A FLOW INJECTION CHEMILUMINESCENCE METHOD FOR THE DETERMINATION OF ALENDRONATE SODIUM BY SUPPRESSION OF Cu(II) ENHANCED LUMINOL-H_2O_2 REACTION

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تم تطوير طريقة جديدة مبتكرة لتقدير أليندرونات الصوديوم باستخدام التوهج الكيميائي و تقنية الحقن السرياني. تعتمد الطريقة على تكوين معقد مع كبريتات النحاس في وسط حمضي، حيث يقوم هذا المعقد بتثبيط التوهج الكيميائي الناتج من نظام أكسدة اللومينول بفوق أكسيد الهيدروجين المحفز بواسطة أيونات النحاس الثنائي. وقد تم دراسة أمثل الظروف الآلية والكيميائية والتي يمكن أن تؤثر على التفاعل وتم الحصول على العلاقة الخطية لمنحنى التعيير القياسي والتي كانت في المدى ٠,٣٢ــ٢,٤ ميكروجرام/مل من تركيز أليندرونات الصوديوم وبحد كشف يساوي ٠,١ ميكروجرام/مل. وكان معامل الارتباط الخطي لست عينات يساوي ٠,٩٩٩١ والانحراف المعياري النسبي لتركيز ١ ميكروجرام/مل يساوي ٠,٨٢%. وقد طبقت هذه الطريقة المقترحة لتقدير أليندرونات الصوديوم في مستحضرات الأقراص الدوائية التجارية.

وقد تم حساب النسبة المئوية للاسترجاع لستة عينات وكان يساوي ٩٩,٩٧ ±٠,٥٩ كما تم مقارنة النتائج إحصائيا مع تلك التي تم الحصول عليها من طريقة منشورة وذلك من خلال اختبارات t و F .

A novel flow injection (FI), chemiluminescence (CL) method has been developed for the determination of alendronate sodium (ALD). The method is based on the formation of a complex with Cu(II) sulphate in acidic medium. This complex inhibits the chemiluminescence response resulting from the oxidation of luminol by hydrogen peroxide catalyzed by Cu(II) ions. The optimization of the instrumental and chemical variables affecting the CL was carefully studied. Linear calibration curve was obtained in the range of 0.32–2.4 μgml^{-1}, with minimum detectability of 0.10 μgml^{-1} (S/N = 3). The correlation coefficient was 0.9991 (n = 6) with relative standard deviation (%R.S.D.) of 0.82% for 1 μgml^{-1}. The proposed method has been applied for the determination of ALD in commercial tablets. The average percentage recovery (n = 6) was 99.97 ± 0.59. The results were compared statistically with those obtained from a published method as revealed by *t*- and *F*-tests.

Keywords: Flow injection; Chemiluminescence; Alendronate sodium; Copper(II) Complex; Luminol, Tablets.

INTRODUCTION

Alendronate sodium (ALD) (sodium salt of 4-amino-1-hydroxy-1,1-biphosphonic acid, Fig. 1) is an aminobiphosphonate drug with potential utility in treatment of diseases characterized by abnormal bone turnover, such as metastatic bone disease, hypercalcemia of malignancy, Paget's disease, periodontal disease and osteoporosis. Unlike earlier biphosphonate compounds (etidronate, clodronate and tiludronate), ALD contain a side-chain primary amino group, which imparts greater potency and specificity [1]. The lack of a detectable chromophore in alendronate, as well as in many other biphosphonate, makes the analytical methods development for this class of compounds challenging. Several methods for the determination of ALD in pharmaceuticals and biological fluids including: spectrophotometric [2-6] fluorimetric [6] voltammetric [7] inductively coupled plasma [8] electrophoretic [9] and chromatographic methods [10-14] were proposed. Most of these methods involve a derivatization step in order to introduce a chromophore into the

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molecule, and they require complex and expensive equipment labour-intensive sample preparation procedure and personal skills. The analytical figures of merit of the above-mentioned procedures are summarized in Table 1.

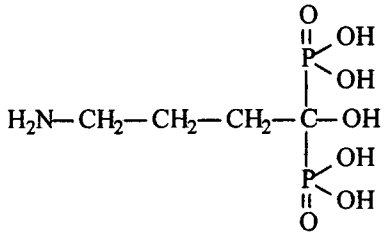

Fig 1: Structure of alendronate, monosodium salt of 4-amino-1-hydroxybutane-1,1-biphosphonic acid.

