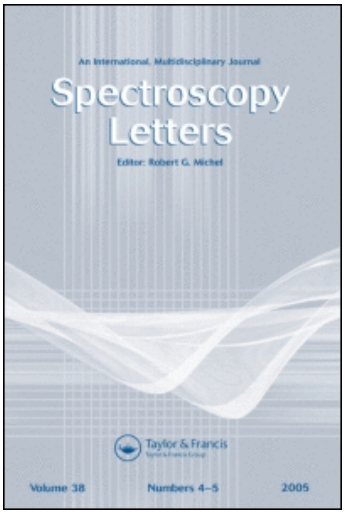

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Spectrofluorometric Determination of Cephalexin in Pharmaceutical Preparations and Spiked Human Urine Using 2-Cyanoacetamide

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Spectrofluorometric Determination of Cephalexin in Pharmaceutical Preparations and Spiked Human Urine Using 2-Cyanoacetamide

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Abstract: A simple, accurate, sensitive, and validated method was developed for the spectrofluorometric determination of cephalexin. The method involves the reaction of cephalexin with 2-cyanoacetamide in presence of 33% ammonia solution. The formed fluorescent product exhibited maximum fluorescence intensity at λ 439 nm, after excitation at λ 339 nm. Different experimental parameters affecting the derivatization reaction were carefully studied and incorporated in the procedure. Under the described conditions, the proposed method was linear over the concentration range 0.04–0.4 μg/mL. The average percent found was 99.6 ± 0.9%. The LOD was 7.76 ng/mL. The proposed method was applied for determination of cephalexin in pharmaceutical preparations as well as in spiked human urine. A mechanism of the reaction is postulated.

Keywords: Cephalexin, 2-cyanoacetamide, spectrofluorometry

INTRODUCTION

Cephalexin 7(D-α-ammino-α-phenyl acetamide-3-methyl-3-cephem-4-carboxylic acid) is a first-generation cephalosporin and one of the most commonly used cephalosporin antibiotics (Scheme 1).

Several methods were reported for cephalexin determination in pharmaceutical preparations. The most recent of these methods include charge transfer complexation reactions with DDQ (2,3-dichloro-5,6-dicyano-*p*-benzoquinon),

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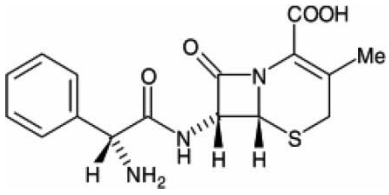


Scheme 1. Cephalexin.

TCNQ (7,7,8,8-tetracyanoquinodimethane),[1] and chloranilic acid.[2] Ascorbic acid was used as a colorimetric reagent after alkaline degradation.[3] 1,2-Naphthoquinone 4-sulfonate reagent was also used for the determination of cephalexin in pharmaceutical and urine samples using solid phase extraction cartridges.[4] Flow injection methods were also applied using tris(2,2-bipyridyl)ruthenium (II)–potassium permanganate systems,[5] formaldehyde–potassium permanganate system,[6] and *o*-phenanthroline iron system.[7]. Polarographic,[8,9] voltammetric,[10] HPLC,[11–14] and micellular electrokinetic chromotography (MEKC)[15,16] methods were also reported. The British[17] and USP pharmacopeias[18] recommend also HPLC methods. Few fluorometric methods were published for the determination of cephalexin, based on the reaction with acetyl acetone–formaldhyde,[19] fluorescamine,[20] or measurement of fluorescence intensity after acid hydrolysis and subsequent alkalinization.[21]

2-Cyanoacetamide (2-CAA) is a fluorogenic reagent; this reagent was used in post–column derivatization in HPLC determinations of catecholamines[22] and carbohydrates[23] and also used for the fluorometric determination of some important pharmaceutical compounds such as prenalterol HCl,[24] oxamniquine,[25] ascorbic acid,[26] aminoglycosides,[27] and 3,4-dihydroxyphenylalanine.[28]

In the current work, 2-CAA is used for the fluorometric determination of cephalexin in presence of 33% ammonia solution.

MATERIALS AND METHODS

Apparatus

The fluorescence intensities were measured using a Perkin Elmer model LS 45 spectrofluorometer equipped with 20-W xenon discharge lamp, excitation, emission grating monochromators, and a 1×1 cm quartz cell. The apparatus is connected by an HP computer.

Materials and Reagents

All the chemicals were of analytical reagent grade, and all the solvents were of spectroscopic grade. Cephalexin monohydrate was kindly provided by

Glaxo-SmithKline (Cairo, Egypt). Cephalexin tablets, suspension, and vials were obtained from commercial sources. 2-Cyanoacetamide 1% aqueous solution and ammonia 33% (w/v) solution were from Merck (Darmstadt, Germany).

