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Original Article

DETERMINATION OF AMILORIDE VIA QUENCHED CONTINUOUS FLUORESCENCE OF AZO DYE USING LOW-PRESSURE MERCURY LAMP TUBE (UV-LIGHT) AND MULTI SOLAR CELLS AT 2 X 90° AS A DETECTORS

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ABSTRACT

Objective: The aim of the method was to develop a novel, simple and rapid fluorometric method determination of Amiloride (AMD) in pure form and pharmaceutical drugs via fluorescence measurements.

Methods: The method depends on fluorescence quenching of 2H-chromene azo dye (2-(4-nitrophenyl)-N-(4-(phenyldiazenyl)-2H-chromen-4-amine) upon adding Amiloride (AMD) using homemade ISNAG 2 X 90° multi solar cell via low-pressure mercury lamp at two significant wavelengths 184.9 and 253.7 nm combined with continuous flow injection analysis.

Results: Under the optimized conditions, the fluorescence quenching linear working range and percentage linearity (r^2%) was (0.03-8 mmol/l) and 98.78 %, respectively. The suggested method was effectively applied to the determination of AMD in two different pharmaceutical drugs and compared with the classical method (UV-vis spectrophotometry at λ=540 nm**).**

Conclusion: The proposed and established method is simple, direct, and efficient. The statistical comparison results using a t-test at 95% confidence interval that was applied to compare the new and classical method showed there are no significant differences between the two methods.

Keywords: Amiloride, 2H-chromene azo dye, Fluorescence quenching, Flow injection analysis

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INTRODUCTION

Amiloride (AMD) [chemically it is known as [3,5-diamino-N-(diaminomethylene)-6-chloropyrazinecarboxamide] is a pyrazine carbonyl guanidine derivative (fig. 1). It is an orally administrated with diuretic and mild antihypertensive properties. AMD was indicated for the inhibition of sodium-potassium exchange in kidneys by blocking the distal membrane to advance the loss of sodium and potassium reabsorption [1, 2]. AMD is generally used therapeutically at most in combination formulations with hydrochlorothiazide [3, 4]. The British certified name of the combination was co-amilozide, the common use of this combination for the treatment of nephrogenic diabetes insipidus [5] and in nitroglycerin therapy [6, 7]. Several analytical techniques have been reported for Amiloride determination, including spectrophotometry [8-10], high-performance liquid chromatography (HPLC)[11, 12], Atomic Emission spectrometry [13] and polarography [14].

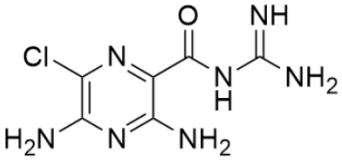


Fig. 1: Chemical structure of amiloride

Azo compounds are the major kind of all synthetic dyes that are widely used in the world [15]. These compounds, with two phenyl rings detached by an azo (-N=N-) bond, are versatile molecules and have much importance in both academic and applied research [16, 17]. Azo dyes are an outstanding class of organic photoactive compounds due to their premium physicochemical features such as optical properties [18, 19], stability [20], and their extensive applications in liquid crystals [21], chemosensors [22] and nanotubes [23]. Chromene (Benzopyran) derivatives play a significant role in the generation of highly efficient fluorescent dyes for synthetic fiber and daylight fluorescent pigments [24, 25].

Fig. 2 shows 2H-chromene azo dye [26], namely (2-(4-nitrophenyl)-N-(4-(phenyldiazenyl) 2H-chromen-4-amine) containing the chromene nucleus as a new derivative of azo dye.

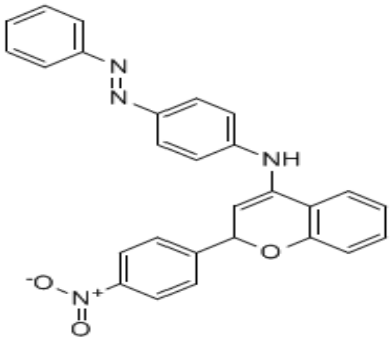


Fig. 2: Chemical structure of the 2H-chromene azo dye

It was observed that 2H-chromene azo dye gives an emission band in ethanol at 425 nm after excitation with 387 nm (a maximum absorbance in ethanol), the fluorescence intensity of this dye was quenched after addition of the amiloride drug as fig. 3.

