Article

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Automated Sequential-injection Chemiluminescence Determination of Glucosamine Sulphate via Luminol-Hydrogen Peroxide System

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A highly sensitive automated sequential-injection chemiluminescence (SIA-CL) method for determination of glucosamine sulphate (GLS) was developed. The goal of the present work is the evaluation of the enhancement effect of the investigated drug glucosamine sulphate on the chemiluminescence reaction between luminol and H_2O_2 in alkaline medium of 1.0×10^{-2} mol L^{-1} sodium hydroxide at pH 11. The experimental conditions affecting the CL reaction such as the sequence of the reagents, concentrations, flow rate and aspirated volumes of reactants were systematically investigated and optimized. Under optimum conditions 50 μL of 1.0×10^{-3} mol L^{-1} luminol, 30 μL of a GLS test solution and 50 μL of 1.0×10^{-2} mol L^{-1} H_2O_2 were used and the luminescing zone was pushed into the detector at a flow rate 100 μL s^{-1}. The proposed method recorded high sensitivity, accuracy and simplicity that could be clarified as linear concentration range 1.0-2000 *n*g mL^{-1} with rectilinear part (r = 0.9992, n = 9) and limit of detection 0.3 *n*g mL^{-1}, along with relative standard deviation 1.3%. It was found that the developed method can be used directly to determine the investigated drug GLS in its pharmaceutical dosage forms and in spiked serum and urine by diluting the samples for a 1000 fold. The obtained results were statistically analyzed and compared with those obtained by the reported method.

Keywords: Chemiluminescence; Sequential-injection analysis; Glucosamine sulphate; Pharmaceutical formulations; Biological fluids.

INTRODUCTION

Glucosamine sulphate (GLS) is one of the most frequently prescribed nutritional supplements for the treatment of osteoarthritis.[1-3] Chemically is known as Bis(2-ammonio-2-deoxy-D-glucose) sulphate. The chemical structure of glucosamine sulphate is shown in (Figure 1). Glucosamine sulphate serves as a precursor for, and inhibits the degradation of proteoglycans (the matrix of articular cartilage); it rebuilds experimentally induced cartilaginous damage; and it has chondroprotective and antiarthritic effects. Furthermore, glucosamine sulfate has very mild anti-inflammatory and antireactive effects on edema-provoking agents including carrageenan, dextran, acetic acid and formalin. Glucosamine sulfate also inhibits in vitro superoxide generation and lysosomal enzymes of the liver.[4]

A number of papers have been published concerning determination of glucosamine sulphate by various analytical methods in different matrixes of pharmaceutical or biomedical interest. Among these methods high performance liquid chromatography,[5-7] liquid chromatography coupled with mass spectrometry,[8-10] capillary zone electrophoresis,[11,12] thin layer chromatography,[13] spectrophotometry[14] and carbon paste and modified nano carbon sensor[15] have been reported.

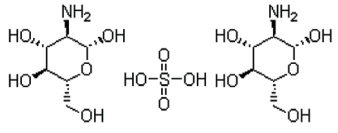

Fig. 1. Chemical structure of glucosamine sulphate.

Chemiluminescence (CL) based on luminol-hydrogen peroxide reaction was considered as the best known systems and widely used in analytical applications as reported in several reviews.[16-20]

Luminol is a diprotic acid (denoted as LH_2) with pKa's of 6 and ~ 13.[21] Luminol exists mostly as LH^- in the pH 11 employed in this study. The possible mechanism of luminol-hydrogen peroxide-GLS CL by analogue[22] involves the following steps:

$$GLS + HO_2^- \rightarrow X\ (\text{Glucosamine intermediate}) + OH^- \quad (1)$$

$$X\ (\text{Glucosamine intermediate}) + LH^- + OH^- \rightarrow GLS + {}^{\bullet}L^-\ (\text{luminol radical}) + H_2O \quad (2)$$

$${}^{\bullet}L^-\ (\text{luminol radical}) + H_2O \rightarrow L + LH^- + OH^- \quad (3)$$

$$L\ (\text{diazaquinone}) + H_2O_2 \rightarrow 3\text{-aminophthalate} + N_2 + \text{light} \quad (4)$$

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From the previous literature survey, no chemiluminescence (CL) method has been developed for the determination of glucosamine sulphate yet.

In recent years much attention has been received especially with sequential-injection analysis (SIA) technique[23-25] due to its high sensitivity and it provides a powerful sample handling in laboratory analysis.