Preparation of Standard Solutions

A stock solution of cephalexin containing 1.0 mg/mL was freshly prepared every day in distilled water and was further diluted with the same solvent to get the working solution (0.1 mg/mL).

Construction of Calibration Graph

Transfer accurately measured portions (0.1–1.0 mL) of the working solution into a set of 25-mL volumetric flasks. Add 2.0 mL of 1% 2-CAA and 2.5 mL of 33% ammonia solution. Heat the reaction mixture in a boiling water bath for 25 min then cool, and make up to volume with distilled water. Measure the fluorescence intensity at λ 435 with excitation at λ 339 nm against a reagent blank. The calibration graphs were obtained by plotting the fluorescence intensities against the concentration of cephalexin; alternatively derive the corresponding regression equation.

Assay of Dosage Forms

Tablets, Capsules, Vials

Weigh and pulverize 10 tablets, mix the contents of 10 capsules, or mix the contents of 10 vials. An accurately weighed amount of the powder equivalent to 20 mg cephalexin was transferred to a 100 mL measuring flask. Add 50 mL distilled water, sonicate for 10 min, filter, then dilute the filtrate with distilled water and continue the procedure as described under "Construction of Calibration Graph". The nominal contents of the drug in each solution were calculated using either the calibration graph or the corresponding regression equation.

Suspensions

Transfer an accurately measured volume of the reconstituted syrup, equivalent to 10 mg of cephalexin, into a conical flask. Add 50 mL distilled water, sonicate for 10 min, then filter. Transfer the filtrate into a 100-mL measuring flask and dilute to the mark with water to obtain a working solution of 0.1 mg/mL. The procedure is continued as described under "Construction of Calibration Graph."

Assay of Spiked Human Urine

In a set of 10-mL centrifugation tubes, spike 5 mL aliquots of urine with varying amounts of cephalexin to give final required concentration, add 5 mL methanol to each tube, and vortex the mixture at 1500 rpm for 5 min.[20] Transfer 1.0 mL of the supernatant into a 25-mL volumetric flask and continue as described under "Construction of Calibration Graph." Carry out a blank experiment adopting the above procedure.

RESULTS AND DISCUSSION

Treatment of cephalexin with 2-CAA in the presence of ammonia solution was found to give a fluorescent product. The formed fluorophore (Fig. 1) exhibits strong fluorescence at λ 435 with excitation at λ 339 nm.

Optimization of the Reaction Conditions

The reaction between cephalexin and 2-CAA was studied in different alkaline media: borate buffer (pH 9–12) and ammonia buffer (pH 9–12). Results in Fig. 2 show that the fluorescence intensity increases with increasing pH values up to pH 11 then decreases; different media such as 0.1 M NaOH and 33% ammonia with the same pH (10.5–11) were studied. The results in Table 1

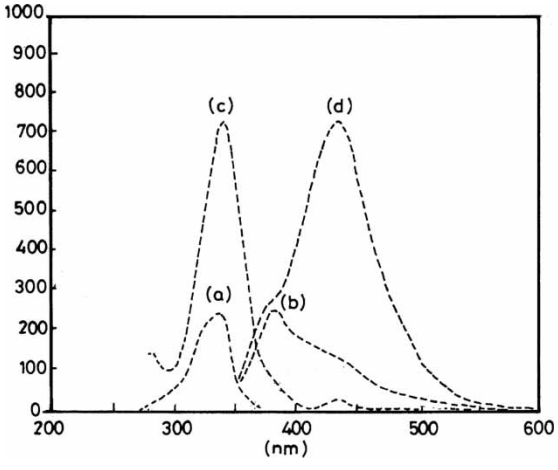


Figure 1. Fluorescence spectra of cephalexin after reaction with 2 mL 1% 2-CAA and 2.5 mL 33% ammonia solution: *a* and *b*, excitation and emission spectra of blank; *c* and *d*, excitation and emission spectra of the reaction product (cephalexin 0.40 μg/mL).

