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Determination of thiol-containing drugs by chemiluminescence–flow injection analysis

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Abstract: A flow injection analysis procedure with chemiluminescence detection for the determination of both thiol-containing drugs and the amino acid cysteine is described. Procedures are based on the inhibition by the drugs of the chemiluminescence generated in the copper-catalysed oxidation of luminol by hydrogen peroxide. The proposed methods were applied to the determination of cysteine, *N*-acetylcysteine, penicillamine, 2-mercaptopropionylglycine and thiouracil in pharmaceuticals.

Keywords: *Chemiluminescence; flow injection analysis; cysteine; N-acetylcysteine; penicillamine; 2-mercaptopropionylglycine; thiouracil.*

Introduction

Thiol-containing drugs are used as therapeutic agents in a variety of pharmaceutical products [1]. Among the more important thiol-containing drugs are penicillamine and *N*-2-mercaptopropionylglycine (drugs frequently used as therapeutic agents in Wilson's disease and rheumatoid arthritis and as efficient antidotes in heavy-metal poisoning), *N*-acetylcysteine (a mucolytic agent), 2-thiouracil (a thyroid depressant) and the amino acid cysteine.

Analytical procedures using chemiluminescent (CL) methods combine the advantages of speed and sensitivity and have been frequently used for the analysis of drugs [2]. It is well known that luminescent methods are capable of much better limits of detection than spectrophotometric methods. This is achieved by the almost complete absence of background emission. Thus, in luminescence spectrometry, trace analysis involves the detection of a small signal above a theoretically zero background. Flow injection analysis (FIA) allows an extreme reproducibility of both sample and reagent mixing. These characteristics are essential for precise CL studies. The oxidation of luminol to generate excited 3-aminophthalate in alkaline solution is one of the most efficient and best known CL reactions. The mechanism of this reaction is not sufficiently established [3–5]. The reaction is catalysed by several metal ions, such as copper [6], Co and Fe [7] and by enzymes: horseradish peroxidase [8], microperoxidase [9] and myeloperoxidase [10]. Several organic compounds inhibit the copper-catalysed reaction. Based on this effect, aminoalcohols and benzene derivatives [11], proteins [12–14] and amino acids [15–16] have been determined.

The aim of this work was to develop a new CL–FIA system for the determination of thiol-containing drugs and the amino acid cysteine based on the inhibition of the copper-catalysed luminol–hydrogen peroxide reaction. Sample injection produced negative peaks because the catalytic activity of Cu(II) decreased due to complex formation between Cu(II) and the thiol-drugs.

Experimental

Reagents

All chemicals were of analytical-reagent grade and the solutions were prepared with doubly distilled water. Luminol (3-aminophthalhydrazide, Fluka, Switzerland) 5×10^{-3} M solution was prepared by dissolving luminol in the buffer solution (pH 10.4) containing 0.1 M potassium hydroxide and 0.1 M boric acid. Hydrogen peroxide 10^{-2} M solution was prepared by diluting a 30% solution with water. Copper(II) 10^{-3} M solution was prepared by dissolving copper(II) nitrate (Merck,

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Darmstadt, Germany) in water. Cysteine (Sigma, USA), *N*-acetyl-cysteine (Sigma), penicillamine (Fluka), 2-thiouracil (Sigma) and *N*-2-mercaptopropionylglycine (Sigma) 10^{-3} M stock standard solutions were prepared in water and kept in dark bottles at 4°C; working standard solutions were prepared by dilution prior to use.

Apparatus

A flow-through detector built in this laboratory was used to detect chemiluminescence. The detector housing contains a PTFE T-piece for the efficient mixing of the reagents placed immediately before the flow cell, a Hellma 170-OS glass flow cell (114 mm^2 area and 1 mm optical path) backed by a mirror for maximum light collection and a R605K photocell for measurement of the emitted light intensity placed in front of the flow cell to measure the light whilst emission is most intensive. No wavelength discrimination is involved. The total electronic circuits, amplification system and the photocell were obtained from a flame photometer and located in a light-tight housing. The CL emission was recorded on an Omniscribe chart recorder.

The flow injection system consisted of a Gilson Minipuls HP4 peristaltic pump, an Omnifit injection valve, 0.5 mm i.d. PTFE tubing and various end fittings and connectors (Omnifit).

