 12, benzo[*a*]pyrene; and 13, 9,10-diphenylanthracene

cholamines.[75] The system has also been used for detection of dansylated amino acids separated by thin-layer chromatography.[76] Detection limits in the fmol region are often obtained for such favourable systems.

Peroxyoxalate systems have some advantages over luminol and similar CL reagents. Their efficiency and therefore sensitivity is greater, and they are not susceptible to metal ion catalysis or effects of oxygen. The detection limit for H_2O_2 is similar to values reported for the luminol system (Table 1), and the system has been applied to the determination of H_2O_2 produced by a photochemical reactor (detection limit, 1.5×10^{-8} M)[77] and to the determination of uric acid[78] or glucose[61] after enzymic generation of H_2O_2.

There are also some complications with peroxyoxalate systems that are not found in the non-sensitised CL and BL systems. The first is that the oxalate esters are generally not water soluble, and are more or less susceptible to hydrolysis. This places some restriction on their applicability to aqueous samples, although this is much less of a problem when used for LC detection. The second is that the luminescence intensity depends on the particular ester, and the "pH" used. Bis(2,4,6-trichlorophenyl) oxalate (TCPO) gives greatest intensity, *i.e.*, reacts fastest, at "pH" 7.5. In bis(2,4-dinitrophenyl) oxalate, the dinitrophenyl group is a better leaving group, so that reaction is faster, CL intensities are greater and measurable luminescence can be achieved at a "pH" as low as 3.5.[68] However, peroxyoxalate systems do exhibit CL in the absence of a sensitiser.[79] Although this is an extremely weak emission, it does provide a measurable background at high amplification, and thus restricts the signal to noise ratio. The intensity is also dependent on a number of experimental factors, such as solvent and reagent purity, solvent composition and, in flow systems, on pulsing originating in the pumps, a phenomenon which might arise from mixing variations. It is not surprising that this noise is greater with the more sensitive oxalate ester, and is usually the factor governing the limit of detection.

Much work is being carried out on improving the properties of oxalate esters used for CL generation. An alternative approach has been to accept the benefits of water insolubility, and to use solid TCPO[42] as the reagent, which dissolves very slowly in the flowing medium.

Table 2. Some direct chemiluminescent determinations

Analyte	Oxidant system	Detection limit/ mol l^{-1}	Reference
Sulphide . .	ClO^-	1×10^{-7}	81
	H_2O_2 - peroxidase	1.5×10^{-9}	82
Adrenaline . .	H_2O_2 - OH^-	6×10^{-7}	80
Humic acids . .	MnO_4^- - OH	0.7*	83
Paracetamol . .	Ce^{IV}	4×10^{-7}	84
Quinones	H_2O_2 - OH^-	1×10^{-5}	85
Hydrazine	ClO^-	*ca.* 10^{-8}	86,87
Morphine	MnO_4^- - H^+	1×10^{-10}	88
Naphthols	MnO_4^- - H^+	5×10^{-7}†	89
Tetracycline . .	Br_2 - OH^-	4×10^{-5}	90
	Dibromodimethyl-hydantoin	1×10^{-6}	91
Sulphenyl esters . .	Iodosobenzene	5×10^{-4}	92
Loprazolam . .	MnO_4^- - H^+	7×10^{-6}	93
Streptomycin . .	MnO_4^- - H^+	*ca.* 10^{-5}	36
Sulphite	MnO_4^- - H^+	1×10^{-6}†	94
	MnO_4^- - H^+	1.5×10^{-8}‡	95

* mg l^{-1}.
† With sensitiser.
‡ With 3-cyclohexylaminopropanesulphonic acid.

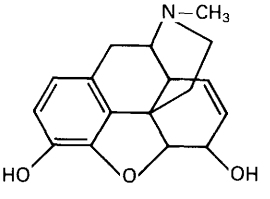
Direct Chemiluminescent Determinations

The determinations described above involved interaction of the analyte with well established CL reactions, often as a catalyst or sensitiser or after a sequence of other reactions. Further development of analytical procedures involving this very small number of CL reactions will require considerable chemical ingenuity, and must be very restrictive to the wider application of CL. The alternative approach, which is gathering momentum, is to seek CL reactions of the analytes themselves. The search is bringing to light a number of unsuspected CL systems, some of which are described below.