The goal of choice for the determination of the investigated drug using sequential-injection chemiluminescence was attributed to the simplicity and high sensitivity with less time consuming. In this method, glucosamine sulphate enhances the CL emission from luminol oxidation by hydrogen peroxide in alkaline solution. This enhanced emission is proportional to the concentration of the studied drug. Also, using SIA-CL the proposed method exhibited good stability and reproducibility. The accuracy of the proposed method was evaluated by comparison with a reference UV- spectrophotometric method.[14]

RESULTS AND DISCUSSION

The factors affecting the enhancing effect of GLS on the CL generated by the oxidation of luminol with hydrogen peroxide in alkaline medium were carefully studied. Different oxidants such as potassium permanganate, potassium perchlorate, potassium dichromate, Cerium sulphate and hydrogen peroxide were carefully examined to select the most suitable oxidizing agent. No CL signal was recorded on using potassium permanganate, potassium perchlorate, serium sulphate and potassium dichromate. While, using hydrogen peroxide exhibits sharp CL signal. Therefore, luminol-hydrogen peroxide-CL system was selected and the effect of luminol and hydrogen peroxide concentration was further investigated and optimized.

Optimization of chemiluminescence conditions

Effect of luminol concentration

Luminol is considered as reducing agent for hydrogen peroxide and also plays an important role in CL reaction. The concentration of luminol should be carefully optimized in order to ensure good CL system stability. The effect of luminol concentration on CL intensity was investigated in the range of 1.0×10^{-5}-1.0×10^{-1} mol L^{-1}. As shown in Figure 2, it was cleared that the CL intensity exhibited significant increase at 1.0×10^{-3} mol L^{-1}. The subsequent experimental analysis of GLS was carried out using 1.0×10^{-3} mol L^{-1} of luminol.

Effect of hydrogen peroxide concentration

The influence of hydrogen peroxide concentration on the CL intensity was investigated using different hydrogen peroxide concentrations in the range 5.0×10^{-5}-1.0×10^{-1} mol L^{-1}. Figure 3 clarified that the CL intensity was increased at 1.0×10^{-2} mol L^{-1}. This can be attributed to the higher concentration of hydrogen peroxide in the presence of luminol could produce the saturation of detector due to the high CL intensity of the blank signal obtained.[26] Therefore 1.0×10^{-2} mol L^{-1} hydrogen peroxide was used for further studies.

Effect of sodium hydroxide concentration

The effect of sodium hydroxide concentration on the CL intensity system was investigated using five different concentrations in the range 1.0×10^{-3}-1.0×10^{-1} mol L^{-1}. As shown in Figure 4, the CL intensity was clearly increased by the increase of sodium hydroxide concentration. The highest CL signal was obtained at 1.0×10^{-2} mol L^{-1}.

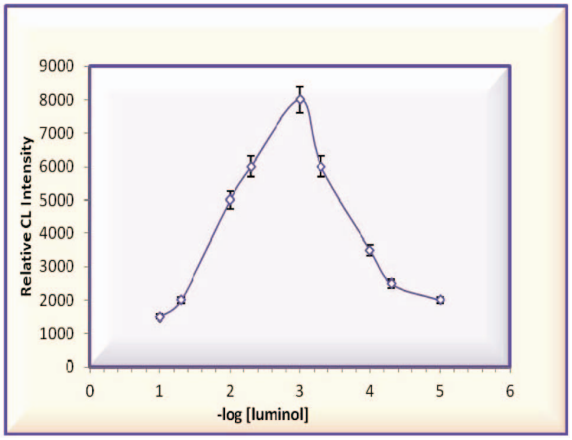

Fig. 2. Effect of luminol concentration, (GLS 1000 *n*g mL^{-1} and Hydrogen peroxide 1.0×10^{-2} mol L^{-1}).

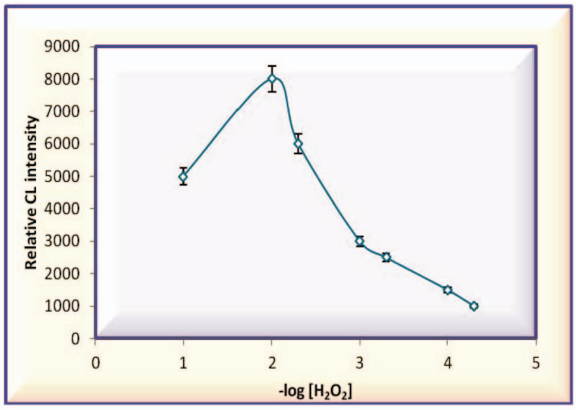

Fig. 3. Effect of hydrogen peroxide concentration, (GLS 1000 *n*g mL^{-1} and luminol 1.0×10^{-3} mol L^{-1}).