Flow system for the determination of the thiol-drugs

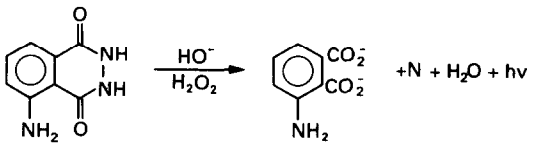
A schematic diagram of the FIA manifold is shown in Fig. 1. A three channel system was used. Luminol (pH 10.4) flowed into one stream and merged with a stream of Cu(II) at a PTFE T-piece. The resulting solution met another stream in which flowed hydrogen peroxide in a second PTFE T-piece placed in front of the flow cell to record the maximum CL intensity. All three carrier streams were pumped at the same flow rate by means of a peristaltic pump and the total flow rate was 7.2 ml min^{-1}. T-pieces were chosen as mixing devices to obtain the most effective and rapid mixing of the reagents. Sample injection (185 µl sample loop) was effected into the luminol stream and produced negative peaks. The CL decrease was recorded and the peak heights were measured. Calibration graphs were obtained by plotting peak heights against the logarithm of the drug concentrations.

Determination of the drugs in pharmaceuticals

Dissolve one capsule of the pharmaceutical preparation in water up to 250 ml in a calibrated flask or carefully grind one tablet to fine powder using a glass mortar and pestle and transfer quantitatively with water to a 250 ml volumetric flask. Dilute to volume with water, filter, discard the first few millilitres and analyse a suitable aliquot by the CL–FIA method. Obtain the concentration of the drug from the peak height using the calibration graph. If an ampoule is used, dilute a suitable aliquot in a calibrated flask and proceed as described above.

Results and Discussion

Luminol is oxidized in alkaline solution to produce the excited-state aminophthalate di-anion which is the light emitter:



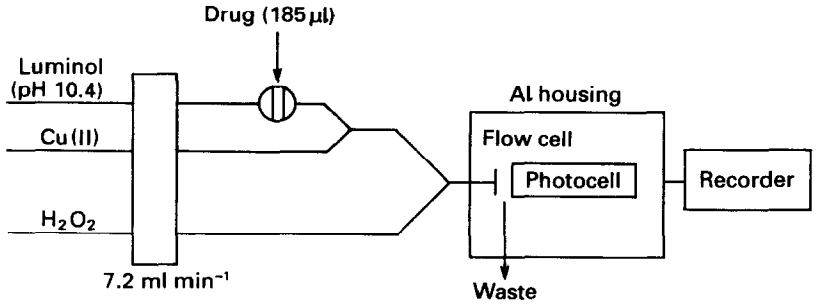


Figure 1
Flow injection manifold for thiol-drugs determination. 5×10^{-3} M luminol in 0.1 M buffer KOH–boric acid pH 10.4; 5×10^{-5} M Cu(II); 10^{-2} M H_2O_2; total flow rate, 7.2 ml min^{-1}; sample loop, 185 µl.

Several experiments were performed to establish optimal CL intensity of the luminol–H_2O_2–Cu(II) reaction before injecting the drugs. The parameters optimized were pH, reagents concentration, sample volume injected and flow rate.

Effect of pH, luminol and hydrogen peroxide concentrations

The kinetics of CL reactions strongly depend on pH. This effect was studied by using 10^{-3} M luminol solutions in boric acid–KOH buffer of different pHs ranging between 9.8 and 10.8. Figure 2(A) shows that pH values below 9.8 lead to almost negligible signals; CL quickly increased until pH 10.4 and again decreased at higher pH values. The maximum intensity was obtained with pH 10.4 and this value was selected. In all pH intervals tested no blank signal was detected.

The variation of CL intensity with luminol concentration in the range 10^{-4}–10^{-2} M (pH 10.4) is shown in Fig. 2(B). The largest emission was reached with a 5×10^{-3} M luminol concentration. Figure 2(C) shows the results obtained varying the oxidant concentration. Values below 10^{-4} M gave no CL emission; with 10^{-2} M H_2O_2, the signal reached a maximum and then decreased rapidly. Therefore, a 10^{-2} M H_2O_2 concentration was chosen. Again, there was no detectable blank signal.

Effect of Cu(II) concentration. Determination of the catalyst

The relationship between CL intensity and copper concentration is shown in Fig. 2(D). CL continuously increased with catalyst concentrations up to 10^{-4} M. Higher concentrations did not produce higher signals. A calibration graph between 5×10^{-6} and 10^{-4} M (0.3–6.3 μg ml^{-1}) of copper was obtained by plotting log CL vs log [Cu(II)] which may be applied to the CL determination of copper.