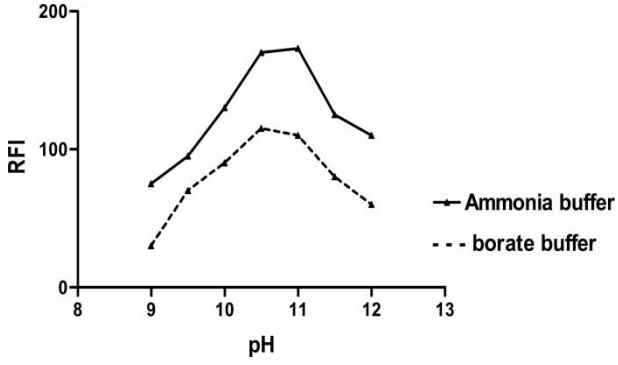


Figure 2. Effect of buffer pH on the fluorescence intensity of the reaction product of cephalexin (0.20 μg/mL) with 1% 2-CAA.

show that the reaction gives the highest fluorescence intensity with 2.5 ± 0.1 mL of 33% ammonia. The difference in response to different alkaline media may be attributed to the change in the medium polarity, which results in some source of physical interaction between buffer and the excited singlet state of the drug molecule.

Regarding the reagent concentration, it was found that 1% 2-CAA gave the maximum intensity. By studying the effect of varying the volume of the reagent, it was found that 2 ± 0.2 mL was enough for maximum fluorescence intensity as shown in Fig. 3.

For the reaction time and stability of the reaction product, it was found that the maximum formation of the fluorophore takes place after 20 min upon heating in a boiling water bath. The formed fluorophore was stable for at least 2 hr at room temperature (Fig. 4).

Table 1. Effect of 33% ammonia and 0.1 M NaOH on the reaction between cephalexin (0.2 μg/mL) and 2-CAA

	RFI	
Volume added (mL)	33% Ammonia	0.1 M NaOH
0.5	170.1	105.7
1.0	200.23	59.66
1.5	220.50	70.87
2.0	240.76	40.20
2.5	250.73	32.65
3.0	230.55	20.39

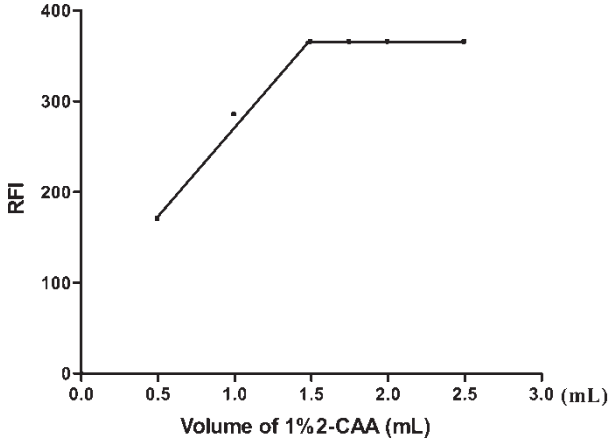


Figure 3. Effect of volume of 1% 2-CAA on the fluorescence intensity of the reaction product of cephalexin (0.20 μg/mL) using 2.5 mL 33% ammonia.

Stoichiometry of the Reaction

The stoichiometry of the reaction was studied adopting the limiting logarithm method.[29] The two straight lines obtained upon using increasing concentrations of the drug while keeping the concentration of the reagent constant (Fig. 5a) and upon using increasing concentrations of the reagent while keeping the concentration of the drug constant (Fig. 5b) gave two slopes with values of 0.874 and 0.877, respectively. Therefore, the molar reactivity

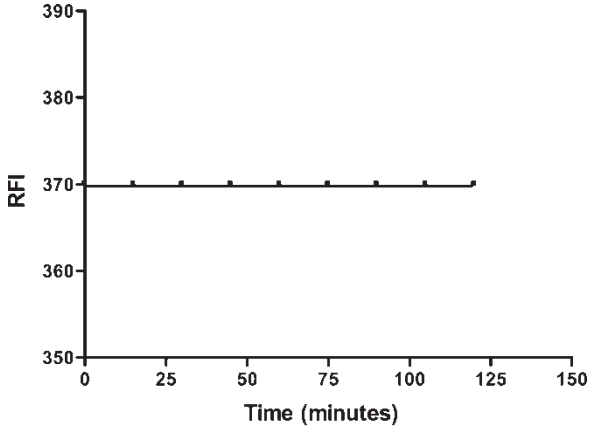


Figure 4. Effect of time on the fluorescence stability of the formed fluorophore of cephalexin (0.2 μg/mL) after reaction with 2 mL 1% 2-CAA, 2.5 mL ammonia in a boiling water bath for 20 min.