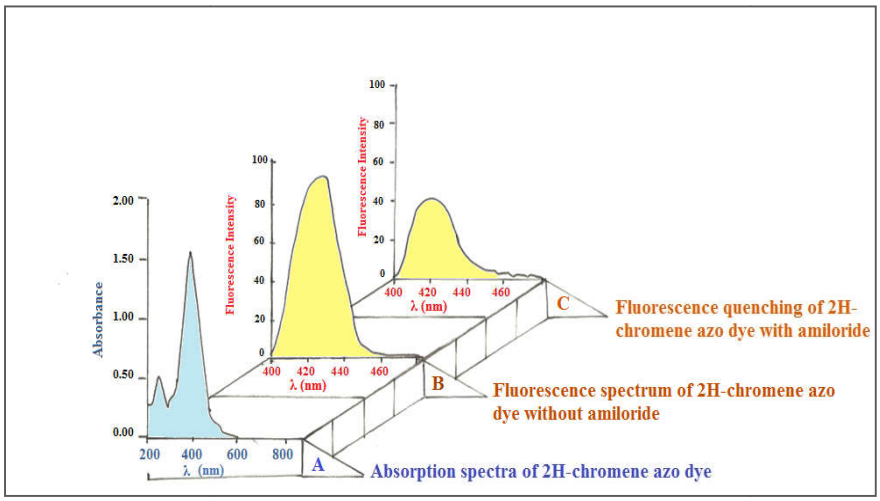


Fig. 3: (A) Absorption spectra of 2H-chromene azo dye in ethanol; fluorescence intensity of 2H-chromene azo dye in ethanol: (B) without amiloride (drug), (C) In the presence of drug

In the present work, a novel fluorometric method has been developed for the determination of Amiloride combined with a flow injection technique using a homemade ISNAG-fluorometer [27]. This method is based on using azo dye (2-(4-nitrophenyl)-N-(4-(phenyldiazenyl)-2H-chromen-4-amine) as fluorescence dye, then the constant fluorescence is quenched by amiloride. The fluorescence quenching was measured by ISNAG-fluorimeter.

MATERIALS AND METHODS

Apparatus and reagents

ISNAG-fluorometer is a homemade instrument [27] was used in measuring the fluorescent response with 4-channels peristaltic pump (Ismatec, Switzerland), valve 6-port medium pressure injection valve (I D E X corporation, USA), sample loop (1 mm ID Teflon, changeable length). The output signals were estimated via a Potentiometric recorder (Siemens, Germany (1-5 V). UV-Vis Spectrophotometer digital double-beam mode (UV-Vis spectrophotometer, UV-1800, Shimadzu, Japan) was also used for classical spectrophotometric methods. SHIMADZU Fluorescence spectrometer to measure fluorescence spectra.

All chemicals were applied to analytical-reagent grade. A standard solution of AMD ($C_6H_8ClN_7O$, molecular weight 229.6 g/mol, 0.02 mol/l) was prepared by dissolving 1.148 g in 250 ml ethanol. 2H-chromene azo dye ($C_{27}H_{19}N_4O_3$, molecular weight 448 g/mol, synthesis previously [26], 0.001 mol/l).

Two pharmaceutical brands of amiloride 5 mg tablets were purchased from a local pharmacy; (Actavis, UK) and (Moduretic, Lebanon).

The flow injection manifold system is composed of one line (fig. 4) represents the carrier stream (fluorescent azo dye solution (0.3 mmol/l) which passes through the injection valve to carry amiloride sample of 100 µl as a sample volume, 1.5 ml/min flow rate and 4 mmol/lconcentration of amiloride) and then passes through the measuring cell. The response of quenched the continuous fluorescence of azo dye was measured by a homemade ISNAG-fluorimeter via low-pressure mercury lamp; it gives two main wavelengths, namely 184.9 nm and 253.7 nm. While the suggested probable mechanism pattern is expressed in scheme 1 [28, 29].