New CL reactions are generally discovered by testing the analyte with a wide range of oxidants (and reductants) under an equally wide range of conditions.[36,80] A typical set of oxidants might be H_2O_2, ClO^-, cerium(IV), IO_4^-, MnO_4^- (H^+ and OH^-) and Br_2, with the possibility of also adding catalysts and of carrying out the reactions at different pH values. There are some guidelines for predicting which analytes are likely to generate CL. For example, if oxidation of the molecule is known to give a fluorescent product (as was the case with morphine, tetracycline and streptomycin, as described below), or if the analyte itself has the type of structure that might lead to fluorescence, there is a possibility that oxidation of the analyte will give CL. Very often, however, the CL reactions discovered in screening tests cannot be predicted; frequently, also, a predicted CL reaction is found not to emit.

A selection of direct determinations based on CL is given in Table 2. The detection limits vary considerably, but many of the procedures use μl volumes of sample solution, so that even relatively insensitive procedures in concentration terms are able to detect down to nmol amounts.

The development of a procedure for morphine is typical.

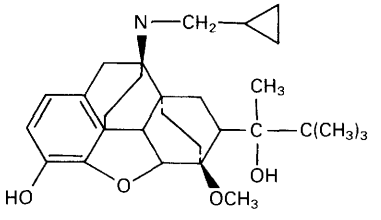


(V)

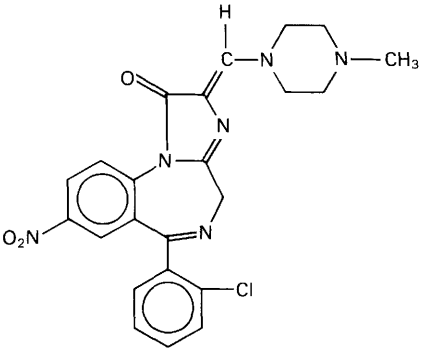
Morphine (**V**) can be determined spectrofluorimetrically after oxidation to a dimeric product, pseudomorphine.[96] It was possible, therefore, that the oxidation reaction would produce CL, as was found to be the case. Acidic permanganate gave the greatest intensity, with tetraphosphoric acid being the best acid.[36,88] A screen of related narcotic analgesics and of other drugs showed that, of the compounds tested, only morphine and some of its derivatives gave intense CL. These results showed that for intense CL to occur, there must be a free OH group at position 3. Therefore, heroin (3,6-diacetylmorphine) and morphine 3-glucuronide, an important metabolite of morphine, do not give intense CL. Interestingly, buprenorphine (**VI**), a drug attracting much attention as a morphine substitute, also gives intense CL, and can be determined on that basis.[97]

The very low detection limit for morphine indicated that CL detection might be suitable for forensic purposes, and thus an LC procedure was developed that could be applied to blood or urine samples. The original method,[98] after sample clean up, used an acidic eluent, which would mix post-column with the aqueous permanganate stream. The acidic eluent made it necessary to use a polymer column. The procedure had an on-column detection limit of 5 ng of morphine. Subsequent modification of the procedure, especially avoiding the use of the acidic eluent by incorporating the acid in the permanganate stream, has decreased the detection limit to 0.08 ng on-column.[99]

Another example of the unexpected occurrence of CL was found during a screen of benzodiazapines for CL in an acidic permanganate medium. Of seven compounds tested, only loprazolam (**VII**) was chemiluminescent.[93] There was no obvious structural reason for this.



(**VI**)



(**VII**)

Conclusions

Chemiluminescence can provide analytical procedures with extremely low detection limits. Better instrumentation will undoubtedly improve this situation further, because many systems are almost free of background luminescence. There will undoubtedly be a great expansion in the number of direct CL determinations that will be possible as more CL reactions are discovered. As with luminescence processes, CL can be affected (quenched or enhanced) by other compounds present in samples, so that useful applications will require such interactions to be understood and controlled. For this reason, the use of CL for LC detection is becoming recognised as a means of avoiding interferences and of achieving an extremely reproducible and very sensitive means of analysis.

I thank those who worked with me as students or visitors, or who subsequently have developed an interest in CL, for the major contribution they have made to this subject. They include J. L. and M. Burguera, A. R. Wheatley, R. W. Abbott, A. T. Faizullah, S. A. Al-Tamrah, A. A. Al-Warthan, A. C. Calokerinos, N. P. Evmiridis and, most recently, A. R. J. Andrews. I also thank the Analytical Chemistry Trust for the award of a studentship to A. R. Wheatley, and the SERC and the Home Office Forensic Service for CASE awards to R. W. Abbott and A. R. J. Andrews. Finally, I thank Dr. P. J. Worsfold, whose parallel, but independent, contributions in this area have done much to enhance our reputation in the area of chemi- and bioluminescence.

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