Effect of flow rate and aspirated volumes of sample and reagents

The effect of flow rate, aspirated volumes of sample and reagents was considered as critical parameters that govern the optimization of SIA instrumental configuration. Reagents and sample zones are the volumes and test solution aspirated. To optimize these parameters a modified simplex program was used to investigate the aspiration times for the determination of optimum CL detection signal. The proposed method was carried out by varying the volume of sample and CL reagents solutions. As shown in Figure 5a, it was found that for sample the CL intensity was increased with the increase of sample zone volume up to 30 µL and then kept unchangeable. This may be attributed to adjacent sample-reagent zones and disperse to each other to form the CL reaction. Moreover for CL reagents the optimum aspirated volume was 50 µL. The time was extended to 30 s for complete flushing through the holding cell with carrier in between analysis cycles. Also the influence effect of the flow rate on CL intensity was investigated in the range of 10-200 µL s^{-1}. It was noticed that the CL intensity was increased with the increase of flow rates. The optimum flow rate was found to be 100 µL s^{-1} and was used for further studies (Figure 5b).

SIA control program and characterization

The SIA control program for automated CL determination of GLS involving all optimized parameters was utilized to perform the calibration, interferent studies and quantification of the investigated drug in its dosage forms and biological fluids. The time recorded by one cycle was about 30 s and hence the sample throughput of 120 h^{-1} could be achieved. The analytical characterization of the proposed method for SIA-injection CL determination of GLS was investigated. As shown in Table 1, the measurable linear concentration range was 1.0-2000 *n*g mL^{-1}, (r = 0.9992) with lower limit of detection 0.3 *n*g mL^{-1}, the regression parameters were calculated from the calibration graph, the reproducibility of the proposed method was tested using nine drug test solutions and the relative standard deviations was 1.3%. The calculated relative standard deviation was less than 5% indicating that the proposed method was suitable for routine analysis of the investigated drug.

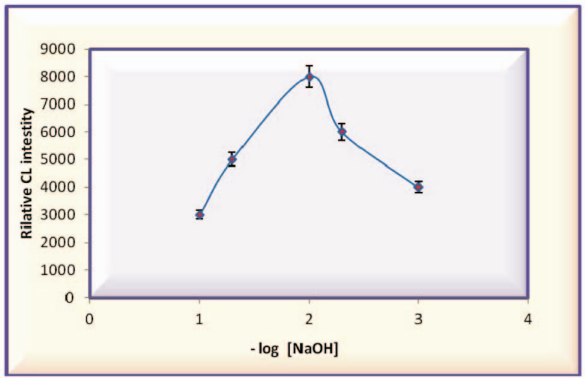

Fig. 4. Effect of sodium hydroxide concentration on CL intensity system (GLS 1000 *n*g mL^{-1}, Hydrogen peroxide 1.0×10^{-2} mol L^{-1} and luminol 1.0×10^{-3} mol L^{-1}).

Interferent study

The effect of interference of some common ions and excipients on the determination of 1000 *n*g mL^{-1} GLS test solution was investigated. The tolerable limit of a foreign species was taken as a relative error not greater than ±5% in the CL signal of GLS. The influence of coexisting species