Chemiluminescence (CL) has been exploited in a number of analytical applications owing to its great sensitivity, ease of use and simple instrumentation [15] However, to our knowledge, no chemiluminemetric method for the determination of ALD has been yet reported. The sensitivity of CL methods for the determination of trace metals is well known [16]. Most such methods are based on the catalysis or inhibition of reactions involving the oxidation of reagents such as luminol, pophine, gallic acid and lucigenin [17].

In aqueous solutions, the most commonly used chemiluminescent species is luminol (5-amino-2, 3-dihydrophthalazine-1, 4-dione), which reacts with hydrogen peroxide in the presence of a catalyst (generally a metal) in alkaline solution to yield 3-aminophthalate in an excited electronic state which returns to ground state with the production of light [18] The light intensity can easily be monitored with a photomultiplier tube with no wavelength discrimination. Metal ion chelators such as citrate, EDTA, and amino acids are known to cause suppression of metal ion enhanced luminol-CL [19] Alendronate sodium (ALD) is reported to form soluble labile copper(II) complex [2,7] and so will suppress the luminescence of the system luminal-H_2O_2-Cu(II). The amount of emission inhibited is proportional to the concentration of ALD; thus the amount of ALD can be determined by measuring the decrease in the chemiluminescent intensity.

This paper presents, for the first time, a flow injection-CL method for the determination of ALD, based on the quenching effect of the formed ALD-Cu(II) complex on the reaction between luminol and hydrogen peroxide catalysed by Cu(II). The proposed method was applied successfully to determine ALD in tablets.

EXPERIMENTAL

Instrument and flow system:

The flow system used for the determination and CL detection of ALD is shown schematically in Fig 2. A Gilson minipuls 3MP4 peristaltic pump (two channels, variable speed) was used to drive the carrier and the reagent streams through the flow system. Each stream was pumped at a constant flow rate using PTFE tubing (0.8 mm id).

The drug solution (300 µl) was injected through the sample injection valve, which allows the mixing of the sample with luminol solution (6×10^{-4} M in 0.1 M Na_2CO_3) and then combination with 1×10^{-3} M H_2O_2 solution just before the detector. The emitted intensity was measured by a photomultiplier tube (PMT, THORN EMI 9789QB), which was operated at 1100 V. The PMT was provided by a stable power supply (500 mV) (THORN EMI, Model PM 288 BN). The signal was recorded by a Yokogawa model 3021 recorder (Yokogawa, Japan). Peak height was measured for each signal and expressed as voltage output of the photomultiplier tube.

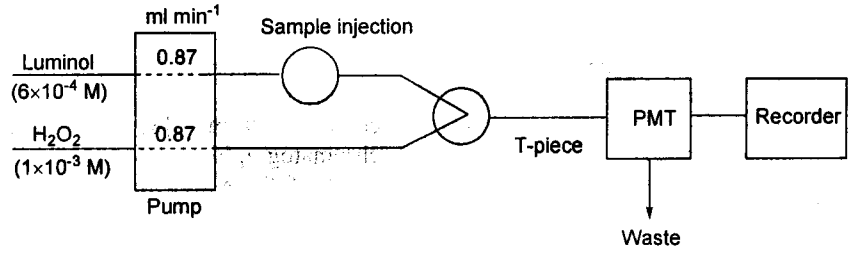

Fig 2: Flow-injection manifold for CL determination of ALD.

Table 1: Comparison of the analytical figures of merit of the proposed FI-CL method with earlier reported methods for the determination of ALD in pharmaceuticals.

Technique	Detection	Detection limit ($\mu g\ ml^{-1}$)	Determination range ($\mu g\ ml^{-1}$)	Relative Standard deviation (%)	Sampling Rate (h^{-1})	Ref.
IC	Conductivity	2	20–80	< 2 4.3	< 6 < 6	(11)
CE	UV/240 nm	—	50–150	< 2	< 6	(9)
IC	UV/220 nm	1	20–80	< 2	—	(12)
ICP	UV/178.3 nm	—	1.7–115	< 2	< 15	(8)
IC	RI	0.4	200–600			(14)
Batch spectro-photometry	290–310 nm	2	8.1–162.5	< 3	—	(4)
Anoding stripping voltammetry	- 153 mV	0.009	0.096–0.288	< 2	—	(7)
UV	240 nm	0.3	1–60	3	60	(6)
Fluorimetry	340/355 nm	0.04	0.13–10	2	30	(6)
Proposed FI-CL method		0.1	0.23–2.4	0.82	50	The proposed method

Reagents and materials:

All reagents used were of analytical reagent grade, and the solutions were prepared with distilled water. The following reagents were used: Alendronate standard solution, 0.15 mM (0.4 mg ml^{-1}) was prepared from alendronate-Na (Merck Research Labs, Rahway, NJ, USA) and dissolved in distilled water. Working standard solutions were prepared by appropriate dilution immediately before use. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was obtained from Aldrich (Milwauke, WI). Luminol stock solution, 1×10^{-2} M, was made by dissolving 0.1772 g of luminol in 100 ml of 0.1 M sodium carbonate buffer (pH 10). This solution was stable for a few months in the refrigerator. Hydrogen peroxide solution was prepared just before use by diluting a measured amount of 30% (w/v) standard solution (BDH, UK) with degassed water. Copper(II) sulphate stock solution, 1.5×10^{-3} mM was prepared from anhydrous copper sulphate (BDH, UK) dissolved in distilled water. Nitric acid stock solution, 1.6 mM was prepared from concentrated nitric acid (optima grade, BDH, UK).

General procedure:

The FI manifold shown in Fig. 2 was used. Blank working solution was prepared by mixing 0.1 ml of $CuSO_4$ solution (1.5×10^{-3} mM) and 1 ml of HNO_3 solution (1.6 mM) in a 25 ml volumetric flask and diluting to the mark with distilled water. Working drug solutions were prepared by mixing different aliquots of stock drug solution (0.4 mg ml^{-1}) with 0.1 ml of $CuSO_4$ solution (1.5×10^{-3} mM) and 1 ml of HNO_3 solution (1.6 mM) in a 25 ml volumetric flasks and diluting to the mark with distilled water. A 300 µl portion of the blank solution was injected into a carrier stream of luminol solution which was then combined with a stream of H_2O_2 solution. The resulting peak height in mV was measured (blank peak). Each working drug solution was injected by the same manner and the resulting peak height in mV was measured. The amount of decreasing in height of the blank peak in mV was calculated and plotted against drug concentration to obtain the calibration curve; alternatively, the regression equation was derived.

Procedure for tablets:

Ten tablets were weighed and finally grounded. A weighed amount of the fine powder equivalent to 10 mg of ALD was dissolved in distilled water by sonication for 10 min. The solution was filtered into a 100 ml volumetric flask and the filtrate was diluted to volume with distilled water. This solution, labeled to contain 100 µg ml^{-1}, was analyzed by the FL-CL procedures as described above. Nominal content of tablets was calculated either from the previous plotted calibration graph or by using the regression equation.

RESULTS AND DISCUSSION

As reported earlier, formation of a complex between ALD and copper (II) ions (K_{form} = 1.67 × 10^4 M^{-1}) has been applied to the spectrophotometric [2] voltammetric [7] and capillary electrophoretic [9] determination of ALD.

In this work, our system monitors the CL resulting from the reaction of Cu(II) with luminol and hydrogen peroxide in an alkaline solution. When ALD forms a complex with Cu(II), the CL intensity is decreased, (Fig.3).This decrease is proportional to ALD concentration.

Optimum conditions for complex formation:

In accordance with a previous report [2], 1.6 mM nitric acid and 1.5 × 10^{-3} mM copper(II) sulphate were used for the formation of ALD-Cu(II) complex.

Configuration design:

A two-line manifold was used for the determination of ALD. The maximum CL intensity was obtained when the Cu(II) sulphate solution was injected into a stream of alkaline luminol and then mixed with H_2O_2 solution prior to the detector. Then the ALD-Cu(II) complex solution was injected instead of the Cu(II) sulphate solution, leading to a decrease in the previous CL intensity.

Optimization of experimental variables:

As the proposed procedure involved CL inhibition, it was decided to maximize blank readings in order to improve sensitivity. A series of experiments were conducted to establish optimum analytical variables for the CL determination of ALD by using the luminal - H_2O_2 - Cu^{2+} reaction in alkaline medium. The parameters optimized included reagent concentration, pH, and some manifold parameters.

Effect of reagent concentration and pH:

Effects of luminol, hydrogen peroxide, and copper(II) sulphate concentrations were investigated to attain the highest blank values. The effect of luminol concentration was investigated in the range 1 × 10^{-6} to 1 × 10^{-2} M, the results showed that the CL intensity was greatest when luminol was 6 × 10^{-4} M, (Fig. 4).

Hydrogen peroxide concentration was studied in the range 1 × 10^{-5} to 1 × 10^{-2} M. It was found that 1 × 10^{-3} M H_2O_2 was suitable; higher concentrations gave higher emission, but the signals were noisy and irreproducible, (Fig. 5).