RESULTS AND DISCUSSION

Optimization of variables using one line manifold system chemical variable

Effect of 2H-chromene azo dye concentration as a chemical variable

A set of 2H-chromene azo dye concentration ranging from 0.05-0.5 mmol/l were prepared as a carrier stream with a flow rate of 1.2 ml/min and 50 µl of 4 mmol/l of amiloride as an injected sample to study the effect of the amiloride solution on the quenching of continuous azo dye fluorescence. The profile shows in the fig. 5. A, B. Table 1 summed up the obtained results, which observed that an increase in fluorescence of azo dye with an increase of azo dye concentration, at the same time, increased quenching effect by amiloride solution. The optimum concentration of azo dye that gave maximum fluorescence, quenching of continuous fluorescence by amiloride, and minimum effect of blank response is 0.3 mmol/l. A higher concentration of azo dye does not include in this study due to the self-quenching [30].

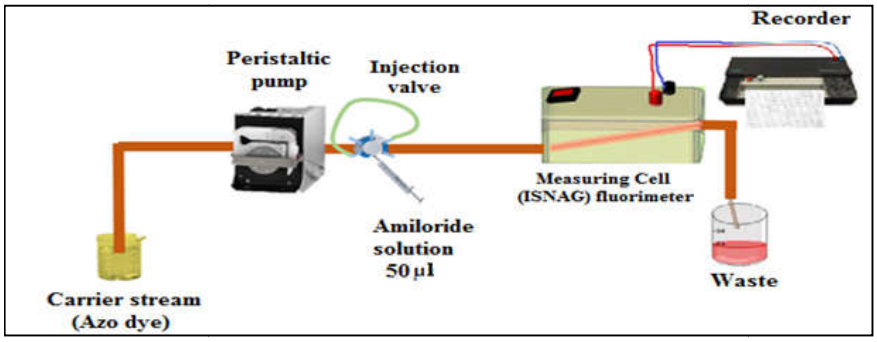
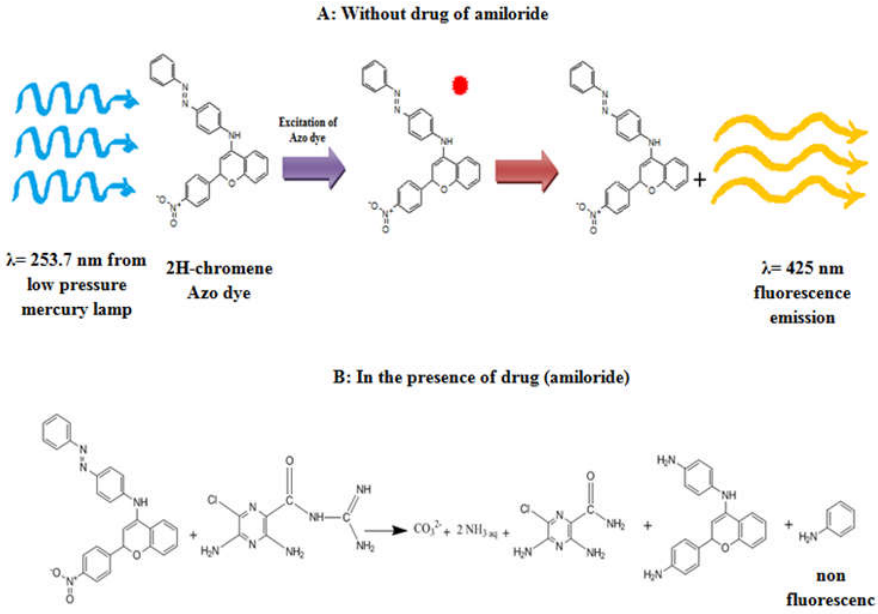


Fig. 4: Manifold system design of quenching of 2H-chromene azo dye fluorescence via the use of amiloride solution as an injected sample