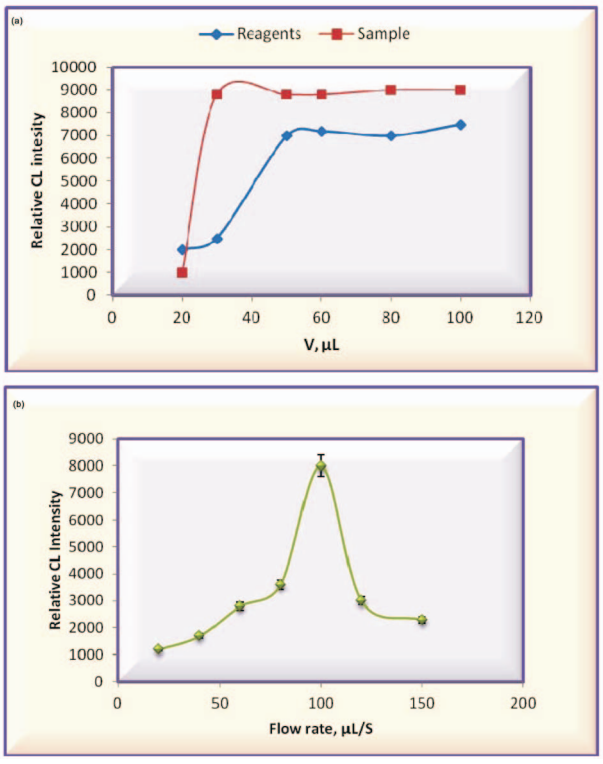

Fig. 5. (a) The influence of aspirated volumes of GLS and reagents (10-100 µL) on the relative CL intensity. Conditions: glucosamine sulphate 1000 *n*g mL^{-1}; 1.0×10^{-2} mol L^{-1} hydrogen peroxide and luminol 1.0×10^{-3} mol L^{-1}. (b) The influence of flow rate on the relative CL intensity. Conditions; GLS 1000 *n*g mL^{-1}; 50 µL 1.0×10^{-2} mol L^{-1} hydrogen peroxide; 50 µL luminol 1.0×10^{-3} mol L^{-1} and pH 11.

Table 1. Analytical results obtained from the determination of glucosamine sulphate using luminol and hydrogen peroxide system

Analytical characteristics	Obtained results
Linear range *n*g mL^{-1}	1.0-2000
Detection limit *n*g mL^{-1}	0.3
Intercept on the ordinate	297.12
Slope	12.404
%RSD for 1000 *n*g mL^{-1} (n = 9)	1.3%
Correlation coefficient, r	0.9992

in human serum on GLS was also examined. The obtained results were listed in Table 2. It can be seen that there is no influence on the determination of GLS in pharmaceutical dosage forms. While, for serum samples main possible interference from ascorbic acid, urea, heavy metal ions such as Fe^{3+}, Co^{2+}, Cu^{2+}, Al^{3+} and Ni^{2+}. The latter ions can be eliminated by addition of EDTA. After 1000 fold dilution the interference from such substances could be greatly minimized to negligible level.

Analytical applications

The proposed SIA-injection CL was used for the determination of GLS in its commercial tablets and biological fluids. The obtained results were presented in Tables 3, 4 and statistically compared by Student's-t test[27] with those obtained from the reported spectrophotometric method.[14] The results did not reveal any significant difference between them. The content uniformity assay for GLS tablets was investigated and the results were presented as the mean % recoveries and standard deviation (98.89 ± 0.8). To clarify further accuracy and precision for the proposed method the results in terms of linear concentration range and lower limit of detection obtained from the determination of the proposed method were compared with those recorded in previously reported methods as summarized in Table 5.

EXPERIMENTAL

Apparatus. SIA system (FIAlab instruments model 3500,

Table 2. Tolerable concentration level of interferents to 1000 *n*g mL^{-1} glucosamine sulphate

Interferents	Tolerable level *n*g mL^{-1}
EDTA, Na^+, K^+, Cl^-, NO_3^-, NH_4^+, CO_3^{2-} and SO_4^{2-}	1000
Br^-, Al^{3+}, Ca^{2+} and Mg^{2+}	500
Glucose, sucrose, lactose, talc, starch	800
Uric acid, ascorbic acid, magnesium stearate, citric acid, oxalic acid	25
Adrenaline, dopamine, cystine, histamine, tyrosine	250
Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, and Cu^{2+}	100

Table 3. Determination of glucosamine sulphate using SIA-injection CL detection in pure form and dosage forms in comparison with reported method

Sample	Taken *n*g mL^{-1}	Found *n*g mL^{-1}	Recovery %	Reported method[14] Taken mg mL^{-1}	Found mg mL^{-1}	Recovery %	Student's t-test	F-Test
Pure solution	1	0.9964	99.64	5	4.95	99.00		
	200	199.95	99.97	8	7.98	99.75		
	400	398.94	99.73	10	9.96	99.60		
	1000	1006.5	100.65	15	14.84	98.93		
	1500	1498.0	99.86	20	20.14	100.70		
	2000	1998.0	99.90	25	24.61	98.44		
	Mean % ± SD		99.96 ± 0.36	Mean % ± SD		99.40 ± 0.79	1.58 (2.23)*	4.77 (5.05)*
Just vitamin 1500 mg/tablet	1	0.99	99.41	5	4.89	97.80		
	10	9.83	98.27	8	7.99	99.87		
	50	49.92	99.84	10	9.96	99.60		
	200	200.54	100.27	15	14.68	97.87		
	400	398.14	99.53	20	19.71	98.55		
	1000	997.23	99.72	25	24.92	99.68		
	Mean % ± SD		99.51 ± 0.68	Mean % ± SD		98.89 ± 0.94	1.31(2.23)*	1.91(5.05)*