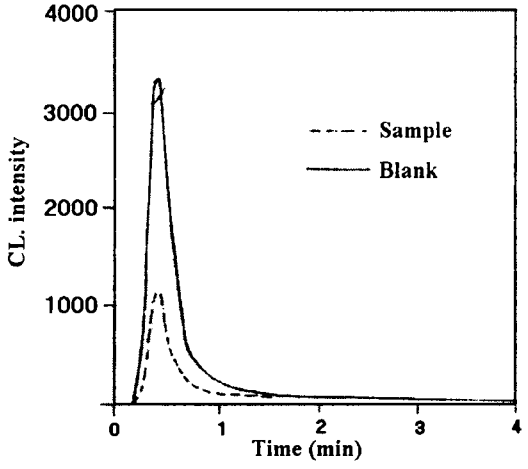

Fig.3: The CL intensity (—) in the absence of ALD, (···) in the presence of ALD, 0.6 µgml^{-1}.
Conditions: 6 x 10^{-4} M luminol, 1 x 10^{-3} M H_2O_2 and 1.5x10^{-3} M copper(II) sulphate.

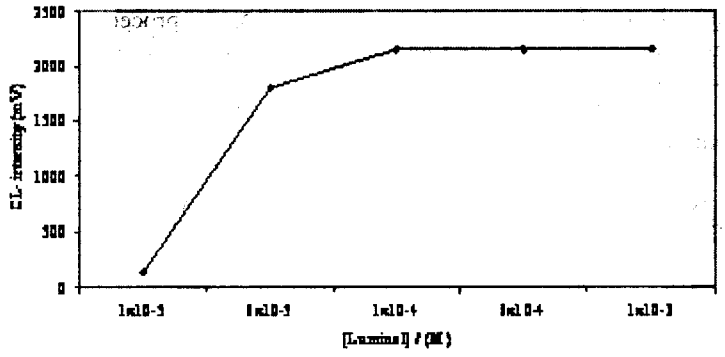

Fig. 4: Effect of luminol concentration on the net CL intensity for 2 μgml^{-1} ALD, 1 x 10^{-3} M H_2O_2 and 1.5 x 10^{-3} M copper(II) sulphate.

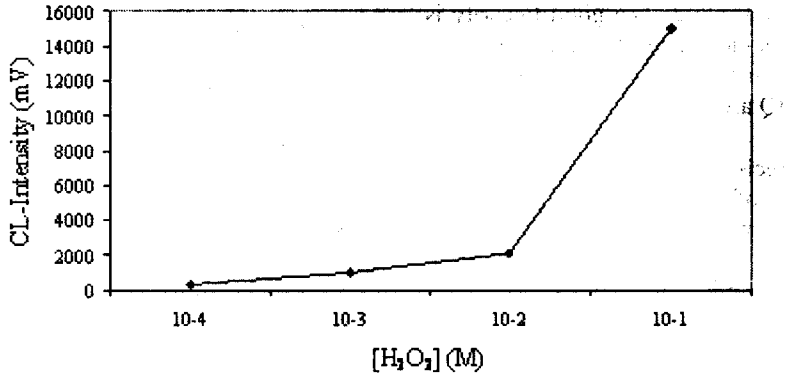

Fig. 5: Effect of H_2O_2 concentration on the net CL intensity for 2 μgml^{-1} ALD, 1.5 x 10^{-3} M copper(II) sulphate and 6 x 10^{-4} M luminal.

Since the conventional CL reaction of luminol with hydrogen peroxide catalyzed by Cu^{2+}, different concentrations of copper (II) sulphate were prepared, $1 \times 10^{-6} - 1 \times 10^{-2}$ mM and their effects on the net CL intensity were investigated. It was found that 1.5×10^{-3} mM copper(II) gave the greatest intensity and was chosen for possible sensitivity.

Since most of the CL systems occur under alkaline conditions, various buffer solutions (0.1 M sodium carbonate, 0.1 M sodium phosphate and 0.1M ammonium citrate) were prepared and used as solvents for the luminol. The pH of each buffer solution was adjusted to different pH values (9.6-10.8), best results were obtained when luminol was prepared in carbonate buffer at pH 10.0.

Effect of manifold parameters:

The manifold parameters studied under the optimized reagent concentrations were, the injected sample volume and flow rate.

Although the CL intensity increased with increasing both the injected sample volume and flow rate, the signal of blank also increased significantly. An injected sample volume of 300 μl and a flow rate of 0.87 ml min^{-1} were recommended in order to reduce the consumption of reagents and to improve the detection limit.