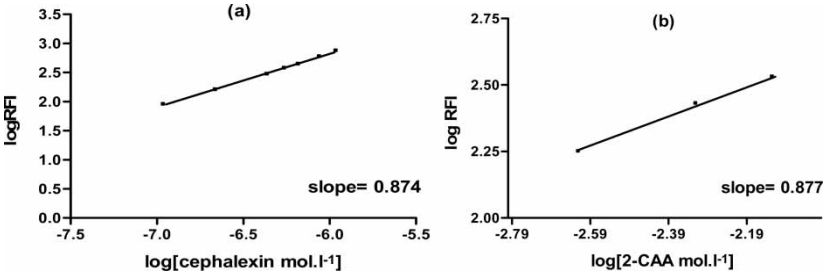


Figure 5. Limiting logarithmic plots for molar ratio: (a) log RFI vs. log [cephalexin] with [2-CAA] kept at 1.25×10^{-3}M; (b) log RFI vs. log [2-CAA] with [cephalexin] kept at 5.47×10^{-3} M.

of the reaction is 1:1, hence the reaction pathway was proposed in two steps: first 2-CAA forms a carboanion in presence of 33% ammonia, followed by a nucleophilic substitution on the carbon atom of the carbonyl group of β-lactam ring with ring opening to give a highly conjugated fluorophore[30] as shown in Scheme 2.

Validation

Linearity

There was a linear dependence of the relative fluorescence intensity on the concentration of cephalexin. Under the described experimental conditions, the fluorescence intensity– concentration plot was rectilinear over the range 0.04–0.4 μg/mL. Linear regression analysis of the data gave the following equation

$$\mathrm{RFI} = 13.887 + 1789.436\,C, \quad \mathrm{r} = 0.9998,$$

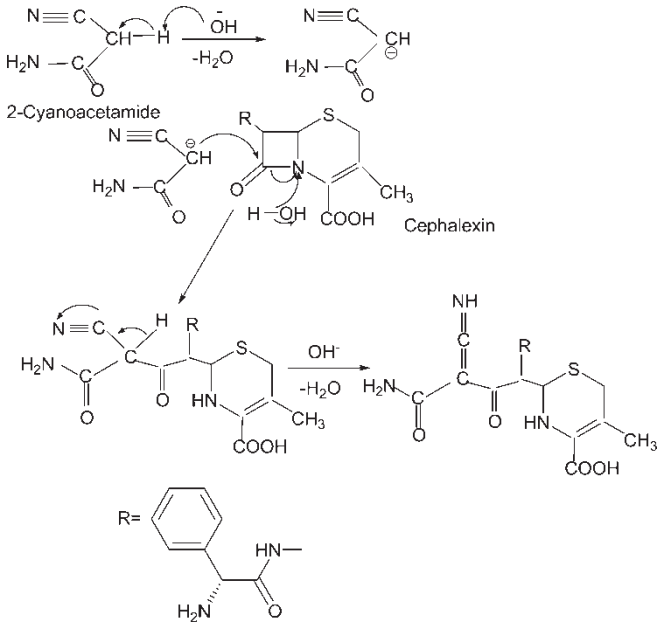
where RFI is the relative fluorescence intensity and C is the concentration in μg/mL.

The good linearity of the calibration graphs and the negligible scatter of the experimental points are clearly evident by the correlation coefficient.

LOQ (Limit of Quantification) and LOD (Limit of Detection)

The LOQ was determined by establishing the lowest concentration that can be measured with acceptable accuracy and precision. Cephlexin can be quantified under these conditions at a concentration of 0.0231 μg/mL.

The LOD was calculated by establishing the minimum level at which cephalexin can be reliably detected, and it was found to be 7.76 ng/mL.[31]



Scheme 2. Proposal of the mechanism of the reaction between cephalein and 2-cyanoacetamide in 33% ammonia solution.

Precision

The results of intraday and interday accuracy and precision of the method are summarized in Table 2. The interday and intraday precisions were examined by analyzing the three concentrations three times for 3 consecutive days. The repeatability of the method is fairly high indicated by the low values of SD and RSD. The interday and intraday accuracy was also proved by the low values of Er% (error percent).

Robustness

The robustness of the method is demonstrated by its capacity to remain unaffected by small but deliberate variation in method parameters.