Scheme 1: Proposed mechanism for quenching of 2H-chromene azo dye fluorescence

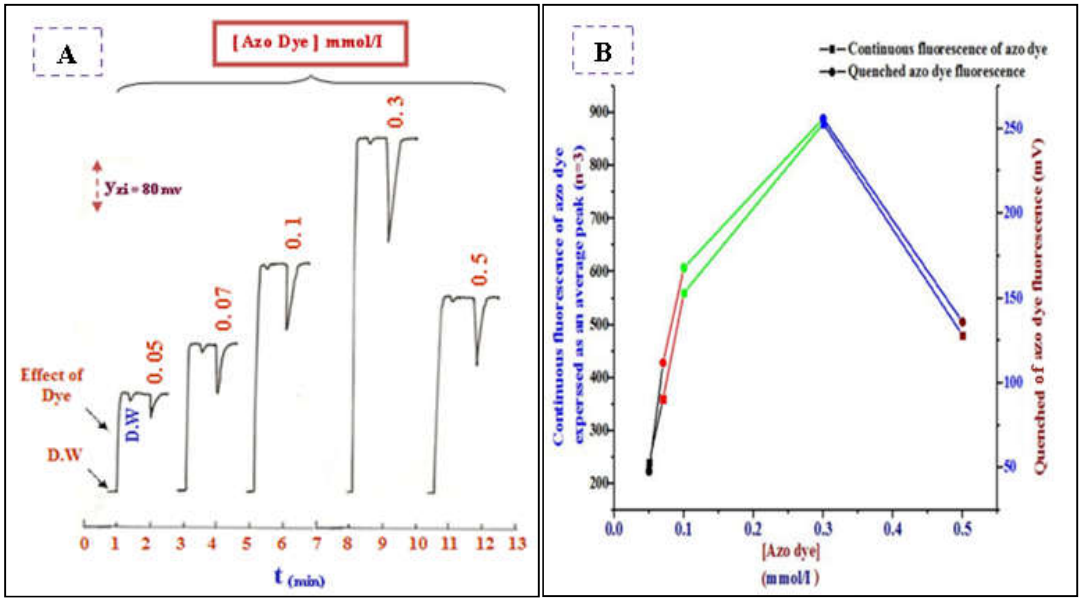


Fig. 5: Effect of 2H-chromene azo dye concentration on fluorescence intensity expressed as: (A) Profile versus time, (B) Increased of fluorescence using low-pressure mercury lamp characteristic emitted light at 184.9 and 253.7 nm, *DW Distilled water as a blank

Table 1: Effect of variation of azo dye concentration for determination of amiloride

[Azo dye] mmol/l	Continuous fluorescence of azo dye described as the average peak heights (n=3) $\bar{y}_i$ in mV	Quenched of azo dye fluorescence $\bar{y}_{Qi}$ (n=3) mV	Confidence interval of the average response[a] (at 95% confidence level) $\bar{y}_{i(mV)} \pm t_{0.05/2,\, n-1} (\sigma_{n-1}/\sqrt{n})$
0.05	240	48	48±1.23
0.07	360	112	112±2.99
0.1	560	168	168±2.12
0.3	880	256	256±2.024
0.5	480	136	136±2.34

Response of blank: 24mV, n= 3 (number of measurements), [a]Data expressed as mean$\pm t_{0.05/2,\, n-1}$ (SEM), $t_{0.05/2,\, n-1}$= 4.303.

Physical parameters optimization

Effect of flow rate

Employing the optimum concentration of azo dye (0.3 mmol/l) and a selected concentration 4 mmol/lof amiloride, 50 µl sample volume, and open valve mode with variable flow rate (0.5-2.0 ml/min) for the carrier stream (2H-chromene azo dye solution). Fig. 6A shows a kind of response profile that was obtained while all results were sorted in table 2. It was can be observed that at a low flow rate, there is an expansion in dispersion and dilution effect due to the distribution of segment (i. e; amiloride) on a larger area [31]. But at the high flow rate result in an increase in peak height, the peak base width decrease, lower at analysis time, and minimize the dilution effect and obtained sharp edges responses [32]. Therefore, the best flow rate was 1.5 ml/min (fig. 6B).