Effect of flow rate and injected sample volume

The effect of flow rate is critical. Too low or too high flow rates result in the absence of CL in the flow cell. Variation of the total flow rate over the range 3–12 ml min^{-1} produced an increase in the CL (see Fig. 3). The increasing sensitivity suggests that the CL reaction is very rapid and completed within the cell; at larger flow rates, more light is emitted per unit time, resulting in greater peak height. The graph is thus the result of a better dispersion/mixing at high flow rates. A total flow rate of 7.2 ml min^{-1} (2.4 ml min^{-1} for each reagent) was chosen because higher rates led to both high pressures in the connectors and excessive consumption of reagents.

The variation of the CL emission with the injected sample volume in the 35–335 μl range was also studied. It was shown that maximum light inhibition occurred with higher sample

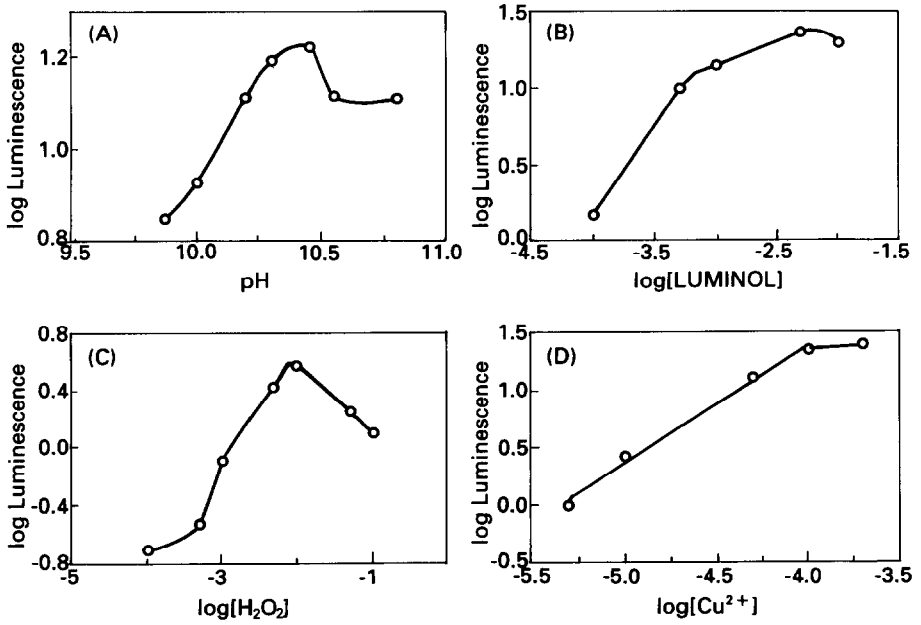


Figure 2
Effect of (A) pH, (B) luminol concentration, (C) hydrogen peroxide concentration and (D) Cu(II) concentration on CL emission. Total flow rate, 7.2 ml min^{-1}; sample loop, 185 μl.

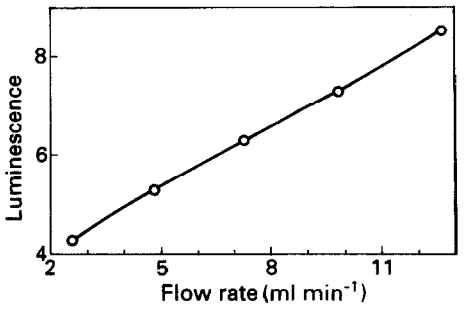


Figure 3
Influence of total flow rate on CL intensity. 5×10^{-3} M luminol; pH 10.4; 10^{-2} M H_2O_2; 5×10^{-5} M Cu(II); sample loop, 185 μl.

loops. However, at larger volumes (higher than 235 μl), the sample formed a well-defined sample plug in the stream; therefore, the mixing of the sample with the reagents was insufficient, and broadening of the peaks appeared. Thus, a 185-μl sample loop was chosen.

Determination of thiol-containing drugs

Once chemical and instrumental variables were optimized to achieve maximum CL emission, the appropriate position to inject the inhibitory sample was tested. Best results (maximum difference between the negative peak of the inhibitory drug and water) were obtained by injecting the sample into the stream of luminol. As discussed below, negative peaks appeared because the catalytic activity of Cu(II) decreased by complexation with thiol drugs. The catalytic activity of a copper complex is maximum when two of the four coordination sites of copper are occupied by a ligand, while no catalytic activity is observed when all four coordination sites are occupied by a ligand [17]. The decrease of the catalytic activity of the copper–drug complex which occurs at high drug concentrations may be attributed to the formation of a complex in which the four coordination sites of copper are occupied by the drug. A similar behaviour has been previously described [15]. However, when the concentration of copper is approximately equal to that of the drug, positive peaks appeared. This effect may be attributed to the formation of a different Cu–thiol complex with two coordination sites occupied and hence maximum catalytic activity obtains. Thus, an increase in the CL was observed.