* Figures in parantheses are the tabulated values of t- and F-testes at 95% confidence limit

Table 4. Determination of glucosamine sulphate using SIA-CL system based on luminol-hydrogen peroxide in humane urine and serum

Urine Sample			Serum sample		
Taken *n*g mL^{-1}	Found *n*g mL^{-1}	Recovery %	Taken *n*g mL^{-1}	Found *n*g mL^{-1}	Recovery %
1	0.99	99.00	1	0.98	98.00
10	9.85	98.50	10	9.95	99.50
50	48.55	97.10	50	48.45	97.12
200	198.80	99.39	200	199.12	99.56
400	395.15	98.78	400	392.52	98.13
1000	985.45	98.54	1000	979.75	97.98
Mean% ± SD		98.55 ± 0.78	Mean% ± SD		98.39 ± 0.96

Table 5. Comparative analytical results relevant to the terms of linear concentration range and detection limit between the proposed SIA-CL injection and other reported methods

Method	Linear range (μg mL^{-1})	LOD (*n*g mL^{-1})	Reference
Proposed SIA-CL injection	0.001-2.0	0.3	-
HPLC	20-1000	-	[3]
Capillary electrophoresis	10-1000	-	[9]
LC/MS	0.05-3.4	50	[7]
HPTLC	0.8-1.2	-	[10]
Spectrophotometry	5000-25000	-	[11]

USA) comprised of a CAVRO XL 3000 syringe pump volume 2.5 mL (Cavro Scientific Instrument Int., USA) and Vici Valco Cheminer RT® 125-0718 eight-port manifolds. Fluorimetric flow detector (UIV lamp switched off) equipped with a lab-made CL module with spiral geometry; the photomultiplier tube voltage was 320 V. Autosampler model ALM 3200. The SIA system involved a holding coil (length 70 cm, i.d. 0.8 mm, PTFE tubing volume 1.2 mL). The same tubing was spirally coiled on a 52 mm × 52 mm Perspex plate, which substituted the secondary filter in the fluorimeter; this CL module had a central inlet, peripheral outlet and the diameter of the spiral was 24 mm. The SIA unit was PC controlled and data acquisition was performed with (FIAlab for windows version 5.9.321) software. Schematic view of the employed SIA manifold is shown in Figure 6. The solution stability monitoring and UV spectrophotometry was performed on UV-Visible Spectrophotometer Ultrospec (model 2100 pro).

Reagents. All chemicals were of analytical reagent grade and distilled water was used throughout the work. Pure grade glucosamine sulphate was kindly supplied from Adwia Co. Egypt. 1.0×10^{-3} mol L^{-1} luminol (Sigma Chemical Co.) stock solution was prepared in 100 mL of 1.0×10^{-2} mol L^{-1} sodium hydroxide (WINLAB). Hydrogen peroxide 30% (WINLAB) was used to prepare 1.0×10^{-2} mol L^{-1} by appreciated dilution using distilled water. The pharmaceutical preparation (Just vitamin® 1500 mg/tablet) was purchased from local drug stores. Urine samples were obtained from healthy volunteers and serum samples (Multi-Serum Normal, Randox Laboratories, UK) were obtained from commercial sources.

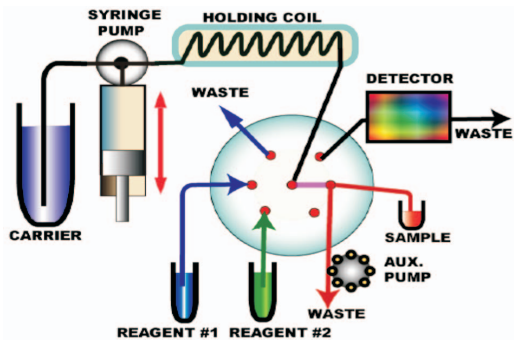

Fig. 6. Schematic diagram of SIA injection system for chemiluminescence determination of glucosamine sulphate; carrier stream (water); reagent 1 (luminol 1.0×10^{-3} mol L^{-1}); reagent 2 (hydrogen peroxide 1.0×10^{-2} mol L^{-1}); sample (glucosamine sulphate 1000 *n*g mL^{-1}).