Possible CL mechanism:

The copper(II) ion can serves as a catalyst for the luminol-H_2O_2 system (16). In the presence of a chelating ligand, the fraction of uncomplexed

copper(II) is decreased leading to a decrease in the CL intensity. The biphosphonate hydroxyl moiety of alendronate sodium serves as a strong binding ligand for copper(II) with the resultant of ALD-Cu(II) complex (1:1 stoichiometery) [2]. If alendronate-Cu(II) complex is arranged to be the rate-limiting reagent, then the amount of the decreased-emission is proportional to the concentration of ALD in the range 0.32□2.4 μg ml^{-1}.

$$H_2O_2 + \text{Luminol} + OH^{\ominus} \xrightarrow{Cu(II)} \text{Aminophthalate} + N_2 + 3H_2O + h\gamma$$

Validation of the proposed method:

Linearity:

Under the optimum experimental conditions, the calibration graph was found to be linear over the range stated in Table 2. The good linearity is evident from the values of the standard deviation of the slope (S_b) and correlation coefficient (r). The LOD and LOQ are also presented in Table 2.

Accuracy and precision:

The accuracy of the method was tested with several synthetic samples of ALD with different concentrations. The results obtained are shown in Table 3, from which it is clear that the recovery is excellent.

The intraday and interday precision of the method was studied by analyzing three different concentrations, each in three replicates. The R.S.D. was 1.10 and 1.60, respectively (Table 2).

Specificity:

As the procedure described is for application to pharmaceutical preparations, the presence of excipients was evaluated. It was found that, common excipients found in the tablets had no effect on the CL response. Citrate, which is present in many pharmaceutical preparations (i.v. solutions), will interfere with the application of the method by the formation of complexes with copper(II).

Application:

The proposed method was applied to the determination of ALD in two commercially available preparations. Table 4 shows the results obtained which are in good agreement with the reference method [4], as the calculated *t*- and *F*-values did not exceed the theoretical ones, confirming the good accuracy of the method [20].

Conclusion:

The first FL-CL assay for the determination of alendronate sodium, an amino-bisphosphonate drug used in the treatment of osteoporosis, is reported. The developed method is applied to the determination of ALD in pharmaceutical samples with good accuracy and precision. Compared to previous reported procedures, the FL-CL assay offer the advantages of low running and instrumentation costs, easy-to-handle reagents, low reagent consumption and waste production, sensitivity, reasonable determination range and significantly increased samples analysis rate.

Table 2: Validation of the proposed FI-CL procedure

Parameter	Value
Linearity range* (μgml^{-1})	0.32□2.4
LOD (S/N = 3) (μgml^{-1})	0.10
LOQ (μgml^{-1})	0.30
Slope ± S.D.	2.180 ± 0.152
Intercept ± S.D.	0.807 ± 0.073
Correlation coefficient (r)	0.9991
Precision ± R.S.D.	
Intraday	99.82 ± 1.10
Interday	100.70 ± 1.60

*n = 6

Table 3: Analysis of alendronate sodium in bulk powder and tablets by the proposed FL-CL and a reported method.

	Proposed method		Reported method Recovery %[4]
	Concentration taken µgml^{-1}	Recovery[a] (%)	
Bulk powder	0.32	98.00	100.14
	0.64	101.7	101.50
	0.80	100.0	99.20
	0.96	99.78	101.50
	1.60	98.67	100.00
	2.40	100.4	
$\bar{X}$		99.76	100.47
± S.D.		1.31	1.10
Variance ratio *F*-test		1.68 (6.26)[b]	
Student's *t*-test		1.00 (2.228)[c]	
FOSA-MAX® tablets (70 mg alendronate sodium/tablet)[d]	0.32	101.0	99.00
	0.64	99.50	99.63
	0.80	100.17	99.52
	0.96	99.87	98.33
	1.60	99.30	99.30
$\bar{X}$		99.76	99.16
± S.D.		0.594	0.520
Variance ratio *F*-test		1.64 (6.39)	
Student's *t*-test		2.13 (2.306)	
Osteomax® tablets (6.53 mg alendronate sodium trihydrate/tablet)[e]	0.32	98.10	
	0.64	99.55	
	0.80	101.00	
	0.96	99.30	
	1.60	99.69	
$\bar{X}$		99.53	99.16
± S.D.		0.93	0.520
Variance ratio *F*-test		3.20 (6.39)	
Student's *t*-test		0.78 (2.306)	

[a]Each is the mean of three determinations.
[b]Tabulated *F*-values at (P = 0.05).
[c]Tabulated *t*-values at (P = 0.05).
[d]Merck Sharp & Dohme Limited, UK. (Lot No.: NC 482220).
[e]El-Amriya Pharm. Ind. (Alexandria, Egypt).

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