Calibration graphs were obtained by plotting peak height vs log [drug] and were linear in the ranges 10^{-4}–10^{-2} M for cysteine, penicillamine and 2-thiouracil and 2×10^{-4}–10^{-2} M for *N*-acetylcysteine and 2-mercaptopropionylglycine (Table 1). The relative standard deviations for 5×10^{-4} M drug concentration (10 determinations) were: cysteine, 1.98%; *N*-acetylcysteine, 1.44%; penicillamine, 1.38%; 2-mercaptopropionylglycine, 1.15%; and 2-thiouracil, 2.06%. The proposed method allows a sampling frequency of about 40 samples per hour.

Interferences

Cysteine is one of the essential amino acids. Interferences by other amino acids on the determination of 5×10^{-4} M cysteine was studied. Copper(II) forms complexes with α-amino acids but the reaction is slow and a large excess of copper is needed to obtain stoichiometric reaction with the amino acid. Table 2 shows the results of the interference of essential amino acids on cysteine determination. As can be seen, the proposed procedure is selective. The tolerance limit was taken as the concentration causing an error of no more than ±3% in the cysteine recovery.

Interference by foreign species was studied for the determination of the thiol-drugs. Since the aim of this work is the determination of the drugs in pharmaceuticals, the effect of common tablet fillers was considered in particular. The results obtained for 5×10^{-4} M penicillamine showed no interference by fructose, lactose, glucose, saccharose, maltose, caffeine, salicylic acid, tartrate, oxalate and

Table 1
Calibration graphs for thiol-drugs

Drug	Calibration graph (h =)	Correlation values (r)
Cysteine	12.44 + 2.426 log[Cys]	0.9984
N-acetylcysteine	8.28 + 1.436 log[AC]	0.9966
Penicillamine	9.26 + 1.824 log[PA]	0.9970
2-Mercaptopropionylglycine	8.37 + 1.551 log[MPG]	0.9968
2-Thiouracil	8.96 + 1.638 log[TU]	0.9982

Table 2
Interference of the most important amino acids on the determination of 5×10^{-4} M cysteine

Limiting molar ratio [amino acid]/[cysteine]	Amino acid
10*	Lysine
7	Glutamine, alanine
5	Leucine, isoleucine, threonine, glycine, valine, arginine, phenylalanine
3	Methionine, asparagine, tryptophan, tyrosine, glutamate, proline, histidine
2	Aspartate, cystine

*Maximum ratio tested.

Table 3
Applications of CL–FIA method to pharmaceutical analysis

Sample (laboratory)	Drug	Content* Certified	Found
Fluimucil (Zambón)	*N*-acetylcysteine (mg per ampoule)	300	295.2
Fluimucil (Zambón)	*N*-acetylcysteine (mg per powder)	100	98.6
Cupripén (Rubió)	Penicillamine (mg per capsule)	250	252.0
Hepadigest (Uriach)	2-mercaptopropionylglycine (mg per tablet)	100	102.1
Sutilan Cusí (Cusí)	2-mercaptopropionylglycine (mg per tablet)	100	97.6

*Average of three determinations.

starch at relationships [interferent]/[penicillamine] up to 10/1. Hence, the proposed method is sufficiently selective.

Applications

The method was applied to the determination of the drugs in certain pharmaceuticals, and results obtained for three separate determinations are given in Table 3. There were no significant differences between the certified and the experimental values.

Conclusions

The copper-catalysed luminol–hydrogen peroxide CL reaction has been shown to be applicable to the determination of certain compounds of clinical interest which contain thiol groups. The inhibitory effect of the thiols on the catalytic effect of copper is the basis of the determination. The CL measurement was readily automated in a flow injection system with good precision and high sample throughput. The method has lower detection limits than spectrophotometric methods due to the absence of background. However, sensitivity is worse than other luminol CL systems because the method is based on the decrease of a signal. The reagents and instrumentation for this analysis are inexpensive and the method appears adequate for pharmaceutical quality control analysis.

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