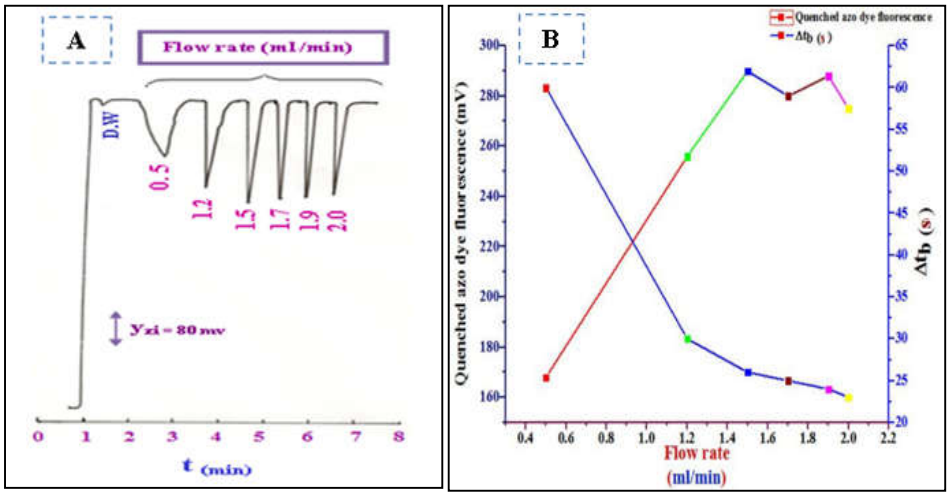


Fig. 6: Effect of the variance of flow rate on: (A) Response profile, (B) Quenching of fluorescence response by amiloride and the width of peak base (Δt_B)

Table 2: Efect of variable flow rate utilizing 50 µl of 4 mmol/lof amiloride via quenching of 2H-chromene azo dye continuous fluorescence

Flow rate (ml/min)	Quenched of azo dye fluorescence $\bar{y}_{Qi}$ (n=3) mV depicted as the average peak heights (n=3) $\bar{y}i$ (mV)	Confidence interval of the average response[a] (at 95% confidence level) ($\bar{y}_i \pm t_{0.05/2,\, n-1} (\sigma_{n-1}/\sqrt{n})$	Δt_b Peak base width (s)
0.5	168	168±1.57	60
1.2	256	256±2.05	30
1.5	290	290±2.11	26
1.7	280	280±2.01	25
1.9	288	288±2.03	24
2.00	275	275±2.23	23

Continuous fluorescence response: 880mV, Response of blank: 24mV, n=3 (number of measurements), Δt_b (s): Time lapse for the quenched of azo dye fluorescence response within estimation cell or peak base width, [a]Data expressed as mean$\pm t_{0.05/2,\, n-1}$ (SEM), $t_{0.05/2,\, n-1}$= 4.303.

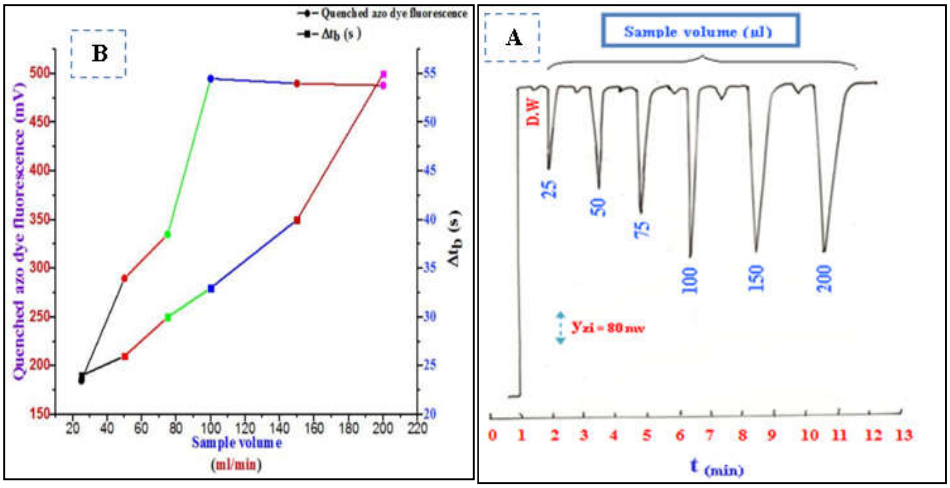


Fig. 7: Effect of variable sample volume on: (A) Profile using quenched fluorescence by amiloride, (B) Quenching of fluorescence response described as the average peak heights (mV) and Δt_B