Analytical application

The validity of the method could be proved by analyzing an authentic sample of cephalexin. The results obtained in Table 3 were in good agreement with those given by the official method. Statistical analysis of the results obtained by both methods by applying the Student's *t*-test and variance

Table 2. Evaluation of the accuracy and precision data of the proposed spectrofluorometric method for the determination of cephalexin

Conc. added (μg/mL)	Conc. found (μg/mL)			
	SD ± mean	% Recovery	RSD%	Er%
Intraday				
0.16	0.159 ± 0.002	99.94 ± 0.89	0.89	0.63
0.20	0.199 ± 0.002	99.74 ± 0.95	0.95	0.48
0.32	0.319 ± 0.002	99.92 ± .0.66	0.66	0.33
Interday				
0.16	0.161 ± 0.001	100.36 ± 0.67	0.67	0.33
0.20	0.200 ± 0.001	100.63 ± 0.69	0.69	0.34
0.32	0.321 ± 0.002	100.14 ± 0.63	0.63	0.31

ratio F-test reveals no significant difference between the performances of the two methods. Statistical evaluation of the regression line regarding standard deviation of the residuals ($S_{y/X}$), standard deviation of the slope (S_b), and standard deviation of the intercept (S_a) gave the values 0.009, 6.9×10^{-4}, and 0.003 respectively.

The simplicity of the method and the stability of the reaction product permitted the determination of cephalexin in its commercial tablets, capsules, suspensions, and vials. The results obtained were in good agreement with those given using the official method (Table 4). Common

Table 3. Application of the proposed method for the determination of cephalexin in pure form

Proposed method			Reference method[20]
μg Taken	μg Formed	% Recovery	
0.04	0.0397	99.25	100.20
0.08	0.081	101.25	100.88
0.16	0.159	99.38	99.58
0.20	0.201	100.50	
0.24	0.239	99.58	
0.32	0.319	99.69	
0.40	0.401	100.25	
X′		99.99	100.22
± SD		0.72	0.62
t		1.38(2.306)	
F		1.23(6.16)	

Each result is the average of three separate determinations. Values between brackets are the tabulated *t* values and F-values at $p = 0.05$.

Table 4. Application of the proposed method for the determination of cephalexin in dosage form

Pharmaceutical preparation	Proposed method			Reference method[20]		
	Added μg/mL	Found μg/mL	Recovery (%)	Added μg/mL	Found μg/mL	Recovery (%)
Ceporex suspension[a] (250 mg cephalexin/5 mL)	0.16	0.161	100.6	0.201	0.201	100.50
	0.20	0.198	99.00	0.30	0.297	99.00
	0.24	0.238	99.20	0.40	0.398	99.50
	0.32	0.322	100.60			
	0.40	0.403	100.80			
X′ ± SD			100.04 ± 0.86			99.70 ± 0.76
t			1.36 (2.45)			
F			1.13 (6.94)			
Ceporex tablets[a] (500 mg cephalexin/tablet)	0.16	0.159	99.40	0.20	0.198	99.00
	0.20	0.203	101.50	0.30	0.302	100.66
	0.24	0.241	100.40	0.40	0.405	99.75
	0.32	0.321	100.30			
	0.40	0.399	99.80			
X′ ± SD			100.22 ± 0.68			99.8 ± 0.83
t			1.92 (2.45)			
F			1.71 (6.94)			
Ceporex vial[a] (1 g cephalexin/vial)	0.16	0.162	101.3	0.20	0.199	99.50
	0.20	0.199	99.30	0.30	0.298	99.30
	0.24	0.241	100.4	0.40	0.402	100.50
	0.32	0.319	99.70			
	0.40	0.397	99.30			
X ± SD			100.04 ± 0.82			99.8 ± 0.64
t			1.06 (2.45)			
F			1.28 (6.94)			

Each result is the average of three separate determinations.

Values between brackets are the tabulated t values and *F*-values at ($p = 0.05$).

[a]Products of Glaxo-Smithkline.

Table 5. Application of the proposed method for the determination of cephalexin in spiked human urine sample

Spiked amount (μg/mL)	% Found
0.08	97.50
0.16	99.30
0.24	102.08
0.32	98.75
Mean ± SD	99.63 ± 2.3

Each result is the average of three separate determinations.

tablet excipients, such as talc powder, starch, lactose, magnesium stearate, and gelatin, did not interfere with the assay.

The high sensitivity attained by the proposed method allowed its extension to the *in vitro* determination of cephalexin in spiked human urine samples. Cephalexin is administered in doses of 500 or 1000 mg resulting in a urine level of 1 mg per mL. About 80% or more of the dose is excreted unchanged in the urine in the first 24 hr by glomerular filtration and tubular excretion. [32] Thus, the proposed method is sufficient for routine estimation of the drug in human urine. The results shown in Table 5 are satisfactorily accurate and precise.

CONCLUSIONS

A simple, accurate, and precise method was developed for the determination of cephalexin in dosage forms and spiked human urine. The method can measure concentration down to 7.76 ng/mL. The method is simple, rapid, and readily adaptable to routine quality control laboratories.

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