Standard drug solution. A stock solution was prepared by dissolving 0.01 g glucosamine sulphate in 100 mL distilled water. The working solutions were prepared by serial dilutions in the range of 1.0-2000 *n*g mL^{-1}.

Procedure. The SIA-injection technique was used for automated handling (flow rate, aspiration, dispensing and mixing) of appropriate defined volumes of standard test solutions and reagents and dispensing the luminescence zones to the detector in pre-programmed measuring cycles. About 500 μL distilled water was aspirated into syringe pump, which was afterwards used as carrier for delivering sample and reagent zones into the flow-through cell for measuring CL signal. Mixture of 50 μL luminol 1.0×10^{-3} moL L^{-1}, 30 μL sample solution and 50 μL hydrogen peroxide 1.0×10^{-2} mol L^{-1} was aspirated into the holding coil through the eight-way injection valve at a flow rate 100 μL s^{-1} and then the mixed solution was flushed continuously into the flow-through cell located in front of detection cell of the photomultiplier tube (PMT). The CL emission was converted to current signal by FIAlab-3500 PMT and output was fed to luminescence analyzer, recorded with a computer via supplied FIAlab software (version 5.9.321). Each measuring cycle was repeated in triplicate and the mean peak height values were used in the evaluation of experiments. All measurements were carried out at ambient temperature of 25 ± 1 ºC. The concentration of the sample solu-

tion was quantified by the relative CL intensity.

Calibration, lower limit of detection (LOD). The calibration curve for the determination of GLS was carried out under optimum conditions with nine standard solutions of GLS covering the range 1.0-10000 *n*g mL^{-1}. For each standard solution three replicate injections were carried out. The relative intensity of CL against the concentrations of the investigated drug was plotted. The linear regression equation was utilized for fitting the curve. The LOD was calculated by the S/N = 3 method as the concentration of GLS giving rise to CL signal exceeding three times the blank signal.

Analytical applications

Determination of glucosamine sulphate in Just vitamin® tablets. Ten tablets (Just vitamin® 1500 mg/tablet) were crushed, finely powdered and homogenized properly. An amount of powdered tablets equivalent to 100 mg GLS was dissolved in 100 mL distilled water, then sonicated for 15 min. This suspension was filtered through membrane filter (pore size 0.5 μm). Serial dilutions in the range of 1.0-1000 *n*g mL^{-1} were prepared and subjected to CL detection. The mean % recovery for each concentration was calculated from the calibration graph.

Content uniformity assay of tablets. Ten individual tablets of GLS (1500 mg/ tablet) were dissolved in 100 mL of distilled water. Then sonicated for 15 min and filtered as cited above in (2.6.1.). 0.01 mL of the filtered solution was transferred into a conical flask and completed to 100 mL to obtain a test solution containing 1500 *n*g mL^{-1} of GLS. The prepared solution was subjected to CL detection and the content uniformity assay of each tablet was evaluated from calibration graph.

Determination of glucosamine sulphate in serum and urine. The determination of GLS in human serum and urine was investigated by using the spiking technique. The pH of serum and urine samples was adjusted to pH 11 using 1.0 mol L^{-1} NaOH. 1.0 mL adjusted serum was spiked with GLS standard drug solution to contain 1.0 μg mL^{-1}. Deproteination of the samples was carried out by excetration process using 20 mL diethyl ether and toluene for serum and urine samples, respectively.[28] The treated solution was centrifuged at 2500 rpm for 10 min. The prepared sample was diluted with distilled water to contain 1.0-1000 *n*g mL^{-1} of GLS and subjected to CL detection. The peak heights of CL signal were recorded and the % recovery was calculated by comparing the obtained results in serum and urine with the same concentration levels of the drug in water.

CONCLUSION

A sequential injection SIA-chemiluminescence method can be satisfactory applied for rapid quantitative and reproducible determination of glucosamine sulphate in its tablets and biological fluids. The method is based on the enhancement effect of the investigated drug for the CL emission generated due to the reaction of luminol and hydrogen peroxide at pH 11. The factors that affect the CL reaction were carefully investigated and optimized. The obtained results revealed that the proposed CL method showed wide dynamic response, low limit of detection and excellent accuracy and precision. No interference was recorded from most additive compounds to GLS formulations. Moreover, the use of automated SIA-injection technique cut down the consumption of reagents and samples.

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