Table 3: Effect of variable volume of sample on quenching of continuous fluorescence

Sample volume (µl)	Quenched of azo dye fluorescence $\bar{y}_{Qi}$ (n=3) mV expressed as the average peak heights (n=3) $\bar{y}i$ (mV)	Confidence interval of the average response[a] (at 95% confidence level) $\bar{y}_i \pm t_{0.05/2,\, n-1}(\sigma_{n-1}/\sqrt{n})$	Δt_b Peak base width (s)
25	185	168±1.57	24
50	290	256±2.05	26
75	335	290±2.11	30
100	495	280±2.01	33
150	490	288±2.03	40
200	488	275±2.23	55

Response of continuous fluorescence: 880mV, n=3 (number of measurements), Δt_b (s): Time lapse for the Quenched of azo dye fluorescence response within measuring cell or peak base width, [a]Data expressed as mean$\pm t_{0.05/2,\, n-1}$ (SEM), $t_{0.05/2,\, n-1}$= 4.303.

Effect of variable sample volume

Adjusting all achieved optimum that was studied in previous sections with a variable volume of sample segment, which extends from 25-200 µl in addition to open valve mode and 4 mmol/l amiloride concentration at flow rate 1.5 ml/min for carrier stream (0.3 mmol/l 2H-chromene azo dye solution) were used. All results are subjected in fig. 7. A, B and tabulated in table 3 which indicate clearly that the optimum sample volume is 100 µl to obtain sharp and highest response profile expressed as a quenched of azo dye fluorescence, but an increase of the sample segment (>100 µl) leads to a decrease in peak heights, which can be probably attributed to the long time period of the sample segment in front of a detector [33]. So that the 100 µl was the most favorable choice to give the highest response for quenched continuous fluorescence of the azo dye molecule.

Calibration curve

A series of concentrations (0.03-10 mmol/l) of amiloride was prepared under the established optimum condition. Each measurement three times was repeated. The variation in quenched fluorescence response of ISNAG-fluorimeter with amyloid concentration was ranging from 0.03-8 mmol/l with linearity percentage (R^2%): 98.79 % for dynamic range. Above 8 mmol/l; correlation coefficient value will deviate to the working range at linearity percentage (R^2%): 97.59 % probably due to the quenching the inner fluorescence in the form of non-radiative thermal energy or internal convention between electronic levels of all fluorescent molecules and losing fluorescence energy and external convention. Fig. 8 shows the variation of response with concentration using linear regression treatment [34] for the newly developed methodology method. While the classical method using a spectrophotometer (the measurement of absorbance at 540 nm using Ce (IV)–Amiloride–H_3O system) with the range of (0.005-7) mmol/l[35].

The linear equation for dynamic range at n=13 is

Quenched of fluorescence (n=3 ($\hat{Y}$Qi)) in (mV) = 116.78±24.13+84.99±10.13 [amiloride] mmol. l^{-1} While for working range at n= 14 is

Quenched of fluorescence (n=3 ($\hat{Y}$Qi)) in (mV) = 126.18±33.19+79.32±18.23 [amiloride] mmol. l^{-1} and confidence level 95%.

The detection limit is calculated from the gradual dilution of the minimum concentration of the used of effective range calibration graph and depends on the values of a slope. Table 4 summarizes the different ways of calculating the limit of detection for the Quenched of fluorescence [34].

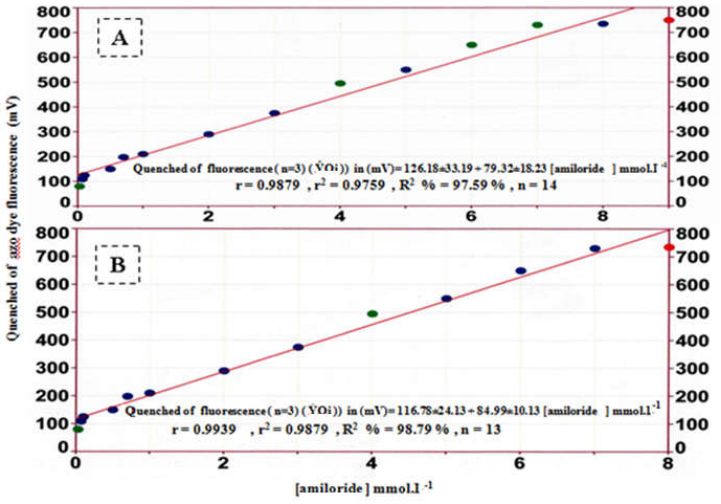


Fig. 8: Calibration graph of quenching 2H-chromene azo dye fluorescence response (mV) with amiloride concentrations (mmol/l): (A) range (0.03-10 mmol/l) and (B) range (0.03-8 mmol/l), n=13 or 14 (working range of calibration graph)

Table 4: Limit of detection for the determination of amiloride (100 µl) at optimum parameter

Practical based on the progressive dilution for the minimum concentration (0.03 mmol/l)	Theoretical formed on the value of the slope $X=3S_B/slope$
0.6888 µg/Sample	0.3242 µg/Sample

X: value of the limit of detection based on a slope, S_B: Standard deviation of a blank (repeated for 13 times).

Repeatability

A repeated eight successive measurements of two concentrations (5 and 7 mmol/l) of analyte gave a variation of RSD % less than 0.5 % this indicates the method was distinguished by high accuracy with good repeatability [33]. Fig. 9 shows a kind of response profile.

Pharmaceutical drug analysis with ISNAG-fluorimeter

The performance of the newly established method (ISNAG-fluorimeter) was evaluated compared with the classical method (UV-vis spectrometer) by the determination of AMD in pharmaceutical drugs from various manufactures (amiloride, 5 mg-Actavis and Moduretic, 5 mg). The suggested method was applied to the analysis of standard addition, each Pharmaceutical drug by preparing a sequence of solutions from each drug via adding 0.2 ml (5 mmol/l) to five (10 ml) volumetric flasks. Then the standard solution (0.01 mmol/l) of AMD is added in various amounts such as (0, 0.1, 0.2, 0.3 and 0.4 ml) and the flasks are diluted to the mark to get the concentration range between (0-0.4 mmol/l), table 5 summarizes the results obtained from the standard addition measurements which were mathematically treated at 95% confidence interval [34, 36].

Utilizing measurable chemometric treatment table 6 was obtained and the results of the two approaches were tried using paired t-test [27, 34]. The final deduction in table 6 displays that there is no significant difference between the newly proposed method and the classical method at confidence interval 95%, as the $t_{tabulated}$ value is greater than the $t_{calculated}$ value; Hence the recently developed method can be applied as a substitution mode for the determination of amiloride in pharmaceutical drugs.

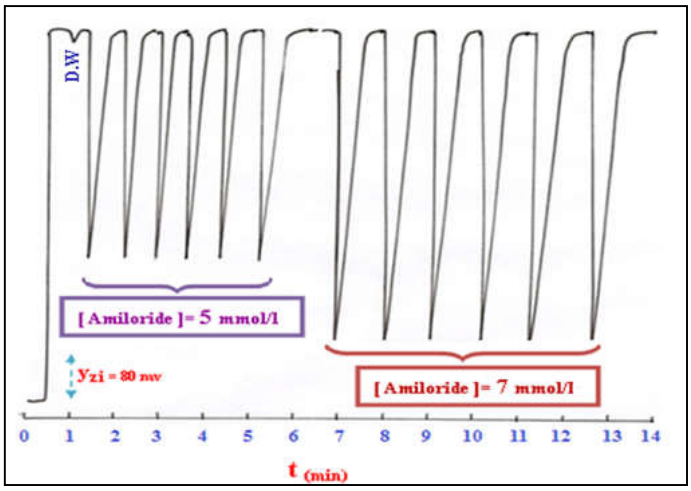


Fig. 9: Repeatability of six successive measurements of 5 and 7 mmol/l of amiloride via the quenching effect

Table 5: Standard addition results for the estimation of amiloride in two different drugs by two methods: Newly developed method using the ISNAG-fluorimeter and UV-vis spectrometer as a classical method

Sample No.	Commercial name, content and company country	Confidence interval for the average weight[a] $Wi \pm 1.96 (\sigma_{n-1}/\sqrt{n})$ at 95% (g)	Sample weight equivalent to 0.0574 g/50 ml (5 mmol/l) of the active ingredient (g)	Theoretical content of the active ingredient[a] at 95% (mg) $\mu \pm 1.96 (\sigma_{n-1}/\sqrt{n})$	$\bar{y}_i$ (n=3) in mV (Newly developed methodology) $\hat{Y}_i$ (mV)= $a \pm S_a t + b \pm S_b t$ [Amiloride] mmol/l						
					UV-Vis-spectrometer $\bar{y}_i$ (n=3) at λ_{max}= 540 nm (Classical method) $\hat{Y}_i = a \pm S_a t + b \pm S_b t$ [Amiloride] mmol/l						
					[Amiloride] mmol/l					Equation of standard addition at 95% for n-2 $\hat{Y}_i = a \pm S_a t + b \pm S_b t$ [x] r, R^2%	Practical content mmol/l in 10 ml and (50 ml) W_i (mg), Rec%
					0.0	0.1	0.2	0.3	0.4		
					$\bar{y}$i for n= 3						
1	Amiloride Actavis (5 mg) UK	0.1195±0.0015	1.37186	5±0.0628	40	90	115	155	200	43±12.34+385±58.12 [Amiloride] mmol/l 0.9957, 99.15 %	0.112, 5.55 5.55, 111 %
										0.79±0.063+8.95±1.15 [Amiloride] mmol/l	5.238, 104.76%
2	Moduretic (5 mg) Lebanon	0.1203±0.0014	1.3810	5±0.0582	167	340	410	500	600	55±14.45+535±48.35 [Amiloride] mmol/l 0.9998, 99.97%	0.102, 5.140 5.14, 102.81%
										0.134±0.04+1.35±0.67 [Amiloride] mmol/l	5.33, 106.67%

Ŷi: Estimated response in (mV) for a newly developed method or absorbance for classic method, X: [AMD] mmol/l, n=3 (number of measurements), r: Correlation coefficient, r^2: Coefficient of determination, R^2 % (Percentage capital R-squared): Elucidated variation as a percentage total variation, [a]Data expressed as mean±$t_{0.05/2,\infty}$ (SEM), S_a: SD of intercept, S_b: SD of slop, $t_{0.05/2,\infty}$ = 1.96 at 95%.

Table 6: Paired t-test for the comparison of the ISNAG (2 X 90 ° multi solar cells) new method with the UV-vis method.

Sample no.	Practical content (mg)		(d) (mg)	$\bar{X}_d$ (mmol/l)	σ_{n-1}	Paired t-test $\frac{\bar{X}_d\sqrt{n^*}}{\sigma_{n-1}}$	Tabulated t-value at 95% confidence interval
	Newly method (ISNAG-fluorimeter)	UV-vis method					
1	5.550	5.238	0.312	0.061	0.355	0.243>12.706	
2	5.140	5.330	-0.190				

n^*: Number of samples, $\bar{X}_d$: An average of the difference between the two methods.

CONCLUSION

In this work, a new method was developed and established for the determination of amiloride in pharmaceutical drugs using ISNAG-fluorimeter-CFI analysis and azo dye as a new reagent. The suggested method based on the quantitative quenching influence of amiloride drug on the native fluorescence of the azo dye. The statistical comparison via the t-test between this newly work and UV-Vis method was in good approval. Hence, this developed method can be used as an alternative method for the determination of amiloride in pharmaceutical drugs.

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AUTHORS CONTRIBUTIONS

This research was done under the supervision of the first author.

CONFLICTS OF INTERESTS

Declared none

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